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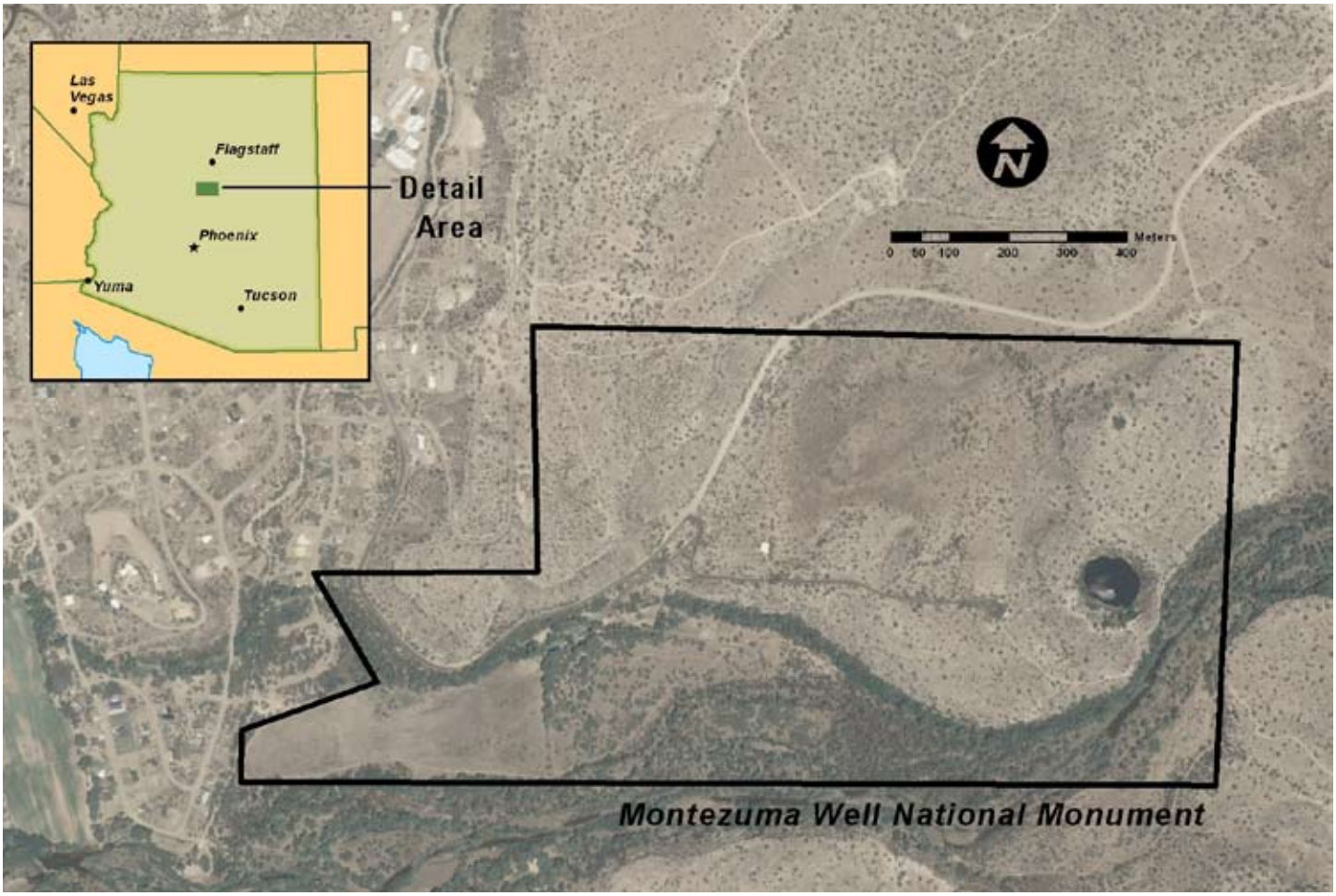
The Ecology of Parasite-Host Interactions at Montezuma Well National Monument, Arizona—Appreciating the Importance of Parasites



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By Chris O'Brien and Charles van Riper III

Prepared in Cooperation with the University of Arizona

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The Ecology of Parasite-Host Interactions at Montezuma Well National Monument, Arizona—Appreciating the Importance of Parasites

By Chris O'Brien[1] and Charles van Riper III [2]

Introduction

Although parasites play important ecological roles through the direct interactions they have with their hosts, historically that fact has been underappreciated. Today, scientists have a growing appreciation of the scope of such impacts. Parasites have been reported to dominate food webs (Bakker and others, 1997; Lafferty and others, 2006), alter predator-prey relationships (Lafferty and Morris, 1996), act as ecosystem engineers (Thomas and others, 1998, 1999), and alter community structure (Poulin, 1999; Wood and others, 2007). In spite of this growing awareness in the scientific community, parasites are still often neglected in the consideration of the management and conservation of resources and ecosystems (Marcogliese, 2004). Given that at least half of the organisms on earth are probably parasitic (Price, 1980; Windsor, 1998), it should be evident that the ecological functions of parasites warrant greater attention.

In this report, we explore different aspects of parasite-host relationships found at a desert spring pond within Montezuma Well National Monument, Arizona (fig. 1-1). In three separate but related chapters, we explore interactions between a novel amphipod host and two parasites. First, we identify how host behavior responds to this association and how this association affects interactions with both invertebrate non-host predators and a vertebrate host predator. Second, we look at the human dimension, investigating how human recreation can indirectly affect patterns of disease by altering patterns of vertebrate host space use. Finally—because parasites and diseases are of increasing importance in the management of wildlife species, especially those that are imperiled or of management concern—the third chapter argues that research would benefit from increased attention to the statistical analysis of wildlife disease studies. This report also explores issues of statistical parasitology, providing information that may better inform those designing research projects and analyzing data from studies of wildlife disease.

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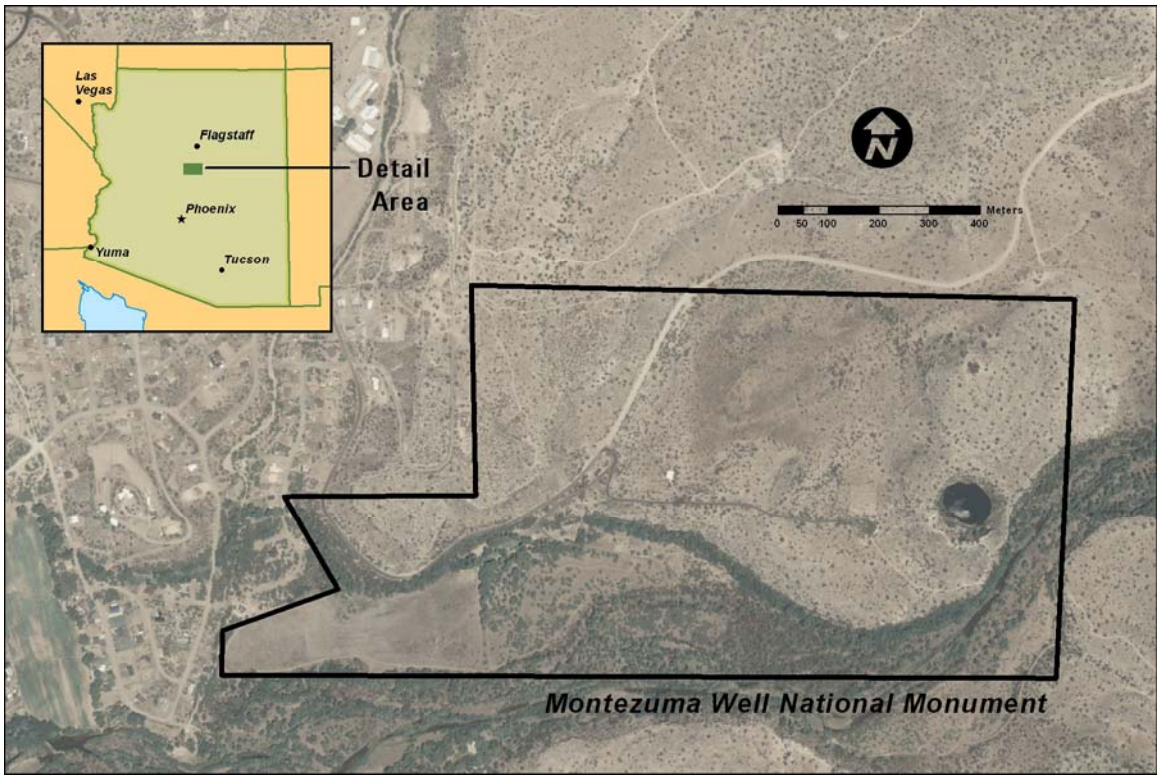

Figure 1-1. Location of Montezuma Castle NM in Arizona and aerial photograph of the Well unit.

In investigating the nature of parasite-host interactions, the role that relationships play in ecological communities, and how human activities alter these associations, scientists usually make inferences by methods of statistical hypotheses testing. This type of hypothesis testing places additional importance on the analysis and interpretation of parasite-host interactions. We address these ideas in this report, focusing on the following questions: (1) How do two parasites with complex life cycles alter the behavior of a novel amphipod host, and how do host and non-host predators respond to infected amphipod prey? (2) Does human recreation affect spatial patterns of infection in an otherwise natural ecosystem? (3) How is hypothesis-testing applied in studies of wildlife disease? (4) What conclusions can we make about the relative usefulness of these methodologies? and (5) How can the analysis and interpretation of wildlife disease studies be improved? Each chapter of this report contains its own literature-cited section, with tables included in appendixes at the end of the full report.

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Chapter 1

Parasites in a Novel Host: Implications for Parasite-Induced Trophic Transmission

Introduction

Behavioral impacts of helminths on crustacean hosts are well studied, but little attention has focused on parasites in planktonic amphipods. Planktonic amphipods exhibit very different behavior from that of benthic amphipods; therefore, we examined patterns of infection of two helminths (acanthocephala: *Corynosoma constrictum* and trematoda: *Microphallus* spp.) in their alternate host, the endemic planktonic amphipod *Hyalella montezuma* and measured the behavior of infected and uninfected amphipods. We also used stable-isotope analysis of nitrogen and carbon to determine whether parasitism altered the habitat preference and/or diet of infected amphipods. Our study produced three major findings: (1) Acanthocephalan-infected *H. montezuma* were strongly male-biased, while samples of uninfected amphipods were not, suggesting either that males are more susceptible to infection or that the parasite is more virulent to females. (2) Acanthocephalan-infected amphipods did not show different activity levels or phototactic and geotactic responses as compared to uninfected amphipods, while trematode-infected amphipods showed reduced activity levels, but no difference in response to light or gravity, suggesting that the trematode had a pathogenic effect upon its host. (3) Although stable-isotope ratios of C and N differed between amphipods from littoral and pelagic zones, we found no differences between trematode-infected and uninfected amphipods, despite the reduction in swimming activity seen in infected individuals. These findings are contrary to most previous studies of helminth-amphipod interactions, and this highlights the need for future studies of parasite-host interactions that emphasize the behavioral differences and phylogenetic relationships of hosts.

Host modification of behavior by parasites with complex life cycles has a rich history (reviewed in Moore, 2002), and current research regarding this phenomenon is vast. Most studies that investigate the behavioral effects of helminth parasites upon their intermediate arthropod hosts report a significant difference in the behavior of parasitized and unparasitized individuals (Poulin, 1994), suggesting that helminths often alter the behavior of their hosts in a way that increases the probability of trophic transmission to a vertebrate definitive host. However, the documented consistency of this pattern reported in the literature has decreased over time (Poulin, 2000).

Amphipods provide a good model for the study of host-parasite modification because they are infected as alternate hosts of helminths with complex life cycles, are easy to culture, and can be manipulated experimentally. However, many studies of the behavioral aspects of parasite-host interactions have focused on amphipods that normally, in the presence of fish predators, associate with dark environments and vegetation in order to conceal themselves. Studies of amphipods have shown that, when infected with helminth parasites, their behavior is altered so that they become more conspicuous to vertebrate definitive hosts (either fish or avian) through changes in swimming behavior, reaction to light and gravity, and/or clinging and evasive behavior (Bethel and Holmes 1973, 1974;

Helluy 1983, 1984; Bakker and others, 1997; Maynard and others, 1998; Bauer and others, 2005; Benesh and others, 2005).

Behavioral modifications have been shown to increase trophic transmission to definitive hosts (Bethel and Holmes, 1977; Bakker and others, 1997) and can result in spatial segregation of infected and uninfected individuals (MacNeil and others, 2003; Wellnitz and others, 2003; Ponton and others, 2005; Miura and others, 2006). Spatial segregation of hosts can result in altered habitat use and/or patterns in diet and these differences can be detected through stable-isotope analyses (Miura and others, 2006). In Montezuma Well, *H. montezuma* uses both pelagic and littoral habitats, and we hypothesized that amphipods would show different isotopic compositions between the habitats. Furthermore, if either parasite altered the behavior of *H. montezuma* that resulted in altered habitat selection, we expected that these differences would be reflected by altered isotopic signatures.

In this study we report the sex ratios and reproductive effort of infected and uninfected amphipods. To test our hypotheses about parasite manipulation of behavior, we measured the swimming activity and behavioral response to light and gravity of infected versus uninfected amphipods. To determine if the trematode parasite altered habitat selection of infected amphipods, the stable C and N isotope ratios in infected and uninfected amphipods from different habitats (pelagic versus littoral) were measured at Montezuma Well. Finally, to measure the effects of parasitism on host and non-host predators, we measured rates of predation of infected and uninfected amphipods by three invertebrate predators, as well as by their waterfowl host predator.

Methods

Patterns of Infection

We measured the sex ratios and brood size of females of 490 amphipods collected using a sweep net in the littoral zone of Montezuma Well in April 2007. Infected amphipods were visually identified and removed from sweep samples along with a representative sample of randomly selected uninfected amphipods. Trematode-infected amphipods were identified by their bright orange color and acanthocephalan-infected individuals were identified by the bright orange cystacanth that was visible through the exoskeleton (fig. 1-2). We were unable to visually identify amphipods that were co-infected with both parasites in the field. Amphipods were sorted into different containers, preserved in 70-percent ETOH (ethanol), and transported to the laboratory, where they were sexed and measured under a dissecting microscope. All amphipods included in the analysis were infected with only one parasite; co-infected amphipods were infected with only one of each parasite.

Figure 1-2. Images of orange trematode-infected (left, top) and uninfected *H. montezuma* (left, bottom), highlighting the color difference in infected amphipods as compared to uninfected conspecifics. A *C. constrictum*-infected amphipod is shown at right; the orange cystacanth is visible through the amphipod's exoskeleton.

Behavior

In order to assess the behavior of infected and uninfected *H. montezuma,* we captured amphipods using a sweep net in the littoral vegetation of Montezuma Well in March 2007. Infected amphipods were visually identified and removed from sweep samples as above, but were kept alive in Well water for transport to the laboratory and measurement of behavior. In the lab, amphipods were housed in Well water that was bubbled with dilute carbon dioxide gas in order to maintain ambient pH, alkalinity, and dissolved oxygen and carbon dioxide of the Well's littoral zone. A light-dark cycle of 12:12 hours was used to mimic the natural photoperiod at the time of capture. All behavioral measurements were conducted within 48 hours of amphipod capture.

Activity levels of infected and uninfected amphipods were assessed by conducting swimming trials in small plastic containers and counting the number of times each individual amphipod crossed the center of the container (Maynard and others, 1998; MacNeil and others, 2003; Benesh and others, 2005). Trial containers consisted of 250-mL Nalgene® water bottles, cut to 5-cm height, with a faint black line drawn in permanent marker across the center of the bottom of the container. Containers were filled with 2 cm of water from the amphipod holding tank, and one randomly selected amphipod was placed in each trial chamber. Amphipods were allowed to acclimate to their new environment for two minutes, then their activity levels were assessed by counting the number of times each animal crossed the line on the bottom of the trial container in a three-minute period. All trials were conducted in the same environment, with artificial light provided by overhead fluorescent lighting; line crossings were recorded on a manual counter.

To measure the depth preference of infected and uninfected amphipods, we filled translucent plastic cylinders (diameter = 7.8 mm, height = 50 cm, with oxygenated, carbonated Montezuma Well water (Bauer and others, 2005). The columns were marked in 5-cm increments from the bottom, and water was replaced with freshly carbonated and oxygenated water as above. An animal was randomly

selected from a common tank, placed in the top of the column, and allowed to acclimate for two minutes, after which time its height above the bottom was recorded every 30 seconds for 5 minutes.

Finally, we also measured the phototaxis of infected and uninfected amphipods using small chambers with dark and light areas. We constructed small plastic containers out of capped, round white plastic PVC pipe. Chambers were 5 cm long, with a diameter of 5 cm; they held 150 mL of water. Half of each chamber was removed by cutting away the top of the plastic tube and cap, and a circular black plastic flap separated the light and dark halves of the pipe, except for a small semicircular slit at the bottom of the chamber. This slit measured 1.5 cm at its deepest point, and was the only place for amphipods to move between the light and dark portions of the chamber. The inside of the dark side of the chamber was painted black, and the open half of the chamber was white. At the beginning of each trial animals were placed directly beneath the semicular slit and allowed to acclimate for two minutes, after which time the location of the amphipod was recorded every 30 seconds for 5 minutes.

After each behavioral trial was concluded, all amphipods were examined under a dissecting scope to identify the number and type of parasite cysts in their body cavities. In addition, amphipods were assigned to 1-mm size classes on the basis of length (base of first antennae to telson) using an ocular micrometer, and they were sexed, using the presence of enlarged posterior gnathopods to indicate a male. All amphipods included in the analysis had only one parasite; co-infected animals had only one of each.

Stable Isotopes

Because we found behavioral differences between uninfected and trematode-infected amphipods, we measured the isotopic composition of carbon and nitrogen in amphipods from different habitats at Montezuma Well. We collected amphipods from both the littoral and pelagic zones at the Well in July 2007, using similar methods as above. Trematode-infected and uninfected amphipods were collected and transported alive to the lab, where they were sexed, measured, dried in a drying oven, and ground manually in a mortar and pestle. To avoid contamination by parasite, tissues were moved and we discarded parasite cysts from the samples before drying.

Stable isotope analyses were conducted by the University of Arizona Environmental Isotope Laboratory. To conduct the analysis, a continuous-flow, gas-ratio mass spectrometer (Finnigan Delta PlusXL) coupled to an elemental analyzer (Costech) was used to quantify stable isotope ratios (δ) of ^{15}N and ^{13}C. Samples were combusted in the elemental analyzer, and standardization was based on acetanilide for elemental concentration, NBS-22 and USGS-24 for $\delta^{13}C$, and IAEA-N-1 and IAEA-N-2 for $\delta^{15}N$. Instrument standard deviation was better than ± 0.09‰ for $\delta^{13}C$ and ± 0.2‰ for $\delta^{15}N$, based on repeated internal standards. For both carbon and nitrogen, stable isotope values are expressed relative to the standard as $\delta‰ = [(Rsample/Rstandard)-1] \times 1{,}000$, where R is the isotopic:nonisotopic ratio.

Non-Host Predation

To examine how altered color and behavior of amphipods might affect predation rates by non-host invertebrate predators, we conducted in situ predation experiments in small Plexiglas cylinders, (Runck and Blinn, 1994) between infected and uninfected amphipods and three predators: the diving bug *Belastoma bakeri,* the endemic waterscorpion *Ranatra montezuma,* and the larvae of the diving beetle *Cybister ellipticus*. Chambers 7.5 cm in diameter and 14 cm long were capped at either end with nylon mesh (100 μm) to confine experimental animals but allow the exchange of dissolved and atmospheric gasses. Twelve invertebrates were captured from the littoral vegetation and placed in 12 individual chambers along with 10 g (wet weight) of the aquatic macrophyte *Potomogeton illinoiensis*. Animals were held for 24 hours, after which time five pairs of parasitized and unparasitized amphipods

were added to each chamber. The chambers were suspended on end in 1-m-deep water in the pelagic region of the Well, together in a plastic crate, and positioned so that the top 5 cm of the chamber extended into the atmosphere to allow animals atmospheric air for respiration. Chambers were incubated for three hours on sunny days between 1000 and 1500 hours. At the end of the experiment the number of amphipods consumed in each chamber was recorded.

Host Predation

We conducted predation trials with domestic captive mallards (*Anas platyrhynchous*) to determine if predation rates were greater on infected amphipods as compared to uninfected conspecifics. Mallards were obtained as adults and habituated to the experimental pool for two hours in the week preceding the trials. Trials were conducted in a 378.5-L tank with an oval top (dimensions 0.65 × 1.21 × 0.6 m deep), filled with 290 L of filtered Montezuma Well water. A large piece of nylon mesh (0.5-mm mesh size) was used to line the tank and aid in later recovery of the amphipods. Water was added to the tank and bubbled with a dilute mixture of CO_2 gas in air to provide dissolved CO_2 and O_2 concentrations similar to those of Montezuma Well. Large whole pieces of *P. illenoiensis,* including the roots, were added to the tank (500 g wet weight), attached by the base of the stalk to the bottom of the tank with bricks. Gas concentrations were allowed to equilibrate for 1 hour, after which time 50 each of uninfected, trematode-infected, and acanthocephalan-infected amphipods were added to the tank and allowed to acclimate for 1 hour. Because ducks were uncomfortable foraging alone, we introduced them in pairs and allowed them to forage for 30 minutes. During the predation trials we observed them dabbling and tipping, much as mallards do in the wild. Tanks were located outside in direct sunlight, and the trials were conducted between 1100 and 1300 hours.

After the foraging period, ducks were removed and rinsed in a smaller pool of water to remove any amphipods clinging to their plumage. The mesh lining of the tank was pulled to near the surface of the tank and searched for amphipods that were clinging to vegetation or swimming about. Large pieces of vegetation were moved to the pool of clean water and shaken vigorously to remove any attached amphipods.

To ensure that amphipod counts were a function of duck predation and not of our ability to find them in the vegetation, we conducted two share predation trials. Tanks were treated as above, except that instead of adding ducks we beat and stirred the surface of the water with a stick for 3 minutes at 5-minute intervals six times. After completion of each share trial, amphipods were collected as above; in both share trials we recovered the full (N = 150) complement of amphipods that were added initially to the tank.

Data Analyses

We used the binomial test and Fisher's exact test to examine differences in sex ratios between infected and uninfected amphipods and multiple regression models to explore the relationship between explanatory variables and behavioral response variables (swimming activity and reaction to light and gravity). We first fit linear models that contained all covariates and two-way interactions, and used likelihood ratio tests to test the explanatory power of individual parameters, removing those with little explanatory power ($P>0.15$), until a minimal covariate-adjusted model was developed. Covariates included sex, length, and gravid (for females). Then a richer model was fit with dummy variables to test for the relationship between infection condition (acanthocephalan, trematodes, or both) and the behavioral response under question, testing for significance with a likelihood ratio test. Models were parameterized so that the reference level for infection condition was always uninfected. Preliminary

analyses suggested differences in males and females; therefore we constructed separate models for amphipods of each sex.

Amphipod responses to light and gravity were measured repeatedly in a longitudinal design over time; therefore, a different modeling approach was warranted. To analyze these data we implemented generalized estimating equations (GEEs) that allowed us to account for the lack of independence between measurements in the experimental design. GEE methods are an extension of generalized linear models that allow the modeling of correlated measurements from observational longitudinal studies (Ballinger, 2004). For measuring response to light, we specified a GEE with a logit link, binomial errors, and a first-order autoregressive correlation structure. For measuring response to gravity, the GEE models had an identity link, normal errors, and a first-order autoregressive correlation structure.

We used ANOVA (Analysis of Varience) to test for differences in $\delta^{13}C$ and $\delta^{15}N$ between habitats and infection conditions, with a separate ANOVA conducted for each element. Explanatory variables included sex, habitat, and infection condition with the trematode parasite. Model selection was conducted as described above.

To analyze invertebrate predation data we used logistic regression to model the odds of predation based on infection status, constructing separate models for each predator. Since two predation trials were conducted per predator, we blocked by trial, as well as by individual chamber. We parameterized the model so that "uninfected" was the reference level, and modeled the odds of predation of trematode and acanthocephalan-infected amphipods as compared to uninfected amphipods. Because logistic regression models exhibited overdispersion, we specified a quasi-likelihood model that allowed us to correct the standard errors of the parameter estimates that are underestimated in a standard binomial model (Ramsey and Shafer, 2002, chap. 21).

To test the hypothesis that capture rates by waterfowl hosts were dependent upon infection condition, we again implemented logistic regression models. We used a blocking variable to control for the confounding effects of individual trials and, as before, we specified a quasi-likelihood model to compensate for overdispersion in the model.

All statistical analyses were conducted using the R package for statistical computing (R Development Core Team, 2006). To implement GEE models, we used the "geeglm" function from the "geepack" library (Yan, 2002; Yan and Fine, 2004).

Results

Patterns of Infection

We found that the sex ratios of *H. montezuma* differed between infected and uninfected amphipods (fig. 1-3). There was little evidence that the sex ratio of uninfected *H. montezuma* differed from 50:50 ($P = 1.0$ from a binomial test), but samples of acanthocephalan ($P<0.001$ from a binomial test) and co-infected amphipods ($P<0.001$) were strongly male biased. There was weak evidence of a difference in sex ratio between uninfected and trematode-infected amphipods ($P = 0.065$, from Fisher's exact test).

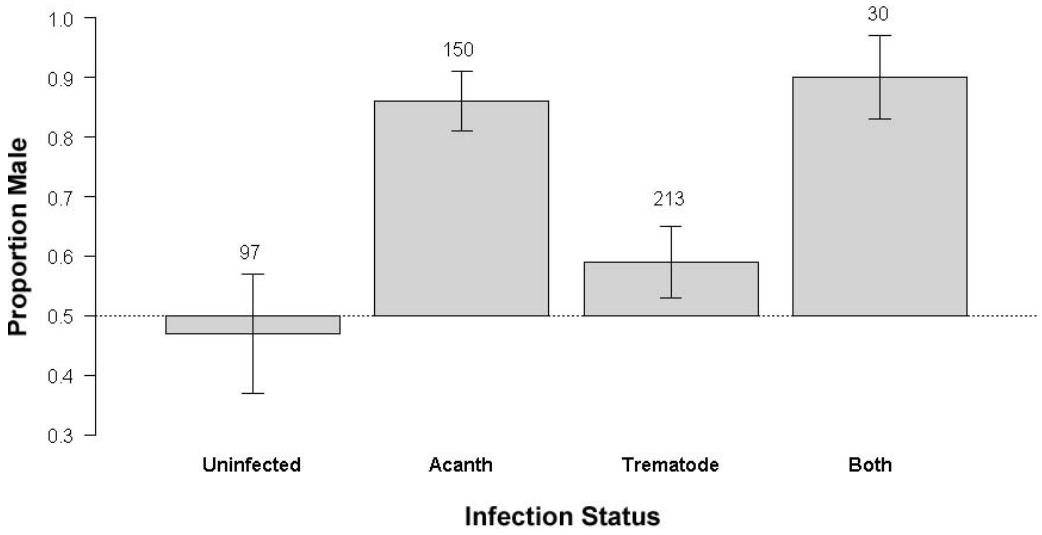

Figure 1-3. Sex ratios of infected and uninfected amphipods from a sample of 490 *Hyalella montezuma*. Error bars are 95-percent confidence intervals, and the dotted line indicates the expected value if the sex ratio is 50:50. Numbers above error bars indicate sample sizes.

We also found variation in the proportion of females that were carrying broods between infected and uninfected amphipods (fig. 1-4). Most of the uninfected females in our sample were gravid (84 percent); however very few acanthocephalan-infected and trematode-infected females were carrying brood (8 percent and 19 percent, respectively). Also, we found that the median brood size of uninfected gravid females was 2.3 times greater (95-percent confidence interval from 1.2 to 2.4) than the median number of eggs of trematode-infected gravid females ($P<0.001$, d.f. = 58, t = 3.754 from a t-test on log-transformed egg number).

Figure 1-4. Proportion of females carrying brood, from the same sample as displayed in figure 1-2. Error bars are 95-percent confidence intervals. Numbers above error bars indicate sample sizes.

Amphipod Behavior

Swimming trials revealed variation in activity between amphipods of different infection condition and sex (fig. 1-5). For males we found weak evidence for significant variation in the swimming behavior of trematode-infected versus uninfected amphipods (table 1-1). Males co-infected with both parasites showed the strongest response, and we estimate that after accounting for the variation in swimming behavior due to size, the activity level of co-infected males was 83 percent that of uninfected male amphipods (95-percent confidence interval from 66 percent to 99 percent).

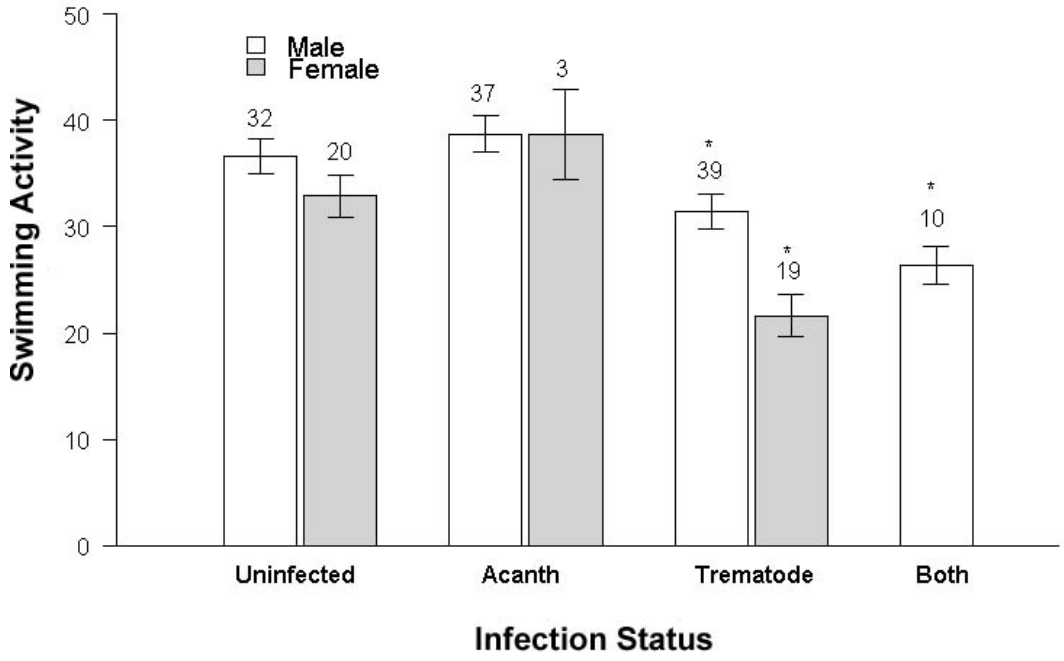

Figure 1-5. Swimming activity (number of line crosses) of amphipods by sex and infection condition. Asterisks indicate significance (P<0.05) from uninfected of same sex. Error bars represent ±1 standard error.; numbers above bars indicate sample sizes. Only trematode-infected and co-infected individuals showed altered swimming activity from uninfected conspecifics.

Although the variation in swimming behavior between infected and uninfected male amphipods was rather weak, we found a different pattern for female amphipods, suggesting that these parasites have pathogenic effects particularly on female hosts. For females, factors that covaried with swimming behavior included infection status as well as the number of eggs carried by a female (table 1-1; chapter 1 tables are found in appendix A). After accounting for variation due to other factors, we estimate that the activity of female trematode-infected amphipods is 56 percent that of uninfected females (95-percent confidence interval from 32 percent to 81 percent).

Overall, amphipods chose the lower portion of the experimental chambers (fig. 1-6), and we found no evidence that covariates explained variation in depth preference among amphipods; the best covariate-adjusted model included was an intercept-only model. There was no evidence of a difference in depth preference between uninfected and infected amphipods, ($\chi^2 = 2.12$, d.f. = 3, P = 0.54663, from a likelihood ratio test).

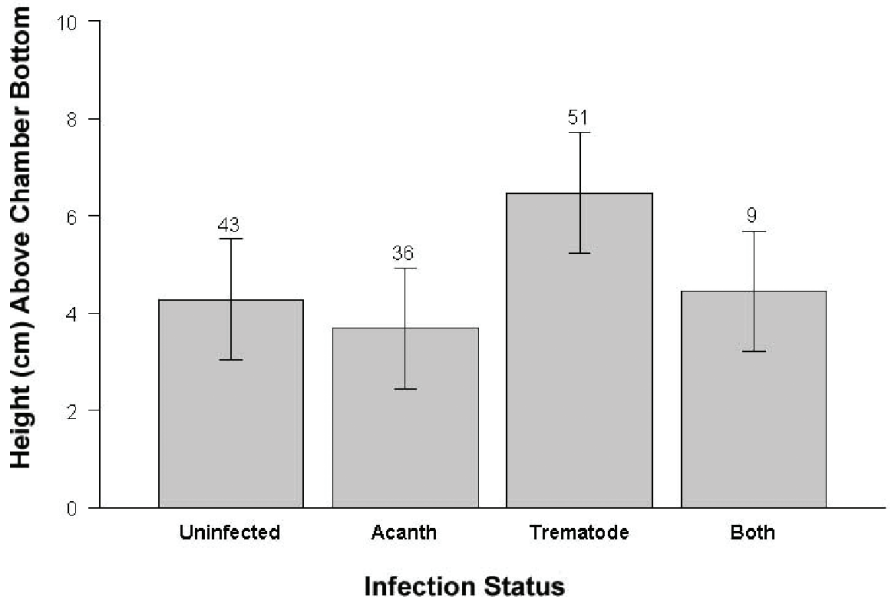

Figure 1-6. Mean height of amphipods above bottom in a 50-cm experimental chamber. Error bars represent 95-percent C.I.; numbers above bars indicate sample sizes. There was no difference in mean swimming height for amphipods of different infection status.

We also found little evidence that uninfected *H. montezuma* displayed phototaxis (fig. 1-7). As with depth preference, little variation in light preference was explained by covariates, and the best covariate-adjusted model included only an intercept term. Likewise, there was no evidence that infection condition explained variation in response to light ($\chi^2 = 3.67$, d.f. = 3, P = 0.299).

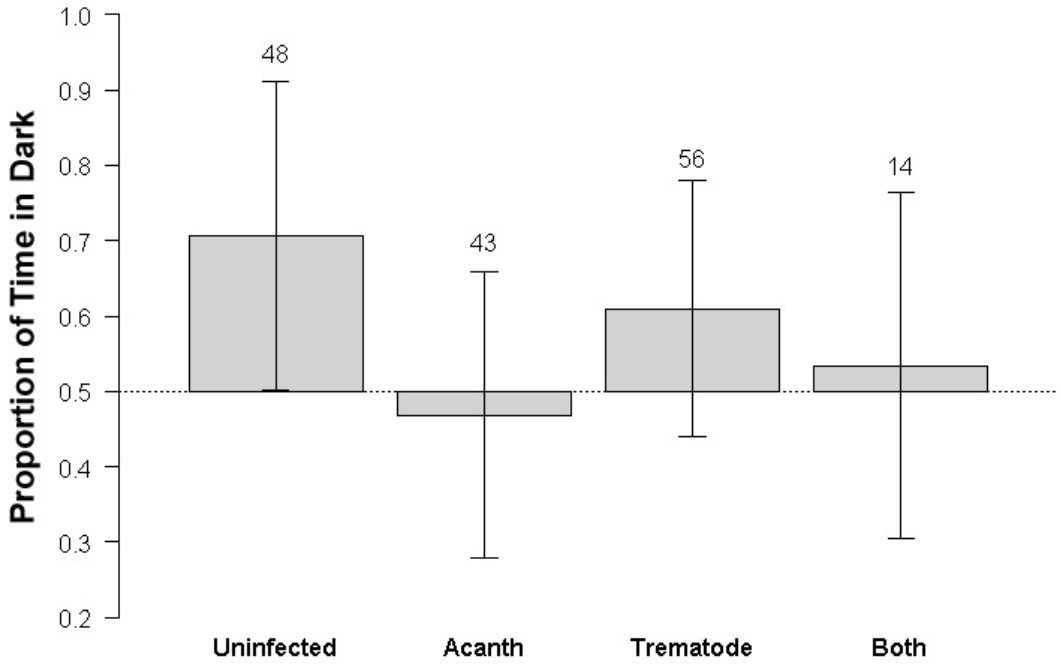

Figure 1-7. Mean proportion of times amphipods were observed in dark side of chamber in experimental trials. The line at 0.5 indicates expected value for no preference. Error bars represent ± 95-percent C.I., and numbers indicate sample sizes.

Stable Isotopes

We found no variation in stable isotope composition of trematode-infected and uninfected *H. montezuma* for both $\delta^{13}C$ and $\delta^{15}N$, but there were differences for both isotopes by habitat (table 1-2). The average stable nitrogen ratios for amphipods in the littoral and pelagic zones (mean ± S.E.) were 6.44 ± 0.056‰, and 6.76 ± 0.104‰, respectively, and values for carbon were -27.68 ± 0.118‰ and -28.20 ± 0.165‰, respectively. Although these differences were significant, the magnitude of the differences was relatively small.

Non-Host and Host Predation

Our predation trials revealed considerable variation in capture rates among the three invertebrate predators, and for *Belastoma*, capture rates differed more than two-fold between trials (fig. 1-8). However, we found no difference in capture rates between infected and uninfected amphipods for all three invertebrate non-host predators (table 1-3).

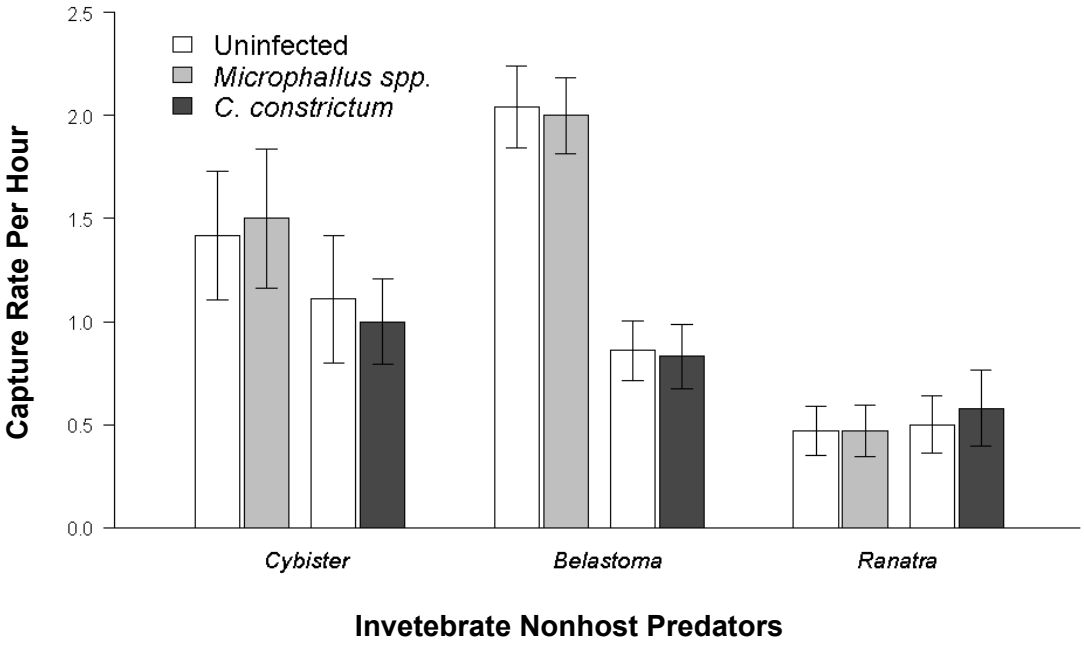

Figure 1-8. Capture rates of nonhost invertebrate predators on uninfected and infected amphipods. Trials were conducted for both *Microphallus*- and *Corynosoma constrictum*-infected amphipods. Uninfected amphipods were used as a control for both sets of trials. Error bars represent ± 1 S.E.

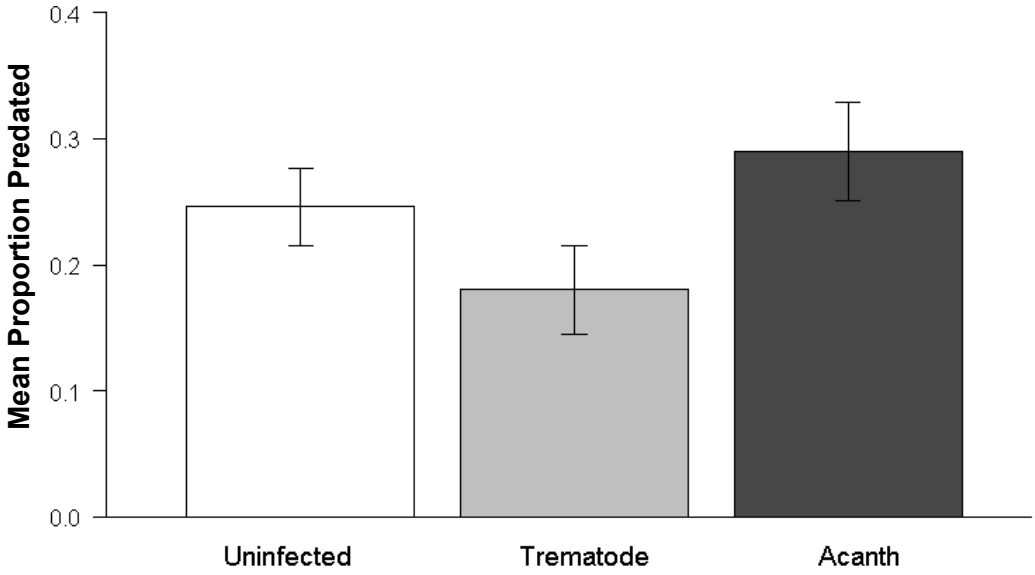

Figure 1-9. Proportion of uninfected, trematode-infected, and acanthocephalan-infected amphipods captured by waterfowl, averaged over three predation trials. Error bars represent ± 1 S.E.

Waterfowl pairs captured between 18 and 44 percent of amphipods in the three trials. Trematode-infected amphipods were captured in a lower proportion than uninfected ones, and acanthocephalan-infected amphipods were captured in slightly higher proportions than uninfected amphipods (fig. 1-9), but these differences were not statistically significant (table 1-4).

Discussion

We found that acanthocephalan-infected amphipods were strongly sex-biased as compared to uninfected amphipods, suggesting that female *H. montezuma* are either less likely to be infected with *C. constrictum* or that the parasite is so virulent that most infected females die in response to infection. Moreover, individual females that were infected with both the trematode and acanthocephalan parasite simultaneously were far less likely to carry offspring than uninfected females, and the brood size of trematode-infected amphipods was significantly less than uninfected ones, suggesting that either *C. constrictum* prevents reproduction or is particularly virulent to gravid females. Other studies have reported different patterns of altered sex ratios of infected crustacea: either male-biased (Thomas and others, 1995; Rauque and Semenas, 2007), female-biased (Gleason, 1987), or no bias in infection with respect to sex (Seidenberg, 1973).

A recent study of the virulence of *C. constrictum* on *Hyalella azteca*, a species closely related to *H. montezuma*, revealed that amphipod survival was not different between uninfected control animals and experimentally infected amphipods with low parasite burdens (mean intensity = 1.9). (Duclos and others, 2006). Animals with higher parasite burdens (mean intensity = 6.2) exhibited increased mortality, and there was a weak effect of sex upon the survival of amphipods, with females exhibiting slightly higher rates of survivorship over time (Duclos and others, 2006). In a study of a microphallid trematode in an isopod host, Hansen and Poulin (2006) report no difference in survival between infected

and uninfected hosts. Although the virulence of parasites might vary between systems, the findings of others suggest either that the patterns we observed are not driven by differential survivorship of males and females or that different mechanisms apply at Montezuma Well. Another possibility is that males are more susceptible to infection because of behavioral or physiological differences. For example, male copepods have been found to be more susceptible to experimental infection (Wedekind and Jakobsen, 1998). Studies on experimentally infected *H. montezuma* are needed to reveal the processes that underlie the patterns we report here.

Our findings, contrary to other studies in the literature (Bethel and Holmes, 1973, 1974; Benesh and others, 2005), indicate that *C. constrictum* does not alter the behavior of its amphipod host at Montezuma Well, which is surprising given the fact that alteration of host behavior is generally considered to be an ancestral trait of acanthocephalans (Moore, 1984). We did find that trematode-infected *H. montezuma* showed reduced activity levels, but no differences were observed in response to light or gravity. Other studies of *Microphallus* have shown that species within this genus can affect their hosts in multiple ways (Helluy 1983, 1984) when the cysts of these parasites are located in the head region of their hosts. However, Hansen and Poulin (2006) found that an undescribed abdominal *Microphallus* increased the activity of its amphipod host, which is opposite of the pattern that we observed.

Because we conducted measurements on naturally infected amphipods, we cannot exclude the possibility that amphipods with reduced activity were more likely to be infected by trematode cercariae, although the fact that we observed differences between males and females argues against this possibility. Furthermore, the fact that there were sex differences in activity levels of infected amphipods, while there were no differences in other behaviors, suggests that, if this reduction in behavior is induced by the parasite, it is likely due to a pathogenic effect rather than an adaptive change by the parasite in order to increase trophic transmission to waterfowl-definitive hosts. Our predation trials support these findings. Experimental infections with *Microphallus* spp. at Montezuma Well are needed to better understand the evolutionary significance of reduced swimming behavior seen in infected *H. montezuma*.

Despite the fact that we found trematode-infected amphipods to have reduced swimming behavior, we found no evidence that this resulted in altered diets, which suggests that infected amphipods do not alter their habitat selection. Trematode prevalence did not differ between habitats, even though waterfowl host density is overwhelmingly greater in the littoral vegetation and snail-first intermediate hosts are only found in littoral vegetation, suggesting that daily amphipod migration serves to equalize infection rates between habitats, even though we assume that transmission would be more likely to occur in the littoral vegetation. This observation further supports our conclusion that trematode-parasitized amphipods are not behaviorally modified by parasites, and that reduced swimming seen in females is a pathogenic effect of infection.

Finally, we failed to find any evidence that infected amphipods were more likely to be predated by either non-host predators or waterfowl host predators, further supporting our conclusion that the behavioral effect of trematodes is a side-effect of infection and not a parasite-induced manipulation. Further tests are needed to understand the effect this parasite has on a range of amphipod hosts it encounters in natural systems.

It is unclear why our study revealed host responses different from those previously reported in the literature, and our findings underscore the importance of experimental infection in the study of these types of interaction. These findings highlight the need for phylogenetic studies that compare the response of related hosts to the same and related parasite taxa.

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Chapter 2

The Influence of Human Visitor Activity on Spatial Patterns of Parasite Infection

Introduction

Although evidence is accumulating that human recreation can directly affect wildlife species in important ways, little is known about indirect cascading effects that recreation can have in ecological communities. For example, parasites with complex life cycles span animal communities, moving from vertebrates to invertebrates and back in a single generation, and the potential exists for impacts of human recreation on wildlife to cascade through animal communities via their parasites.

In this chapter we link human recreation to patterns of disease, by testing the hypothesis that human recreation in a National Monument affects spatial patterns of waterfowl habitat use, which in turn affects the prevalence of a trematode parasite in an invertebrate host. We found that waterfowl chose to forage in areas more distant from visitor-use paths at Montezuma Well (a desert spring pond), and these areas supported greater rates of parasite infection in the initial year of our study. In the second year, when visitor numbers were lower, waterfowl did not show as strong a pattern of differential space use and disease prevalence across the surface of Montezuma Well did not show a spatial variation. Furthermore, an observational study and a randomized experiment supported our hypothesis that human recreation directly affects the foraging location of waterfowl. These findings are among the first to demonstrate that human recreation can indirectly affect spatial patterns of wildlife disease, and our results have important implications for the management of areas that are maintained both for preservation and for human leisure activities, especially in systems where disease is ecologically important.

It has been demonstrated that human recreation directly affects wildlife in complex and profound ways (reviewed in Boyle and Sampson, 1985; Knight and Gutzwiller, 1995; Blanc and others, 2006). However, there is little information on how patterns of human recreation can indirectly affect animal communities by cascading from directly affected wildlife to other community members (Gutzwiller, 1995; Cole and Landres, 1995). Human recreation has the potential to indirectly affect patterns of disease in invertebrate hosts (Cort and others, 1960); however, no studies have yet documented such cascading effects.

In this study we used a parasite-host relationship in Montezuma Well, an aquatic ecosystem contained within a detached portion of Montezuma Castle National Monument, as a model system to investigate the hypothesis that human recreation could alter spatial patterns of disease in invertebrates indirectly, through direct alterations to the foraging location of vertebrate hosts. We measured spatial patterns of infection of the endemic amphipod *Hyalella montezuma* with the digenic trematode *Microphallus* spp. This trematode passes from a waterfowl to a snail, then on to an amphipod host. The life cycle is completed when the amphipod is consumed by the waterfowl host and the parasite can again become reproductive in its vertebrate definitive host.

We measured the prevalence of infection in different areas of Montezuma Well, as well as spatial patterns of waterfowl use, every 2 weeks over a 2-year period. Additionally, we conducted a

randomized experiment by presenting waterfowl with a controlled disturbance and recording the response of foraging waterfowl. Finally, we performed an observational longitudinal study to quantify the direct effect of actual visitors on patterns of waterfowl distribution at Montezuma Well.

We found that the prevalence of disease in amphipods varied spatially across Montezuma Well. Although the strength of this pattern was greater in year one of our study, in both years areas of elevated disease prevalence in amphipods corresponded to areas with greater waterfowl abundances that were, in turn, far from paths used by recreating visitors. The year when the pattern of differential infection was the strongest was the same year in which waterfowl abundance favored the far side of the Well, especially early in the season. Furthermore, during that same time period the number of visitors was greatest, suggesting that human visitors, by affecting the space use of waterfowl, indirectly affected patterns of wildlife disease in areas furthest from visitor paths.

Finally, results of a randomized experiment showed that waterfowl responded directly to the presence of human disturbance. We found that waterfowl density on the side of the Well far from the experimental disturbance increased by almost 40 times over that of the foraging population before the disturbance, demonstrating a strong experimental effect. In an associated observational study, we also documented a positive, albeit small effect of visitor number on patterns of waterfowl use. While the correlation of average visitor numbers on waterfowl spatial distribution was relatively weak, we did observe strong negative responses of waterfowl to individual visitors, especially those who arrived first in the morning or those accompanied by boisterous children or loud dogs.

Across the continent, and indeed the world, the numbers of humans recreating in protected areas is growing; as the numbers of recreators increase, the indirect impacts of those visitors on those natural ecosystems will likely increase as well. Human recreation will continue to have a growing impact in protected areas, which leads to the likelihood that indirect impacts by visitors, such as we found in our study, will continue to increase. Our results indicate that indirect effects do occur, and further studies are needed to document these effects, especially in systems where parasites play keystone roles. More studies of this type will allow us to better understand how human recreation can affect wildlife, the communities they are a part of, and the ecosystems they inhabit. They will allow us to better balance the needs of humans and wildlife in our protected areas.

There is little information on how human recreation patterns can indirectly affect trophic levels of animal communities by the cascading of impacts from wildlife species directly effected to other species throughout the community that are indirectly effected (Gutzwiller, 1995; Cole and Landres, 1995). Human recreation has the potential to indirectly impact patterns of disease in invertebrate hosts (suggested by Cort and others, 1960), because parasites can traverse animal communities, passing back and forth from vertebrate to invertebrate hosts. Therefore, the relationships between parasites with complex life cycles and their hosts provide model systems in which to study indirect effects of human recreation.

Digenic trematodes have complex life cycles and must pass through at least one invertebrate alternate host in addition to their vertebrate alternate host. In the following pages we report the findings from the study of a trematode that passes from an avian host to snails, and then to amphipods, before returning to the definitive host to complete the life cycle (fig. 2-1). The fact that this host traverses a simple food-web during the course of one generation in a federally-protected aquatic ecosystem makes it a prime subject for the study of cascading indirect effects of human recreation.

Mounting evidence suggests that avian definitive-host distribution can affect spatial patterns of infection in alternative hosts (Hetchinger and Lafferty, 2005; Fredensborg and others, 2006), and studies have correlated human activity with disease prevalence (Bustnes and Galaktionov, 1999). Also, scientific literature substantiates that human recreation can affect the spatial location of foraging

waterbirds (Madsen, 1995; Mori and others, 2001). However, we are aware of no studies that have linked human recreation to spatial patterns of parasitism in invertebrate hosts.

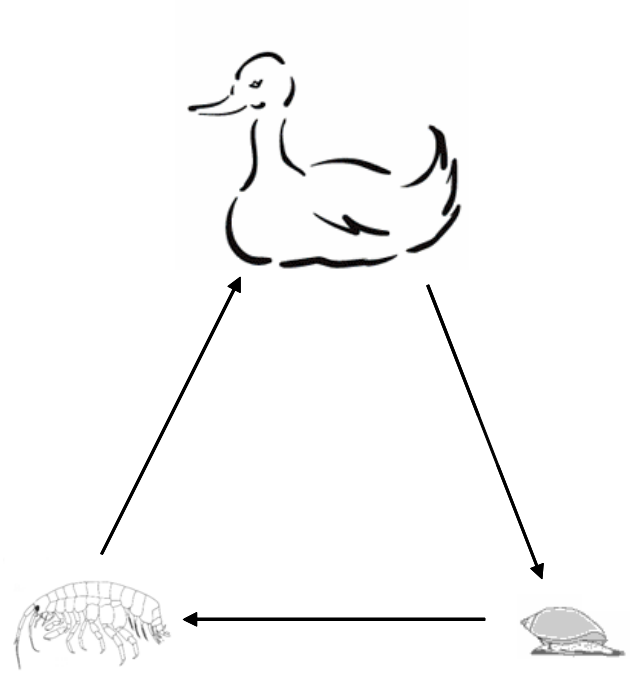

Figure 2-1. Life cycle of the *Microphallus* trematode parasite investigated in this study. Represented: waterfowl, top; snail, lower right; amphipod, lower left. The parasite is reproductive in the waterfowl host. In its first intermediate host (snail) the tremadote is present in sporosyst, rediae and cercariae forms. Free-swimming cercariae then leave snail tissue and penetrate the second intermediate host (amphipods) and encyst as a resting form, the metacercariae. The parasite passes to the definitive host and develops into a sexually producing adult when the definitive host (waterfowl) consumes an infected amphipod.

Parasites are known to play important roles in ecosystems (Thomas and others, 1999; Marcogliese, 2004); therefore, it follows that impacts to parasite-host dynamics could potentially have significant effects at the community level. Because disease transmission increases with increased host density (Bustnes and others, 2000), altered spatial patterns of disease in alternate hosts could increase disease prevalence in wildlife species that serve as definitive hosts. Therefore, identifying links between human recreation and spatial patterns of disease could have significant conservation implications. Gaining information on the indirect impacts of human recreation on spatial patterns of disease will also be important for those concerned with understanding and mitigating the effects of human recreation in otherwise natural ecosystems, particularly in national parks and other protected areas, where impacts from recreation may constitute the most significant component of human disturbance.

In this study we test the hypothesis that human recreation, by altering the foraging location of an avian definitive host, can affect spatial variation in the prevalence of infection in an amphipod that serves as a second alternate host of a trematode parasite. To test this hypothesis we monitored levels of human visitation, measured the abundance and location of foraging waterfowl relative to areas of human recreation, conducted a randomized experiment to directly assess the role of human presence on foraging location in waterfowl, observed visitors and the response of waterfowl to them, and monitored spatial and temporal patterns of infection in an amphipod second alternate host. To our knowledge, this is the first study that suggests human recreation can alter spatial patterns of disease in a natural ecosystem.

Methods

Study System

We investigated the effects of human recreation on patterns of parasitic infection in Montezuma Well, a spring-pond system in central Arizona. Montezuma Well, a detached portion of Montezuma Castle National Monument, is administered by the National Park Service (NPS) and as such is managed primarily for recreation (no hunting is allowed) and preservation of the natural and cultural resources. The Well itself is a limnocrene (surface area = 0.55 ha) with extremely elevated dissolved carbon dioxide and alkalinity (both >500 mg/L); it is unstratified throughout the year, with relatively constant water temperature (range: 18.0°C–24.8°C) and pH (range: 6.3-6.9; Boucher and others, 1984). The unusual water chemistry excludes fish (Cole and Barry, 1973) and has structured a unique aquatic invertebrate community that is noted for its high rate of endemism (four described endemic species) and for the apparent lack of taxonomic groups found in neighboring waters, such as Trichoptera, Lepidoptera, Megaloptera, Neuroptera, and Anisoptera, and the general rarity of Chironimidae and Ephemeroptera (Blinn and Sanderson, 1989). In this system, a *Microphallus* trematode parasite infects a waterfowl definitive host, a gastropod first intermediate host, and a second intermediate host, the endemic amphipod *Hyalella montezuma*. Waterfowl definitive hosts are only present at the Well in large numbers during the winter months.

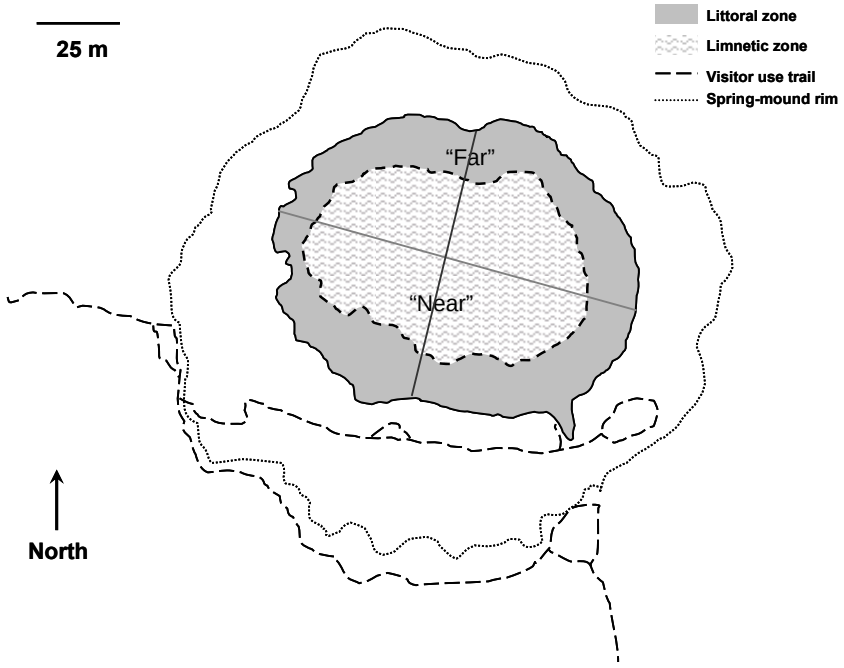

Figure 2-2. Line drawing of Montezuma Well, delimiting different habitat types within the aquatic ecosystem, as well as visitor-use trails and sampling units used in this study. "Near" and "Far" sides of the Well represent halves of the aquatic habitat that are near and far, respectively, from the visitor paths.

For the purpose of this study, we divided the surface of Montezuma Well into four quadrants and oriented them such that two of them encompassed the side of the Well far from visitor paths ("far side," fig. 2-2), while the other two encompassed the side near visitor paths ("near side"). Each quadrant contained both pelagic and littoral habitat, resulting in our observational units having eight habitat/quadrant designations.

Patterns of Human Recreation and Waterfowl Foraging

To measure the levels and patterns of human recreation, we used daily visitor data collected by the National Park Service for Montezuma Well, data estimated with a traffic counter that counted the number of cars. The counter was reset monthly, and the numbers of visitors arriving by tour bus were recorded separately by Park staff. We used the number of cars entering the park each day to estimate visitor number, multiplying each car by 2.8 to estimate the total number of passengers (Butch Street, written commun., 2008, and added to this the number of daily bus passengers to estimate the total number of individuals visiting on a daily basis during our study period.

To quantify patterns of waterfowl foraging, we recorded the number of foraging waterfowl within each of the eight observational units (fig. 2-2). We collected data every 2-7 days between 08:00 and 11:00, over two 9-month periods between September 1 and May 6 of 2003-4 and 2004-5.

During the spring of 2005 we conducted a randomized experiment to measure the response of waterfowl (mainly ducks) to human presence. On eight different mornings, before visitors arrived at the Well (07:30-08:00), one observer traversed the rim three times on either the near or far side (randomly chosen), in view of foraging waterfowl, recording waterfowl locations before and after the experimental disturbance. On the near side of the Well the observer walked along the visitor path; while traversing the opposite side he used the rim. The paths taken on both sides of the Well are similarly situated relative to the water and to the ducks foraging therein. We computed a synthetic response variable for analysis derived from the difference in waterfowl number on far versus near sides relative to the observer both before and after experimental disturbance.

To further explore the effects of visitors and waterfowl at Montezuma Well, we conducted an observational study in 2007. We observed visitors and waterfowl locations for 3 hours each morning from November 10 to 12. We recorded the maximum number of visitors present on visitor paths in view of waterfowl for 5-minute periods from 08:00 to 11:00 each morning. At the end of each 5-minute period we enumerated the total number of waterfowl present on far and near sides of the Well. As in the experimental study, our response variable was the difference in waterfowl numbers between sides of the Well (far-near) relative to the visitor paths (fig. 2-1).

Patterns of Infection

To quantify spatial and temporal patterns of infection, we sampled the endemic amphipod *H. montezuma* over the same 2-year period that we monitored waterfowl spatial patterns, collecting amphipods every weeks by lowering a plankton net (mesh size = 250 μm, opening 30 cm) to the benthic sediments and retrieving it rapidly. Two samples were randomly taken in each observational unit for a total of 16 samples per monitoring period, and we recorded the total water depth for each sample. *H. montezuma* undergoes a daily migration from the limnetic to littoral zone (Blinn and Davies, 1990). To prevent changes in amphipod density over the course of the day from affecting our results, all plankton tows were conducted between 09:00 and 11:00.

Because infected *H. montezuma* show overall altered color from their uninfected conspecifics (Figure 1-1), infected and uninfected individuals are readily distinguished from one another in the field.

We separated infected and uninfected amphipods and preserved them separately in 70-percent EtOH. In the lab we verified the infection status of preserved infected amphipods under a dissecting microscope and noted the sex, number of trematode cysts, and the size of infected amphipods. Because we have never observed trematode-infected amphipods <4 mm (C. O'Brien, unpub. data), we only included amphipods greater than this size in our analysis. We calculated prevalence as the number of infected/total number of amphipods >4mm in each sample.

Statistical Methods

All statistical analyses were conducted using the R package for Statistical Computing, ver. 2.5.1 (R Development Core Team, 2005). To visualize the difference in patterns of waterfowl use of space over time for each year, we used a robust, locally weighted regression (Cleveland, 1979) on scatterplots of the proportion of waterfowl using the far side of the Well over time for both years of the study, implemented with the "lowess" function in R. This function is a nonparametric smoothing method that allows for the visual perception of patterns in a scatterplot that might otherwise be difficult to detect (Cleveland, 1981). We excluded from the analysis those days in which no waterfowl were observed.

Because our measures of waterfowl and trematode prevalence were collected in a longitudinal design over time, we implemented generalized linear mixed models (GLMM) to avoid temporal pseudoreplication that occurs between repeated measurements of the same experimental units (Crawley, 2007). To implement the GLMM models we used the "lmer" function in the "lme4" library of R (Bates, 2007).

We modeled waterfowl space use, abundance, and trematode infection all as functions of fixed effects of interest and random effects of time and spatial location of plots. When modeling the binomial response of trematode infection, we specified binomial errors and a logit link (logistic regression). When modeling waterfowl abundance and space use, we corrected for differences in area of the various observational units by adding area as a covariate. Because waterfowl abundance showed evidence of overdispersion, we specified the quasipoisson family in the GLMM models. For all models, we assessed the significance of fixed effects of interest using likelihood-ratio tests, and we used Markov chain Monte Carlo methods (R function "mcmcsamp", chain length = 100,000; Bates, 2007) to estimate the 95-percent confidence interval for significant ($P<0.05$) parameter estimates of fixed effects from the fitted GLMM models.

Finally, to analyze the results from the randomized experiment, we used a paired-samples t-test on the difference in waterfowl number on the far side versus near side of the Well. The pairs of values consisted of the difference in number before and after disturbance for N = 8 replicates. For analysis of the observational longitudinal study of visitors and waterfowl space use, we implemented linear mixed models (LMM) with the nlme library (Pinheiro and Bates, 2000) in R. We modeled the response variable (difference in number on far and near sides of the Well) as a function of fixed effects (intercept + log (visitor number)) and random effects of time and day. Because residuals from the model exhibited strong temporal autocorrelation (first serial autocorrelation coefficient = 0.78, second serial autocorrelation coefficient = 0.52), we specified a continuous autoregressive process, using time as a continuous time covariate in the LMM model, using the correlation argument to the lme function (Pinheiro and Bates, 2000).

Results

Patterns of Human Recreation and Waterfowl Foraging

We found that visitor number varied dramatically both within and between years (fig. 2-3). Visitation during our 2004-5 observation period was lower than in the 2003-4 period, especially during October, November, January, and February. Mean (± 1 SE) daily visitation numbers were 958 (±2.9) visitors/day during 2003-4 and 859 (±3.4) visitors/day during 2004-5.

We found that waterfowl abundance varied significantly by year, habitat, and location (table 2-1; chater 2 tables are found in appendix B). Waterfowl abundance was almost two times greater on the side of the Well far from visitor influence. Additionally, we found that waterfowl abundance was 14 times greater in the littoral vegetation than in the open water. Finally, there was variation in abundance between years. In 2003-4 waterfowl abundance was more than 30 percent greater than in 2004-5 (table 2-1). Because waterfowl abundance and visitor number were higher in the first year of the study, we tested to see if there was a difference in the overall proportion of waterfowl using the far side of the Well between the two years; we found no evidence for such a pattern ($P = 0.41$, $\chi^2 = .63$, d.f. = 1).

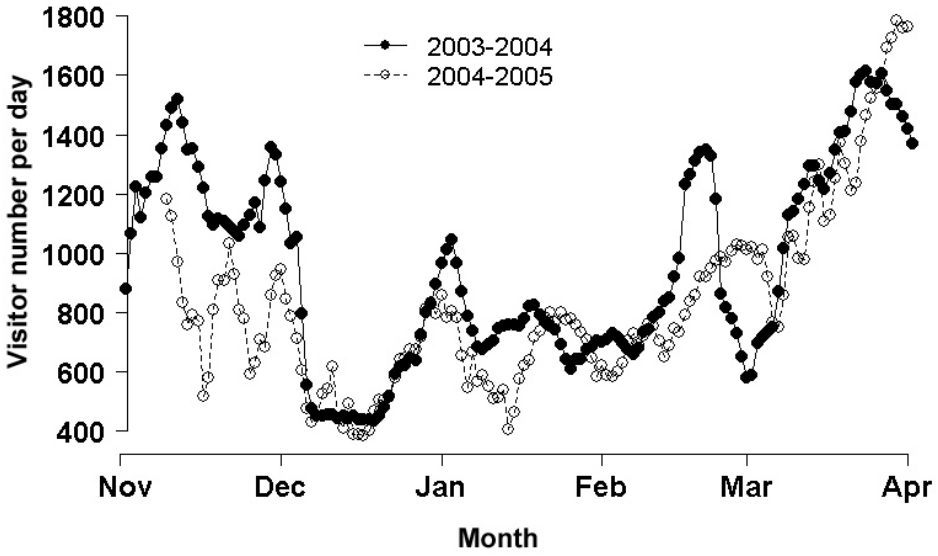

Figure 2-3. Estimated visitor numbers at Montezuma Well over our study periods (Sept.-June) in 2003-4 and 2004-5. Values plotted represent the mean daily visitor numbers from a 7-day running average over two sequential winter seasons.

Although we found no difference in space use patterns between years, graphical analysis suggests qualitative differences between the years, as well as variation throughout the sampling periods by year (fig. 2-4). Throughout the 2003-4 season, waterfowl use favored the far side of the Well; however, in the second year of the study, waterfowl favored the near side (or early in the season, in October and November, showed no preference). The lack of preference corresponds to lower visitor use

during those same two months in 2004-5 (fig. 2-2), suggesting that visitors affected waterfowl use of space to a greater degree in 2003-4 than in 2004-5.

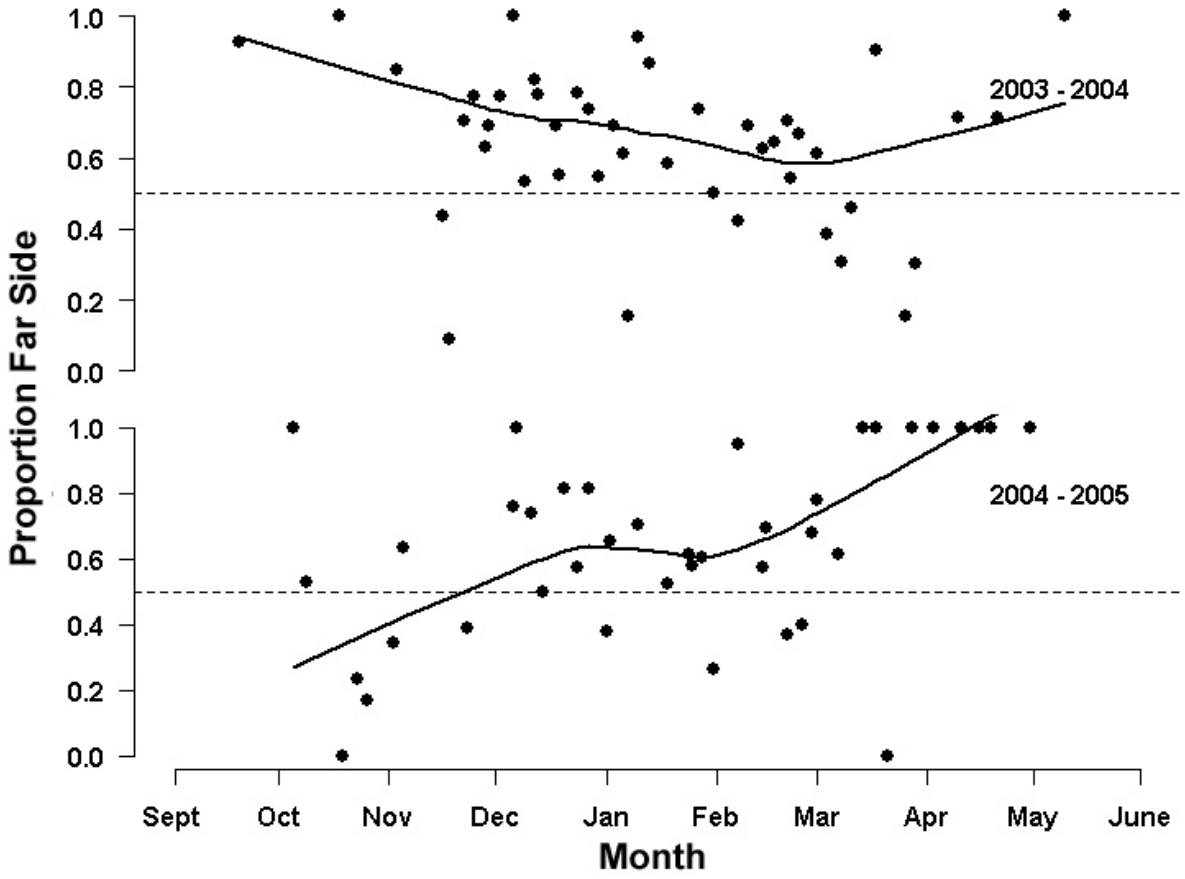

Figure 2-4. Patterns of waterfowl space use over time during the two study periods in 2003-4 and 2004-5. Y-axis is the proportion of waterfowl on the far side of Montezuma Well. The pattern of use was estimated with robust locally weighted regression.

Results from a randomized experiment suggest that human presence has a strong impact on space use by waterfowl. In each of the eight replicates, the number of waterfowl decreased near the observer (fig. 2-5), and this reduction was strongly significant ($P<0.001$, $t=5.7$, d.f. = 7, from a paired samples t-test). We estimate that experimental disturbance reduced the number by 17 animals/ha (95-percent confidence interval from 10 to 24) on the side of the Well near the observer.

We found a direct link between visitor number and location of visiting waterfowl (P = 0.024, t = 2.28, d.f. = 107 from a linear mixed model), and we estimate that for a one-unit increase in the visitor number, median waterfowl number increases on the far side of the Well by 0.67 waterfowl (95-percent confidence interval from .09 to 1.25 waterfowl). Despite the relatively weak relationship between visitor number and waterfowl, our observations revealed that some visitors had disproportionate disturbance effects upon waterfowl. For example, the first visitor of the day flushed waterfowl across

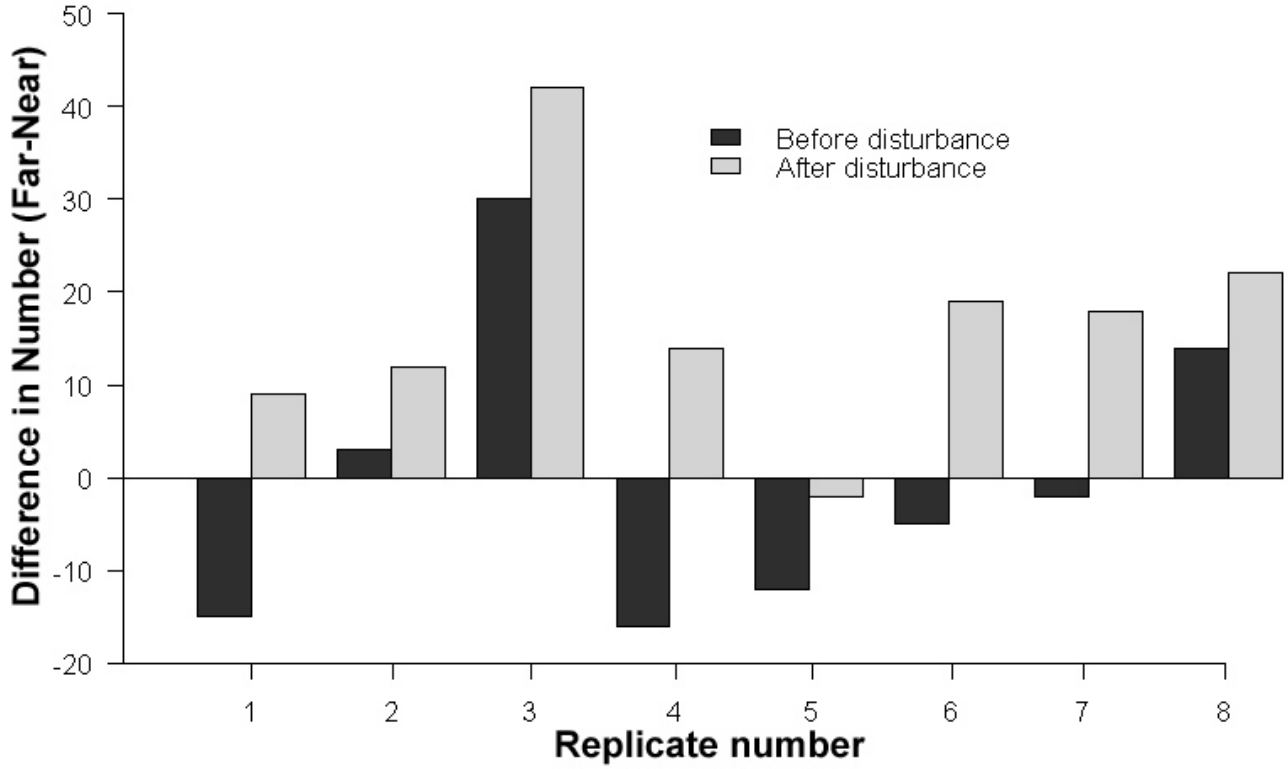

Figure 2-5. Results from experimental disturbance of foraging waterfowl at Montezuma Well. Pairs of differences in waterfowl numbers are plotted for before and after experimental disturbance, for each of eight replicates. Positive values indicate greater numbers of waterfowl far from the observer, and in every trial the observer decreased waterfowl number in his or her proximity.

the Well on all three days, increasing the difference in waterfowl abundance on far and near sides of the Well by 28, 40, and 44 ducks, respectively (fig. 2-6). These findings correspond to the results from our experimental disturbance, and we noted other individuals who had disproportionate effects on the movement of waterfowl; these individuals included groups of boisterous children (3 cases) and a leashed, barking dog (1 case) on the lower path (fig. 2-6).

Patterns of Infection

Throughout the study period the prevalence of trematode infection in *H. montezuma* was low, never exceeding 1 percent of animals infected for a given sampling period, and prevalence was similar between years, peaking several months after waterfowl abundance peaked (fig. 2-7). Because of the lag time of several months between maximum waterfowl number and maximum prevalence, we used lagged weekly average waterfowl abundance (lag = 98 days) to explain temporal variation in prevalence with GLMM models. We found that mean waterfowl abundance explained a significant amount of the variation in temporal patterns of trematode prevalence ($P<0.001$, $\chi^2 = 69.46$, d.f. = 1) and estimate that for every increase of one unit in median waterfowl abundance, the odds of infection in amphipods increased by 1.02 times (95-percent confidence interval from 1.00 to 1.04 times).

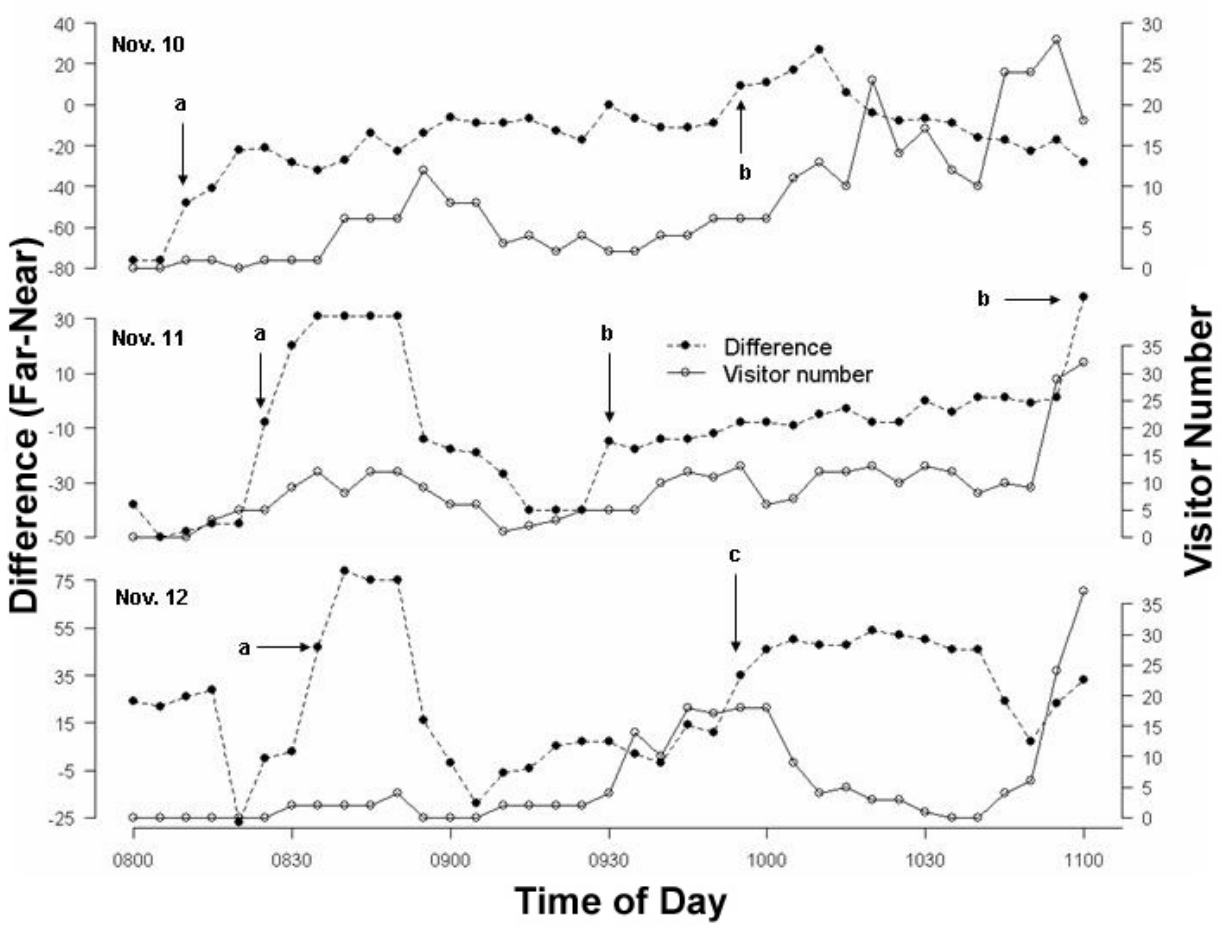

Figure 2-6. Plot of visitor number and corresponding waterfowl space use of Montezuma Well over a 3-day period in November 2007. Left y-axis represents the difference in waterfowl abundance on far and near sides of the Well every 5 minutes between 0800 and 1100. Right y-axis represents maximum number of people on lower and upper paths over preceding 5-minute period. Letters indicate individuals or groups that disproportionately affected waterfowl location: a = first visitor(s) of the day on upper path, b = groups with two or more loud children on lower path, and c = individual with leashed, barking dog on lower path.

Because the difference in infection rates between far and near sides of the Well depended upon year (fig. 2-8), we analyzed factors that explained variation in the odds of infection separately by years (table 2- 2). Although waterfowl numbers were greater in the littoral zone, we found no difference in the odds of infection by either habitat or depth for either year (table 2-2). In the first year of the study we found that the odds of infection differed between sides, but this pattern did not continue in 2004-5 (table 2-2). The magnitude of the difference in the odds was substantial during 2003-4; we estimate that the odds of infection were 1.8 times greater on the far side (95-percent confidence interval from 1.16 to 3.35) than on the near side. Finally, despite the fact that waterfowl abundance was reduced in the second year of the study, in a separate test of the difference in the odds of infection between years, we did not find a corresponding difference in the odds of infection with respect to year ($P = 0.292$, $\chi^2 = 1.11$, d.f. = 1).

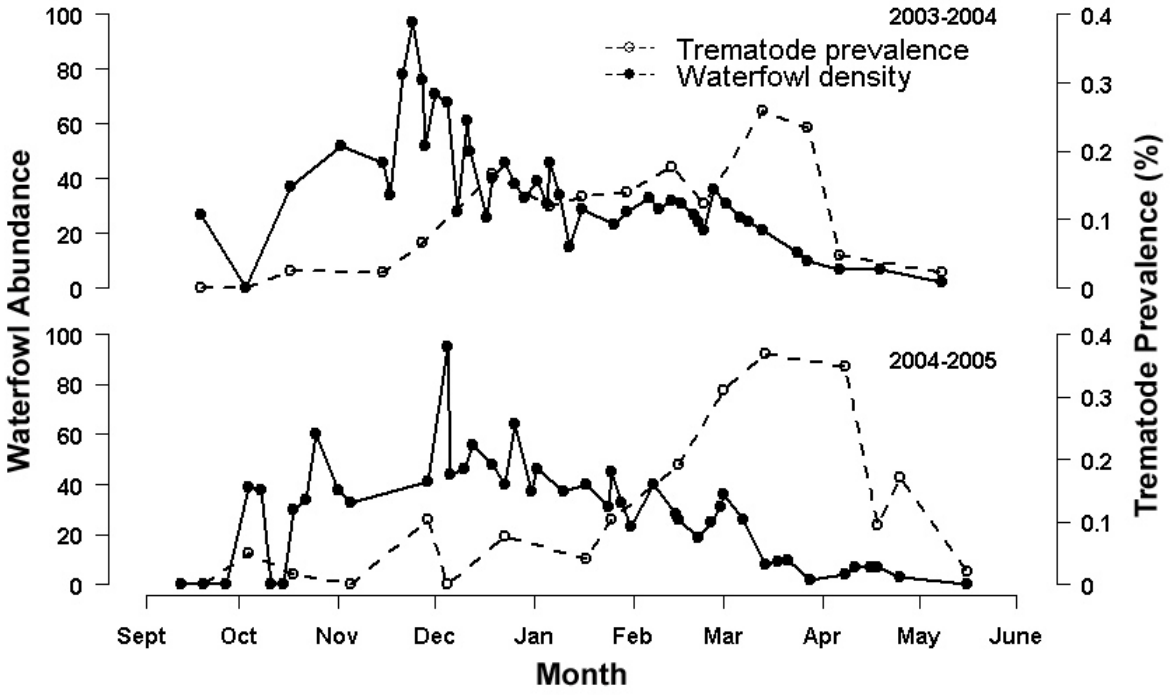

Figure 2-7. Plot of waterfowl abundance and *Microphallus* trematode parasite prevalence in amphipods over the two study periods, September to June 2003-4 and 2004-5, at Montezuma Well.

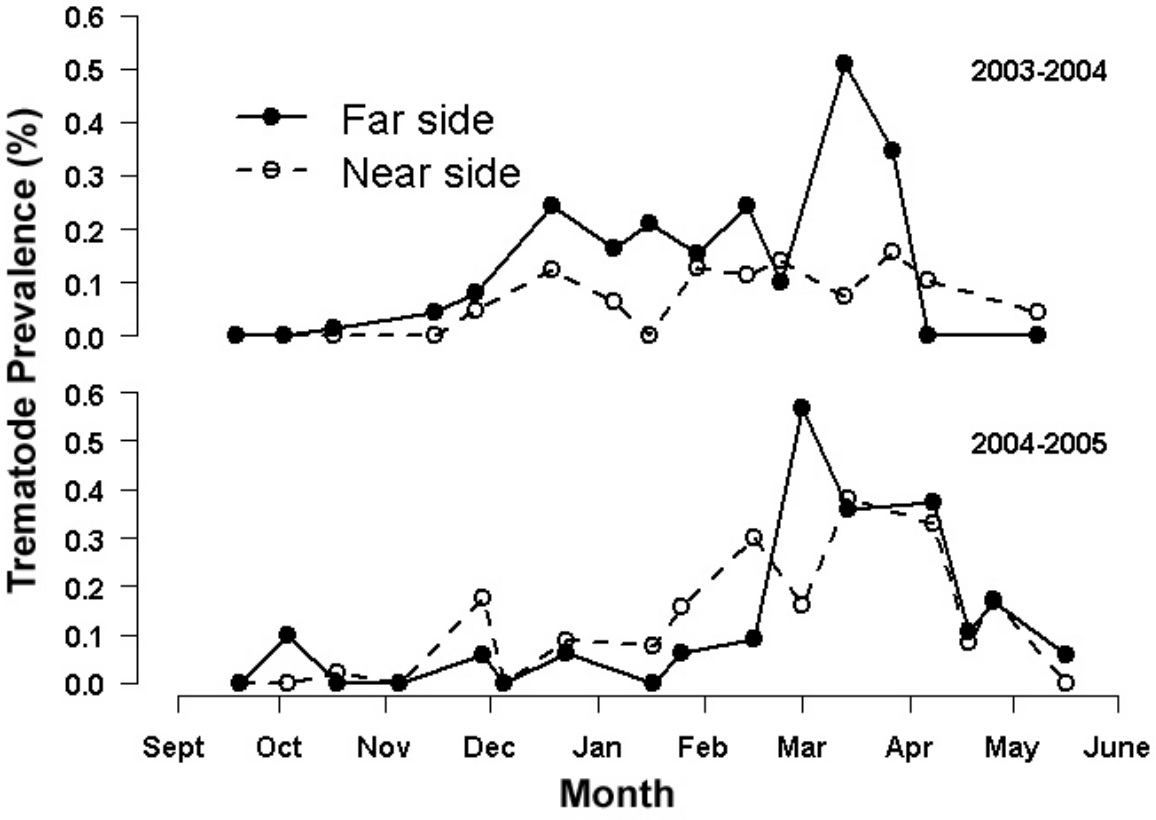

Figure 2-8. Trematode *Microphallus* parasite prevalence in amphiods in far and near sides of Montezuma Well over the two years of our study (2003-5).

Discussion

This study is the first to demonstrate that human recreation can affect spatial patterns of infection in invertebrate hosts. Several lines of evidence in our research support this conclusion: (1) We found that waterfowl responded negatively to experimental human presence, increasing the density of waterfowl far from the observer, and that there was a direct relationship between actual visitor numbers and waterfowl space-use patterns. (2) We found that increased waterfowl abundance was associated with an increase in the odds of trematode infection. (3) For one of the two years of the study, we found elevated prevalence of trematode infection in invertebrates in areas that were far from disturbance by recreating humans; and in a second year, when spatial variation was not detected, visitor numbers were lower, as was the proportion of waterfowl foraging far from visitor paths (at least early in the season).

Patterns of Human Recreation and Waterfowl Foraging

Results from our experiment demonstrate that waterfowl responded strongly to experimental disturbance—at least early in the morning before they had become habituated to visitors, and observational data supported this finding, suggesting that avian response to recreating visitors may depend upon past experiences. Furthermore, results from our observational study provide evidence that

modeling waterfowl as a function of average visitor number is probably not the best approach. Our observations demonstrate that waterfowl respond directly to individual visitors differentially, suggesting that individual-based models might be more appropriate (Judson, 1994; Huston and others, 1988) in assessing human impact on waterfowl behavior.

Patterns of Infection

In 2003-4, infection rates were almost two-fold greater on average in areas far from human disturbance, whereas during 2004-5 this pattern was not as evident. To explain this, we point to a reduction in the proportion of waterfowl using the far side of the Well in 2004-5 from 2003-4 that corresponds to reduced visitor numbers in 2004-5 from those of 2003-4. Since there is a lag time of about three months between peak waterfowl abundance and peak disease prevalence, we argue that this difference is a result of low early winter visitor numbers. The low levels of visitiation during the 2004 early winter period could explain the lack of difference in infection by sides of the lake that occurred later in the year when visitation increased and waterfowl were more confined to the far side of the Well. However, we realize that our evidence is circumstantial, and our study is lacking a causal link between actual visitors at the Well and spatial patterns of infection. An alternate hypothesis that could explain the lack of evidence of a difference in 2004-5 could be a lack of statistical power in our analysis. Our measured rates of parasitism are very low, considerably less than 1 percent. Because tests of hypotheses on proportions close to zero or one have reduced statistical power (Cohen, 1977; O'Brien and others, 2009), it is possible that we simply failed to detect a difference in 2004-5 because of a lack of statistical power.

We also did not detect a difference in disease prevalence between the littoral and limnetic zones of the Well, despite the fact that waterfowl abundance was more than 18 times greater in the littoral than in the limnetic zone. Furthermore, because there is only habitat in the littoral zone for the snail first intermediate host of the trematode, we expected that prevalence would be greater in the littoral zone. The lack of difference that we observed can possibly be explained by the behavior of the *H. montezuma,* which exhibits daily migration between the limnetic zone and the littoral vegetation (Blinn and Davies, 1990).

Implications of Human-Induced Patterns of Infection

Evidence from this study demonstrates that human recreation has a cascading effect that ultimately alters spatial patterns of disease in a natural system. We suggest two important implications of our findings that may be useful in the conservation and management of natural ecosystems.

First, it has been established by both theoretical work (Anderson and May, 1978) and empirical studies (Arneberg, 2001) that local increases in host density can increase transmission back to hosts through density-dependent processes. Our findings demonstrate that human recreation caused a shift in waterfowl foraging locations, resulting in increased waterfowl densities in those areas furthest away from visitor use areas. Coupled with an increased prevalence in the amphipod host at the furthest side of the Well, increasing waterfowl densities could increase prevalence and intensity of disease in this definitive host. Trematodes have been known to have pathogenic effects on waterfowl (Wobeser, 1981), and higher waterfowl densities would create the possibility for increased levels of transmission in waterfowl. Additionally, as waterfowl move through natural migratory processes, increases in disease prevalence and intensity in one avian community could affect communities elsewhere, as on the more northern breeding grounds. We suggest that our results demonstrate that the cascading effects of human recreation can occur and that, in systems where human recreation is common and wildlife disease is

widespread, overcrowding of wildlife due to human recreation in protected areas should be considered in the management and conservation of those areas (Lebarbenchon and others, 2007).

Secondly, we know that parasites and disease can have ecosystem- or community-level effects (Poulin, 1999; Thomas and others, 2005; Collinge and Ray, 2006). In a system where parasites have a strong ecological effect (Thomas and others, 1999), altering the spatial distribution of parasites could have strong effects on the plants and animals that interact with infected hosts. The cascading effects of increased animal densities that are caused by human visitation in protected systems has not been given much consideration by wildlife managers (Lebarbenchon, 2007). When processes that negatively affect wildlife are instigated by human recreation, they become a legitimate concern for those managing protected ecosystems for recreational opportunities.

Human recreation continues to grow in protected areas, which makes it likely that indirect impacts by visitors on wildlife will continue to increase. Our study results suggest that these indirect effects can occur and that further study is needed to document these effects at the community level, especially in systems where parasites play important ecological roles. Studies of this type will allow us to better understand how human recreation can affect wildlife, the communities they are part of, and the ecosystems they inhabit. These studies will also provide critical information with which land manager can better balance the needs of humans and wildlife in our protected areas.

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Chapter 3

Making Better-Informed Decisions in the Analysis of Wildlife Diseases: Hypothesis Testing, Power Analysis, and Estimating Observed Effects

Introduction

The increasing importance of wildlife diseases in conservation efforts places additional importance on research study design, data analysis, and interpretation. In this chapter we explore issues pertaining to the design and analysis of wildlife disease data with regard to hypothesis testing, reflecting upon statistical power, sample sizes, the relative costs of type II/type I errors, and parameter estimation. To explore these ideas, we present: (a) results from a literature review of the Journal of Wildlife Diseases (JWD), and (b) findings from a computer simulation that estimates the type II error rate for specific statistical techniques that have been used in JWD. In addition, to illustrate the benefit derived from using parameter estimates, we present the reanalysis of previously published data. In a review of existing literature, we found that a large majority of studies published in JWD between 2000 and 2005 that included hypothesis tests used chi-squared analysis on prevalence data, and only 19 percent of the studies reported parameter estimates that would allow the reader to interpret the magnitude of the observed effect size. Furthermore, 10 percent of the 591 studies that we reviewed had pooled sample sizes ≤40, and many had potentially high costs of type II relative to type I errors. Results from a computer simulation suggest the possibility that many articles published in JWD from 2000 to 2004 lacked sufficient statistical power; this possibility, coupled with our review of studies that ignored high costs of type II errors, clearly points to the need for researchers to increase attention to statistical power. Finally, in our data reanalysis we demonstrate that the presentation of parameter estimates would allow researchers to better estimate the magnitude of their observed effect sizes and to more accurately assess the biological significance of their findings. We conclude with general guidelines that we hope will assist wildlife disease researchers in the design of future studies and in statistical analysis of their data.

Many empirical studies have demonstrated that disease can play a crucial role in the conservation or demise of threatened species in particular ecosystems (van Riper and others, 1986; Berger and others, 1998). Disease has been called one of the four “mindless horsemen of the environmental apocalypse” (Wilson, 1992). Because of the growing awareness about wildlife diseases as they effect the conservation of species of concern, researchers are placing a greater emphasis on the analysis of wildlife disease data, which in turn places additional importance on research-study design, data analysis, and interpretation.

While recent criticisms of traditional hypothesis testing have emerged (Berger and Berry, 1988; Cohen, 1994; Johnson, 1999), the large majority of studies still employ methods of hypothesis testing (Fidler and others, 2006). Therefore, in this study we explore issues in the design and analysis of wildlife-disease data with regard to hypothesis testing, reflecting upon statistical power, sample sizes, the relative costs of type II errors, and parameter estimation. In this report, we present the results from

our literature review of the Journal of Wildlife Diseases, our findings from the computer simulation, and the reanalysis of previously published data.

Disease is being increasingly recognized as an important, and perhaps crucial, element in the management and conservation of wildlife species (Deem and others, 2001; Tompkins and Wilson, 1998). In the rapidly changing world of wildlife disease, researchers are increasingly being called upon to measure the effects of disease on small, and often endangered, wildlife populations. To document patterns of wildlife disease in wildlife populations, scientists often quantify the rate or degree of disease or parasitic infection and analyze these data using classical techniques of statistical hypothesis testing.

Two common measures of wildlife disease reported in the literature are prevalence and abundance. Prevalence is the proportion of individuals in a sample that are infected with a parasitic disease (Bush and others, 1997). In its raw form, the prevalence measure constitutes a dichotomous response variable (infected/not infected). Given that a random sample of the host organism is collected, prevalence estimates can be representative of the disease status of a host population. Alternatively, abundance is a count of the number of parasites or disease units that are found in a single host (Bush and others, 1997), taking a value of zero or greater. Averaged across all individuals in a random sample, abundance estimates the mean number of parasites or disease units carried by a single animal within a population.

Ideally, random sampling can be used in wildlife disease studies to estimate the prevalence and abundance of parasites within animal populations, and statistical hypothesis testing can be used to assess how these measures of disease vary with different factors (for example, sex, age, time of year, levels of human impact). The analyses of these data are complicated by the fact that both prevalence and abundance data violate assumptions of standard linear models, such as analysis of variance (ANOVA) and linear regression. Prevalence, which is a proportion bounded by 0 and 1 requires either a transformation (the arc-sin and logit transformations are common), the application of contingency table analysis (such as chi-squared or log-linear models), modeling the response as a binomial variable with logistic regression, or employing nonparametric methods. Parasite abundance, bounded by zero and infinity is strongly positively skewed (fig. 3-1*A*). This distributional pattern can be accurately described by the negative binomial, a model that incorporates an overdispersion term (k) that accounts for overdispersion, or the degree to which the variance exceeds the mean (Crofton, 1971). In contrast to the negative binomial, a poisson model (in which the variance = mean) can also be used to model parasite abundance (Wilson and others, 1996; fig. 3-1*B*). In a literature review of 269 measurements of parasite abundance, Shaw and Dobson (1995) found that in 268 of the studies the variance exceeded the mean, thus suggesting a widespread pattern of parasite aggregation in hosts.

Because of the nonnormal distribution of parasite numbers in hosts, the logarithmic transformation has historically been employed to normalize data before the application of linear models. Alternatively, generalized linear models (GLM) allow the user to explicitly model the nonnormal error structure and nonconstant variance of negative-binomially distributed abundance data and offer a more robust alternative to traditional linear model methods for hypothesis testing of abundance data (Wilson and Grenfell, 1997).

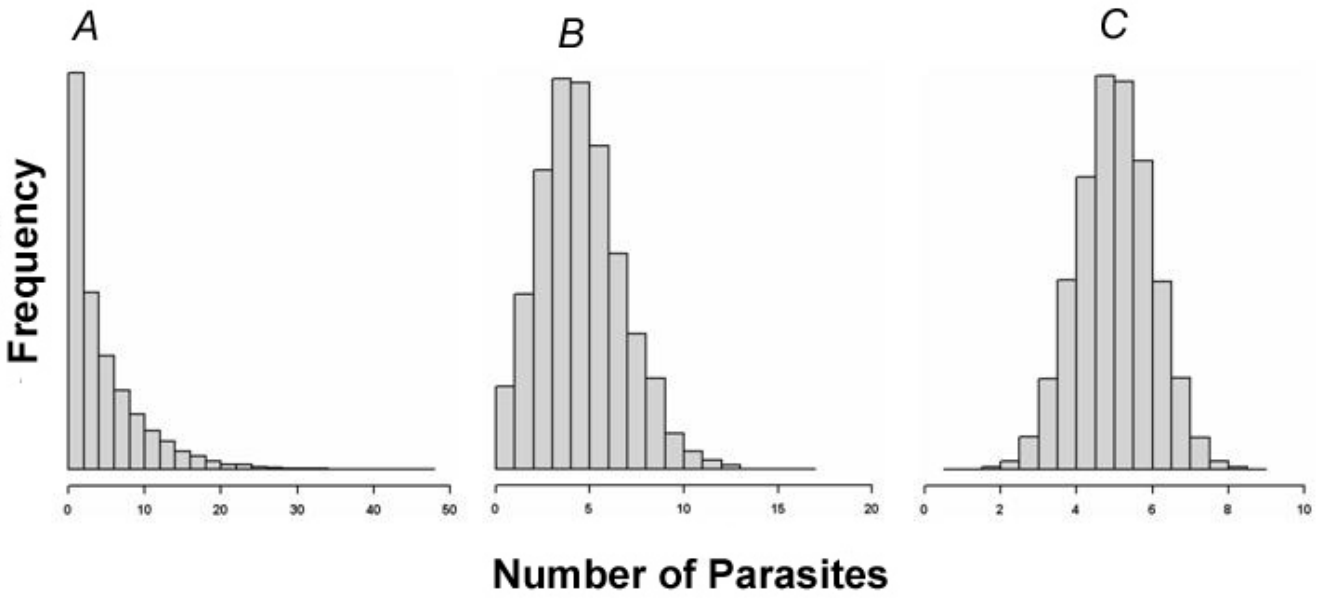

Figure 3.1 Examples of three distributions: the negative binomial (*A*), poisson (*B*), and normal (*C*). For all three distributions, mean = 5. For negative binomial, variance = 30, k = 1; for poisson, variance = 5; for normal, variance = 1.0.

Type I and Type II Errors

Because the material presented herein will make repeated reference to statistical concepts of error, we first review definitions of type I and type II error. The probability of a type I error (α) is the probability of rejecting the null hypothesis when the null hypothesis is true. This error type has traditionally been of primary interest to biologists. An accepted benchmark used for determining statistical significance is $\alpha<0.05$, a standard convention, which while popular, is somewhat arbitrary (Johnson, 1999).

By contrast, a type II error (β) is the probability of accepting the null hypothesis when the null hypothesis is false, and statistical power ($1-\beta$) is the probability of correctly rejecting the null hypothesis. An example follows: If anthropogenic effects truly increase the prevalence of disease in a species of concern, and the study fails to detect that effect, a type II error has been committed. A standard convention is $\beta\leq0.20$, however there are practical arguments for minimizing the probability of β well below 0.2 (Di Stefano, 2003; see below).

Statistical theory dictates an inverse relationship between type I and type II error—decreasing acceptable levels of one error type increases the probability of making an error of the other type. Therefore, when conducting statistical inference it is important to consider both type I and type II errors (Cohen, 1977), and choosing which type of error to minimize should depend upon the situation.

Biologists have traditionally sought to minimize type I at the expense of type II errors; however, in conservation applications the consequences of a type II error may actually outweigh those of a type I error (Dayton, 1998), leading to a concept known as the precautionary principle (Peterman and M'Gonigle, 1992; Kriebel and others, 2001). Under the precautionary principle, type II errors should be minimized under certain conditions, because if we fail to detect impacts that are occurring, we run the risk of continuing a harm of which we are unaware. This has implications when studying an organism

that is locally rare or threatened, and we argue that the consideration of relative costs of type I and type II errors is an important step in the design and implementation of a study of wildlife disease.

Recently, some scientists have expressed a growing concern about the strong reliance on hypothesis testing in the biological sciences (Johnson, 1999), and many alternatives to a hypothesis-testing approach have been proposed (Fidler and others, 2006). Despite this ongoing debate, and because science still overwhelmingly embraces statistical hypothesis testing (for a specific example, see Fidler and others, 2006), we attempt to understand how researchers can apply hypothesis testing more efficiently and effectively in the study of wildlife diseases. While not covered here, methods such as information theoretic approaches (Burnham and Anderson, 2002), Bayesian statistics (Johnson, 1999), and equivalence testing (Hoenig and Heisey, 2001) are three proposed alternatives to traditional hypothesis testing that may be preferable in the analysis of wildlife disease data; researchers are urged to familiarize themselves with these methods and, when appropriate, apply them in their research.

In this chapter we report the results of several exercises that demonstrate how hypothesis testing can be used more effectively in wildlife disease research: (1) Literature review: To understand how data are currently analyzed and presented in our field, we reviewed the Journal of Wildlife Diseases to look at the common methods of analysis of prevalence and abundance data, pooled sample sizes, estimates of the magnitude of the observed effects, and the relative costs of type I and type II errors. (2) Computer simulation: To assess the power of statistical models used in JWD, we report the results from a computer simulation study that estimated the probability of type II errors associated with a variety of common techniques used on prevalence and abundance data in JWD. (3) Data reanalysis: Finally, to demonstrate how reporting parameter estimates and associated confidence intervals can increase information transfer and inform the researcher and reader about the biological significance of data on wildlife diseases, we present the results of a reanalysis of previously published data that compared the prevalence of blood parasites in different populations of birds (Super and van Riper, 1995).

Materials and Methods

Literature Review

We reviewed five years (2000-2004) of papers published in the Journal of Wildlife Diseases (JWD) that tested hypotheses about differences in either the prevalence or abundance of wildlife diseases and parasites. We included all studies that measured either the prevalence and/or the abundance of macroparasites (such as helminths, ectoparasites) that were determined from visual counts, as well as studies of microparasites or disease agents that were determined through seroprevalence tests or counts of parasites per unit volume (such as viruses, bacteria, blood hematazoa).

For the 70 studies identified in this search, we tabulated the types of analyses conducted, whether the magnitudes of the observed effects were estimated, and the total number of animals (pooled sample size, N). We also scored each study for the relative costs of type I and type II errors, according to the philosophy of the precautionary principle (Peterman and M'Gonigle, 1992; Kriebel and others, 2001). Because determining the relative costs of errors in the studies of others is subjective, we identified those studies in which we perceived that there was at least a possibility that costs of type II errors could exceed costs of type I errors—for example, if a type II error could result in the authors failing to detect a true harm to an endangered species. All studies published in JWD were scored as to whether or not the authors rejected their null hypotheses. We chose to include this assessment to raise awareness about the possibility of the high costs of type II errors in the study of wildlife diseases.

Computer Simulation

Because computer simulation provides a robust method for estimating type II error rates, especially for nonnormally distributed data (Crawley, 2002), we used computer simulations to estimate β associated with different statistical techniques of both prevalence and abundance data. To do this, we took random samples from two hypothetical populations with different mean prevalence or abundance and conducted a hypothesis test that reported evidence for or against the null hypothesis of no difference ($\alpha<0.05$) between the two populations. For prevalence data sets we estimated type II error rates generated by the chi-squared test for independence (Ramsey and Shafer 2002, section 19.3), Fisher's exact test (Ramsey and Shafer 2002, 19.4), log-linear regression (Ramsey and Shafer, 2002, chap. 22; Nelder 2000), and logistic regression (Ramsey and Shafer 2002, chap. 20). We conducted tests over a range of pooled samples sizes (N = 20–1,000), and raw effect sizes (difference in prevalence between the two simulated populations, which ranged from 0.01 to 0.4). Because the power of hypothesis tests on prevalence data depend on the location of the proportion relative to 0.5 (Cohen, 1977), we completed two sets of comparisons—one in which the base proportion was 0.5, another in which the base proportion was 0.1.

For abundance data sets we estimated β generated by t-tests (Ramsey and Shafer, 2002, chap. 2), t-tests after log-transformation (Ramsey and Shafer, 2002, chap. 3), the non-parametric Wilcoxin test (Ramsey and Shafer, 2002, chap. 4), and negative binomial regression (a GLM with negative binomial errors and a log link function; Venables and Ripley, 2002, section 7.4). These analyses used the same sample sizes as above, and raw effect size (differences in mean abundance) ranged from 1 to 500. Because type II error rates vary with k (the aggregation parameter of the negative binomial), we conducted three sets of comparisons for three different values of k (0.3, 1, 1.5), which span the range observed in most studies of parasites in wildlife hosts (Shaw and Dobson, 1995; Shaw and others, 1998). In each simulation we generated a pair of random samples with different mean prevalence or abundance and conducted a hypothesis test, repeating this 10,000 times. We calculated the type II error rate as the proportion of the 10,000 tests in which a type II error was made ($P\geq0.05$).

To compare type II error rates for testing hypotheses of difference in prevalence versus difference in mean abundance, we took random samples from two negative binomial distributions with means of 1 and 2, respectively, for three values of k (0.3, 1.0, 1.5). We then conducted a statistical hypothesis test for difference in mean abundance between the two samples, converted abundance values to prevalence and computed sample prevalence, and conducted a hypothesis test for difference in prevalence between the two samples using log-linear regression. These simulations were calculated for a range of sample sizes with a balanced design (N = 20–1,000), and the probability of a type II error was computed as above.

Data Reanalysis

We reanalyzed a dataset from a previously published JWD paper to show the advantages of estimating observed effect size. Super and van Riper (1995) tested the predictions that the prevalence of avian hematazoan parasites is different on coastal islands than on the California mainland and that prevalence of disease differs between resident and migratory bird communities. Super and van Riper (1995) used chi-squared contingency tables to conduct their analyses. In contrast, we used log-linear models to analyze the 2 × 2 tables. Our method, while being more statistically powerful than chi-squared tests, was chosen primarily because it provides a parameter estimate that allows the wildlife disease researcher to infer the magnitude of the observed effect that factors have upon the prevalence of disease. In the 2 × 2 contingency tables of Super and van Riper (1995), we treated “infected/not infected” as a response (Nelder, 2000), and “island/mainland” and “migratory/resident” as independent

dummy variables, and tested the null hypothesis of independence between the response and the independent variable of interest by comparing the sum of the squared deviance residuals with the chi-squared distribution for 1 degree of freedom (Crawley, 2002). We then estimated the magnitude of the effect of the independent variable upon the response with the parameter estimate of the interaction term between the two factors, which gives the odds ratio of the two factor terms (Nelder, 2000), and tested the significance of the parameter with a Wald's chi-squared test.

We chose to use log-linear models because our simulation results revealed that this method is more powerful than others, but we could have implemented other models that also allow for the inference of observed effects. For example, with 2×2 tables the inference from log-linear models is identical to that from logistic regression (Nelder, 2000). We could have also directly estimated the odds ratio from the 2×2 table and computed confidence intervals using the binomial distribution, although this method tests a hypothesis of homogeneity rather than independence, and sampling schemes can dictate the appropriate analysis (Ramsey and Shafer, 2002, section 19.2).

All simulations and statistical analyses were conducted with the R package for Statistical Computing (R Development Core Team, 2007), and we used the rnegbin and glm.nb functions from the MASS library to take random samples and test hypotheses regarding the negative binomial distribution (Venables and Ripley, 2002).

Results

Literature Review

From 2000 to 2004, inclusive, 591 articles were published in the JWD. Of these, 70 papers tested hypotheses regarding differences in mean abundance or prevalence. The following results are from a review of these 70 studies. The majority of studies (96 percent) reported prevalence, and 20 percent of the studies reported both prevalence and abundance (table 3-1; chapter 3 tables are found in appendix C). Differences in mean prevalence were most commonly tested by JWD authors using chi-squared contingency tables (63 percent). Nonparametric tests (Kruskal-Wallis, Wilcoxin-Mann-Whitney) were the most common method (41 percent) used for testing differences in mean abundance (table 3-1).

Because the number of factors and factor levels in the studies that we examined varied widely and designs were rarely balanced, we recorded a pooled sample size for each study, which ranged from 12 to 63,451. The distribution of sample sizes was strongly right skewed, and 28 percent of studies had pooled sample sizes (N) of <100; 10 percent of studies had $N \leq 40$ (fig. 3-2). The median sample size was 216.5.

We also found that authors in JWD were inconsistent in providing estimates of the magnitude of the observed effect in their studies, even when their methods generated these results. Only 19 percent of studies provided parameter estimates from linear models (or GLM) or from odds ratios computed from the binomial distribution.

We also evaluated each study for the relative costs of type II and type I errors, and counted those in which there was potential for the consequence of a type II to exceed that of a type I error. We found that in 30 percent of the 591 studies published in JWD, the potential cost of type II errors exceeded the cost of type I errors. This suggests that in the analysis of data on wildlife disease, more attention should be given to the power of statistical tests and to balancing the probability of type I and type II errors relative to their potential costs.

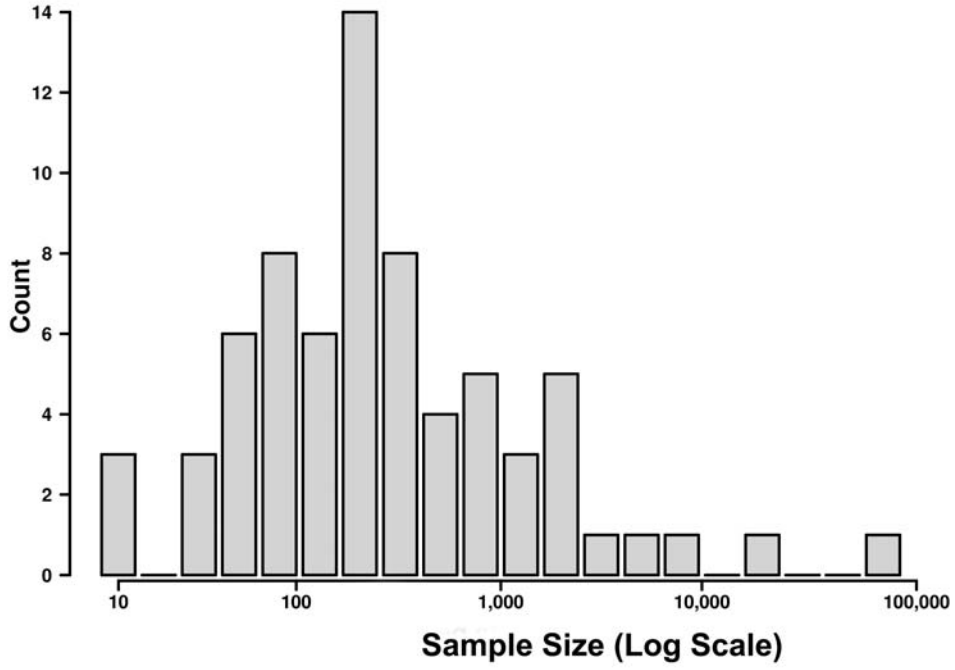

Figure 3-2 Histogram of sample sizes from 70 studies from Journal of Wildlife Diseases. Values represent pooled sample sizes, not those for each grouping variable. We adopted this convention because designs were rarely balanced and the number of factors varied.

Computer Simulation

Our simulated data revealed that the analysis of prevalence data generated high probabilities of type II errors, especially for small pooled sample size and small effect sizes (fig. 3-3). For small pooled sample sizes (N = 20), log-linear regression produced the lowest type II error rates. The probability of type II errors decreased with increasing sample size and was smaller when comparing two proportions that are both closer to 0.5 (fig. 3-3, right) than when comparing two proportions that are far from 0.5 (fig. 3-3, left). Our findings indicate that large sample sizes are necessary when comparing groups using prevalence; N <200 produce high probabilities of type II errors, except when effect size is very large (fig. 3-3). Therefore, in order to minimize $\beta \leq 0.20$, sample sizes must equal or exceed N = 200 when the raw effect size ≥0.17 and when prevalence is far from 0.5. When prevalence is close to 0.5, β was ≤0.20 for the raw effect size ≥0.13.

Similar to our findings for the analysis of prevalence data, error rates for abundance data showed that hypothesis tests comparing mean prevalence between two groups also generated high type II error rates, especially for small pooled sample sizes, small effect sizes, and small values of the aggregation parameter k (fig. 3-3). A GLM with negative binomial errors produced the lowest type II error rates with pooled sample size <100. The probability of type II errors decreased as the aggregation parameter (k), sample size, and effect size increased (fig. 3-2). Highly aggregated samples (k = 0.3) produced type II errors rates >0.2, except when pooled sample size ≥1,000. For more moderate values of k (1.0 and 1.5) N = 200 resulted in low values of β. Effect size had less of an effect on type II error rates in the analysis of abundance than in the analysis of prevalence (figs. 3-3 and 3-4) and was more pronounced for k>0.3 with abundance data.

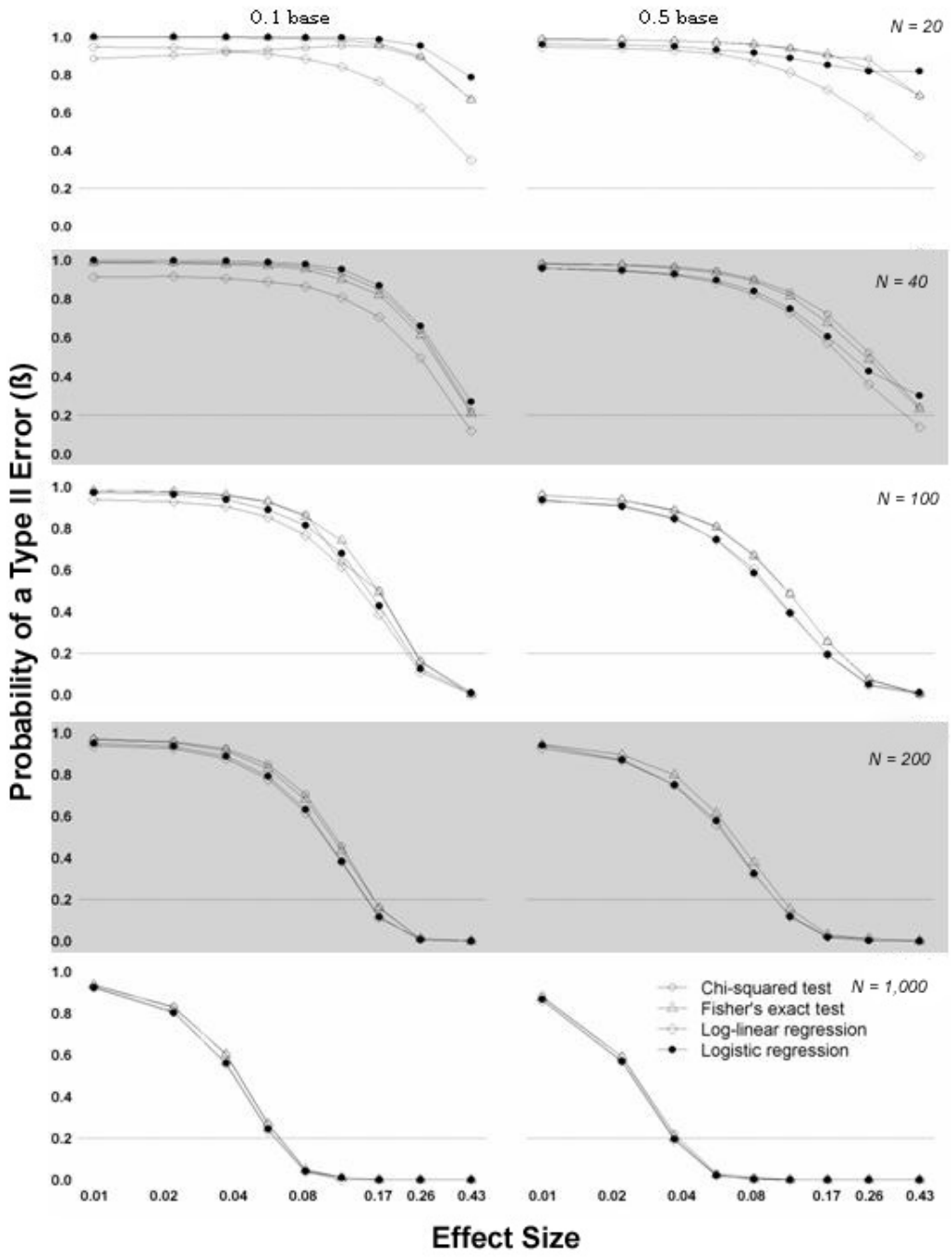

Figure 3-3. Probability of type II errors (y-axis) plotted for increasing sample sizes (vertically) and increasing effect sizes (x-axis) for two different base proportions for five different statistical methods. The effect size represents the difference in mean prevalence between the two populations. Horizontal lines indicate $\beta = 0.20$, the generally accepted upper limit of beta. Sample sizes given are pooled N, for a balanced design comparing two groups (for instance, N = 200 corresponds to a test comparing two samples, each of size 100).

We also directly compared the statistical power of hypothesis tests of prevalence and abundance from the same data set. Our comparison showed that the analysis of abundance is always more powerful than the analysis of prevalence (fig. 3-5), at least when N<1,000.

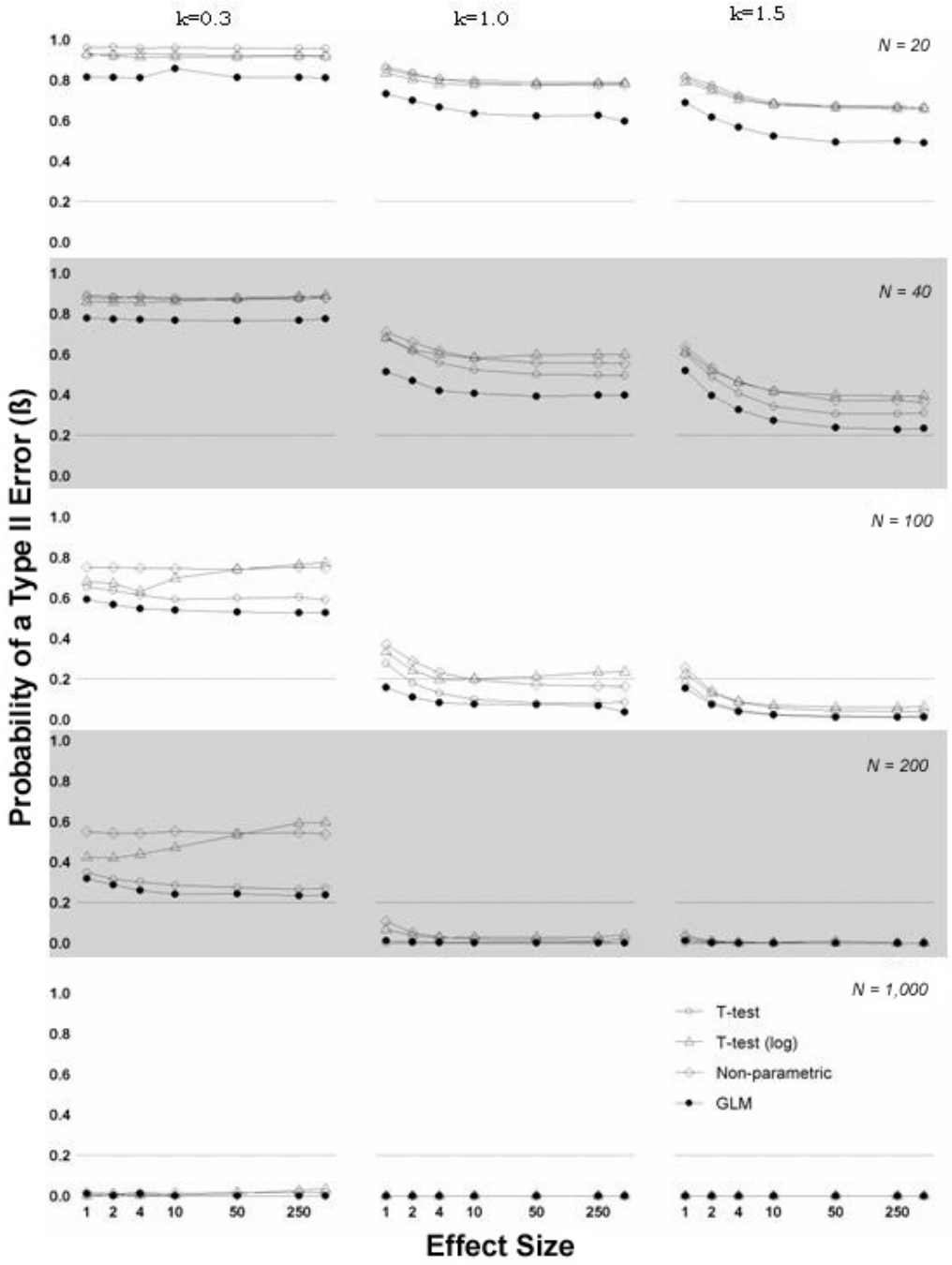

Figure 3-4. Probability of type II errors (y-axis) by effect size (x-axis), plotted for four different statistical techniques for a range of pooled sample sizes and aggregation parameters (k). Horizontal lines indicate β = 0.20, the generally accepted upper limit. The x-axis represents the difference in mean abundance (effect size) between two populations. Sample sizes given are pooled N, for a balanced design, comparing two groups.

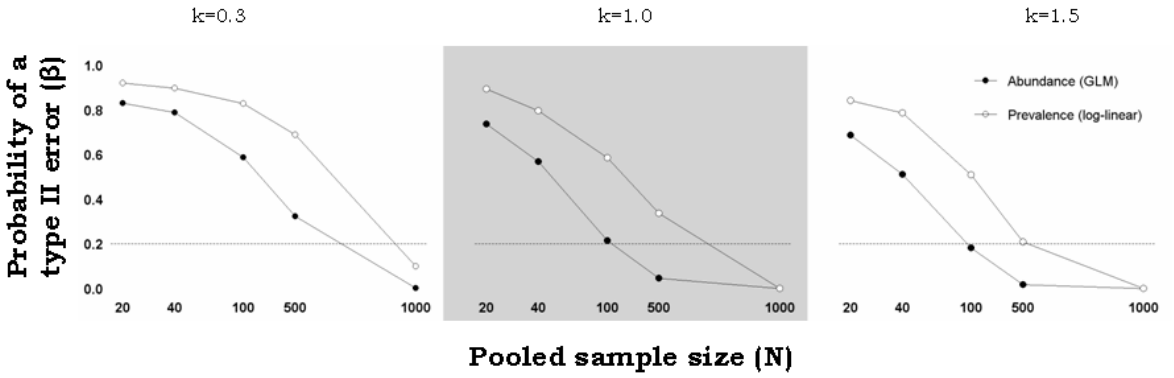

Figure 3-5. Probability of type II errors (y-axis) for increasing sample sizes (x-axis) for the analysis of abundance data using a GLM and of prevalence data using log-linear regression. For all three simulations, samples were randomly drawn from two populations with means = 1 and 2 for k = 0.3 (left), k = 1.0 (middle), and k = 1.5 (right). The corresponding prevalences were 0.36 and 0.46 (left), 0.49 and 0.66 (middle) and 0.53 and 0.72 (right). Horizontal line indicates β = 0.2. Sample sizes shown are pooled N, for a balanced design, comparing two groups.

Data Reanalysis

Super and van Riper (1995) used chi-squared contingency tables for tests of independence to test the hypotheses that "there is no significant difference of hematozoan prevalences between passerine birds found in island versus continental coastal scrub communities" and "there is no significant difference in hematozoan prevalences between resident breeding versus migratory non-breeding birds in California coastal scrub communities." Analyses with chi-squared tests led them to reject both null hypotheses.

We came to the same conclusion as the original authors. However, our methods allowed us to estimate the magnitude of the effect of geographic location and migratory status on hematozoan parasite prevalence—something they could not do with chi-squared analyses. Like Super and van Riper (1995), we found that birds on the mainland site of Palomarin were more likely to be infected than birds from San Miguel Island ($P<0.001$, deviance = 126.2, d.f. = 1), but because our method provided an associated parameter estimate, we were additionally able to estimate that the odds of infection with hematozoan parasites at Palomarin were 9.9 (95-percent confidence interval from 6.1 to 17.1) times those of the odds of infection at San Miguel island ($P<0.001$, $z = 8.754$, from a Wald's test). When we restricted the analysis to resident breeding species only, as did Super and van Riper (1995), we found the same effect ($P<0.001$, deviance = 193.7, d.f. = 1), but additionally, we could estimate that for breeding birds only, the odds of infection at Palomarin are 57 (95-percent confidence interval from 23.3 to 184.7) times those of the odds of infection at San Miguel Island ($P<0.001$, $z = 8.754$).

We also compared the prevalence of hematozoan infection for resident versus migratory birds at the two different sites. Like Super and van Riper (1995), we generally found that the odds of infection for migratory birds varied by migration status at the island site ($P<0.001$, deviance = 24.11, d.f. =1), but we were also able to estimate that the odds of infection for migrant birds were 13.3 (95-percent confidence interval from 4.6 to 48.4) times greater than the odds for resident species ($P<0.001$, $z = 4.42$). On the mainland, there was also a difference in prevalence between migratory and resident birds ($P<0.001$, deviance 50.66, d.f. = 1); however, the pattern was reversed. Our findings indicate that the

odds of infection for migrants were 3.4 (95-percent confidence interval from 2.4 to 4.8) times less than the odds of infection for resident species.

In summary, our findings were similar to those of Super and van Riper (1995); however, our methods were more informative, allowing us to estimate effect sizes—an important step toward understanding statistical results in a biological context.

Discussion

Literature Review

Of the 70 articles in JWD that we reviewed, most used Pearson's chi-squared test of independence for contingency tables when analyzing count data for disease prevalence. We argue that techniques other than Pearson's chi-squared test would be more appropriate because chi-squared tests are one of the least informative of statistical tests (because of the lack of an estimated parameter that allows the user to describe the degree of dependence between the variables of interest; Ramsey and Shafer, 2002). Chi-squared tests are also limited by their ability to only determine independence between sets of variables and homogeneity of proportions. In the study of wildlife disease, the scientist is often interested in measuring infection as a response that is a function of one or more explanatory variables. Other methods, such as logistic regression and log-linear regression, allow the user to explicitly model the probability of infection given one or a number of explanatory variables, and associated parameter estimates can provide inference into the magnitude of these effects. In addition, as we have shown here, log-linear regression has greater statistical power than other techniques. Small sample sizes should dictate the use of this technique, all else being equal.

In our review of JWD papers we found that some articles reported data for studies in which pooled sample sizes were very small; 3 of the 70 articles reviewed had pooled sample sizes of less than 15. At these sample sizes, for small- to intermediate-effect sizes, the probability of type II errors approaches 100 percent. Under these situations, statistical hypothesis testing becomes meaningless, especially if there is any cost to committing a type II error. We argue that when sample sizes are very small other tests may be preferable—simply reporting descriptive statistics with associated confidence intervals, using methods more suited to such small sample sizes (for example bootstrapping methods; Efron and Tibshirani, 1993), or waiting to publish results until larger sample sizes become available. When dealing with critically endangered species, small sample sizes are often unavoidable. We believe that the findings presented herein demonstrate that hypothesis testing may not always be the best way to understand limited datasets.

Much of the scientific literature ignores the balancing of statistical errors with the real-world costs of type I and type II errors, and statistical methods often arbitrarily reduce the probability of a type I error at the expense of increasing type II errors (Di Stefano, 2003). Our literature review revealed that these concerns have also been largely overlooked in JWD, and that in at least a portion of the studies we investigated the potential costs of making type II errors could equal or outweigh the cost of type I errors. Making a type II error is failing to detect an existing harm that could lead to further endangerment of that species, and when focusing on a species of conservation interest, wildlife disease researchers have a greater responsibility to reduce the chance of this error.

We recognize that our efforts to accurately assess the relative costs of type II and type I errors in the work of others may be imperfect; the researcher is eminently more suited to evaluate these relativities in her or his own work. However, we hope that by addressing this issue here, disease researchers will take these issues into consideration when planning future studies.

Computer Simulation

From our study, we have generated basic guidelines for sample sizes in the analysis of prevalence and abundance data. Our computer simulation revealed that below N = 200, analysis of prevalence data lacks statistical power except for the largest effect sizes. For abundance data, there is also low power below N = 100. These findings, combined with results from our literature review, suggest that at least some of the articles published in JWD lacked sufficient statistical power. Furthermore, this conclusion may be conservative. First, our simulations used balanced sample sizes with one two-level factor in the design. A number of studies we reviewed in JWD had unbalanced designs, which are inherently less powerful. Also, for a given pooled sample size, as the number of factors increases from more than one, statistical power decreases. For these reasons, lack of statistical power may be more common than our study demonstrates. Our simulations did not attempt to duplicate the complexity of statistical design often used in JWD; therefore, we recommend that researchers conduct their own prospective power analyses before implementing a study design.

Our simulation results demonstrate that analyzing parasite count data is always more powerful than analyzing prevalence data, at least for N<1,000. When these counts are possible and feasible, such as in the study of macroparasites, abundance data should always be analyzed using the most appropriate methods. Furthermore, independently analyzing abundance and prevalence data from a given dataset is useful because abundance and prevalence describe the disease dynamics of host wildlife populations in different ways.

We found that, for abundance data, negative binomial regression is more powerful than some alternative methods, a finding also reported by Wilson and others (1996). However, this technique does have shortcomings and cannot be a panacea for the analysis of abundance data. For example, this method may not be suited for models in which a single dispersion parameter is fit to multiple combinations of terms in a complex model. When models are simple and sample sizes large, we recommend alternative techniques such as more complex nonlinear maximum likelihood methods and bootstrapping (Wilson and Grenfell, 1997; Newey and others, 2005). Analysis of dispersion also allows the user to account for variation in the dispersion parameter between combinations of model terms (Shaw and others, 1998).

Data Reanalysis

Our analysis of the contingency tables in Super and van Riper (1995) came to the same general conclusion made in their paper published in JWD. The goal of our data reanalysis was not to find fault with the original paper, but to show that alternative methods could provide further insight into those research findings. We reanalyzed the data to verify that chi-square tests will not always be the most informative statistical tool, and that researchers should estimate the observed effect size if at all possible. We argue that the use of an alternative method allows for a more informative exploration of the data. Instead of simply answering the question, "Does the prevalence of blood hematazoa depend upon geographic location?" our additional analyses allowed us to address a more complex and perhaps more biologically meaningful question: "To what degree does the prevalence of blood hematazoa depend upon geographic location?" We believe that if, instead of geographic effects, we were interested in the role of an anthropogenic effect on the prevalence of disease in an endangered species, it would be important to know not only if an effect exists, but also how large that effect is. Finding the size of the effect could be accomplished simply by estimating the difference between an anthropogenic treatment and control, then computing a confidence interval of the difference.

Understanding the biological importance of a statistically significant finding is important in bringing relevance to research in wildlife diseases, as is the interpretation of findings that fail to reject

the null hypothesis. The use of parameter estimates and associated confidence intervals can help in both cases (Steidl and others, 1997; Steidl and others, 2000). In our data reanalysis, estimates of the differences in the prevalence of disease between different geographic areas allowed us to interpret the magnitude of the observed effect, which then led to a discussion of the biological importance of that effect. Because wildlife disease workers collect data that contain biological information, it is incumbent on researchers to use these data to come to biological, not simply statistical conclusions (Steidl and others, 2000). Furthermore, when statistical inference fails to detect a difference, issues of statistical power come into play. Because assessing the power of a test retrospectively can be problematic (Gerard and others, 1998), confidence intervals should be used to guide inference when researchers fail to reject null hypotheses (Steidl and others, 1997; Gerard and others, 1998).

Finally, researchers need to keep in mind that unreliable or biased numbers work just as well as reliable ones when conducting statistical hypothesis tests. Many newer methods are being developed to increase the diagnostic reliability of estimating and analyzing measures such as prevalence (Senar and Conroy 2004; Heisey and others, 2006; Jennelle and others, 2007). While we have not addressed these methods in this paper, we recommend that wildlife disease researchers explore and implement new and emerging statistical techniques as the study of wildlife diseases plays a growing role in the conservation of wildlife species.

Conclusions

In summary, we offer several suggestions regarding the analysis of wildlife disease data: (1) Consider statistical power when designing and analyzing data in wildlife disease studies. If the costs of type II errors are potentially high, ensure that it is feasible to collect enough data to adequately answer the question at hand, and then use the statistical tests that have the most power. In determining necessary sample sizes, use the general guidelines provided in this paper or, better yet, use prospective power analysis to estimate the power of the proposed study. (2) If possible, collect and analyze data that will allow analysis of parasite abundance. Analysis of abundance is not only more powerful for a given sample size than analysis of prevalence, it also allows one to describe disease dynamics in an alternative way. (3) When possible, use statistical techniques that provide parameter estimates of the effect size observed in the study. Report parameter estimates along with confidence intervals, which allows both researchers and readers to assess the biological significance of reported findings.

In researching wildlife disease, scientists are faced with a wide array of statistical options for analyzing their data, and many of us strive to develop research programs relevant to wildlife management. With the complex dictates of research design comes the added responsibility to use appropriate statistical techniques and to maximize information transfer between the scientist and the user of scientific information. We hope that the discussion of statistical techniques in this chapter will help to inform and improve the design of future studies and the analysis of their data and interpretation.

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Appendix A. Chapter 1 Tables

Table 1-1. Parameter estimates generated from linear models of factors that affect swimming activity in male and female amphipods. Top: Factors related to the activity level of male amphipods. Results are from a multiple linear regression model (F = 3.86, d.f. = 4,113, P = 0.006). Bottom: Factors related to the activity level of female amphipods. Results are from a multiple linear regression model (F = 11.18, d.f. = 2,36, P < 0.001). Standard errors, test statistics, P-values and confidence intervals given in the table are those for the individual parameter estimate.

MALES

Factor	Parameter estimate	SE	t	P	95% C.I. Lower	95% C.I. Upper
Parasite						
Acanthocephala*	5.58	3.49	1.59	0.110	-1.33	12.49
Trematode*	-6.13	3.04	-2.01	0.046	-12.14	- 0.11
Both*	-8.013	4.68	-1.71	0.090	-17.28	1.26
Length	5.21	2.66	1.96	0.052	-0.058	10.48

* As compared to uninfected

FEMALES

Factor	Parameter estimate	SE	t	P	95% C.I. Lower	95% C.I. Upper
Parasite						
Trematode*	18.55	4.08	4.54	< 0.001	-26.84	-10.26
ln (Egg number)	-6.65	2.04	-3.26	0.002	-10.78	-2.51

* As compared to uninfected

Table 1-2. Parameter estimates generated by linear models of factors that affect δC and δN values in amphipods. Top: δC values varied by habitat, but not by sex or by parasitic infection. Results from an ANOVA model (F = 6.48, d.f. = 1,22, P = 0.018). Bottom: δN values varied by habitat, and there was weak evidence for variation with respect to sex. There was no difference between trematode-infected and uninfected amphipods. Results from an ANOVA model (F = 5.48, d.f. = 2,21, P = 0.01). Standard errors, test statistics, P-values and confidence intervals given in the table are those for the individual parameter estimate.

$\delta^{13}C$

Factor	Parameter estimate	SE	t	P	95% C.I. Lower	95% C.I. Upper
Limnetic*	-0.52	0.2	-2.54	0.018	-0.94	-0.1
Infected†	-0.20	0.2	-0.97	0.34	-0.62	0.22

* As compared to littoral

† As compared to uninfected

$\delta^{15}N$

Factor	Parameter estimate	SE	t	P	95% C.I. Lower	95% C.I. Upper
Limnetic*	0.32	0.11	2.85	0.009	0.088	0.56
Infected†	-0.13	0.11	-1.1	0.28	-0.36	0.11
Male‡	0.19	0.11	1.682	0.11	-0.045	0.4286

* As compared to littoral

† As compared to uninfected

‡ As compared to female

Table 1-3. Analysis of deviance tables from logistic regression models that tested the effect of two blocking variables (trial and chamber) and parasite infection status on the odds of capture by three nonhost invertebrate predators. For all three models the "parasite" term was nonsignificant, demonstrating that amphipod infection condition does not alter the odds of capture by the invertebrate predator.

Cybister

Factor	d.f.	Deviance	D.F	Residual Deviance	P
Experiment	1	0.17	46	83.15	0.745
Chamber	11	16.14	33	66.81	0.562
Parasite	2	0.19	44	82.95	0.944

Belastoma

Factor	d.f.	Deviance	D.F	Residual Deviance	P
Experiment	1	24.63	46	106.02	<0.001
Chamber	11	47.83	33	58.12	0.001
Parasite	2	0.09	44	105.94	0.972

Ranatra

Factor	d.f.	Deviance	D.F	Residual Deviance	P
Experiment	1	1.43	46	80.75	0.310
Chamber	11	26.43	33	54.11	0.059
Parasite	2	0.20	44	80.55	0.931

Table 1-4. Parameter estimates and associated hypothesis tests from quasi-likelihood logistic regression revealed no differences in the odds of capture of infected and uninfected amphipods

Factor	Parameter Estimate	S.E.	t	P
Trial				
Trial 2*	1.730	0.408	-1.340	0.249
Trial 3*	8.490	0.307	6.960	0.002
Parasite				
Trematode†	1.371	0.305	1.037	0.358
Acanth.†	0.601	0.326	-1.566	0.192

* As compared to trial #1

† As compared to uninfected

Appendix B. Chapter 2 Tables

Table 2-1. Parameter estimates of fixed effects, standard errors, 95-percent confidence intervals and p-values, test statistic values, and degrees of freedom for results from generalized linear mixed model of factors influencing waterfowl abundance. Values of parameter estimates and 95-percent confidence intervals are exponentiated to back-transform the log-transformed response variable and represent the multiplicative change in mean waterfowl number.

Factor	Parameter estimate	SE	95% C.I. Lower	Upper	P	χ^2	d.f.
Side[a]	1.80	0.12	1.49	2.25	< 0.001	27.6	1
Habitat[b]	13.7	0.2	9.97	17.11	<0.001	46.2	1
Year[c]	1.37	0.09	1.27	1.47	<0.001	69.8	1

[a] Far as compared to near

[b] Littoral as compared to limnetic

[c] 03-04 as compared to 04-05

Table 2.2 Parameter estimates of fixed effects, standard errors, 95-percent confidence intervals and p-values, test statistic value, and degrees of freedom for results from generalized linear mixed model of factors influencing the odds of trematode infection in the amphipod *Hyalella montezuma* for the two study periods, 2003-4 and 2004-5. Values of parameter estimates and 95-percent confidence intervals (C.I.) are exponentiated to back-transform the log-transformed response variable and represent the multiplicative change in the odds of infection. Lines are 95% C.I. for significant factors given in the text.

Year	Factor	Parameter estimate	SE	P	χ^2	d.f.
2003-2004	Side[a]	1.84	0.31	0.04	4.21	1
	Habitat[b]	1.03	0.84	0.97	0.00	1
	Depth	0.99	0.09	0.90	0.02	1
2004-2005	Side[a]	1.06	0.61	0.91	0.01	1
	Habitat[b]	0.62	1.35	0.22	1.49	1
	Depth	1.22	0.14	0.37	0.79	1

[a]Far as compared to near

[b] Littoral as compared to limnetic

Appendix C. Chapter 3 Table

Table 3-1 Summary of analysis type and statistical techniques of 70 papers presenting research in which the authors conducted hypothesis tests about difference in mean prevalence or abundance in the Journal of Wildlife Diseases from 2000 to 2004.

Analysis type	Count	% of total
Prevalence	67	96
Abundance	17	24
Both	14	20
Total	70	
Prevalence		
Two-sample t-test/ANOVA	4	5.7
G-test/log-linear	5	7.1
non-parametric rank test[a]	2	2.8
Chi-squared/Fisher's exact	44	63
Logistic regression	15	21
Total	70[c]	
Abundance		
Two-sample t-test/ANOVA	4	24
GLM - negative binomial	1	5.9
non-linear regression	1	5.9
non-parametric rank test[b]	7	41
None	4	24
Total	17	

[a] Kruskal-Wallis

[b] Kruskal-Wallis, Wilcoxin Mann-Whitney

[c] This number greater than total number of studies that analyzed prevalence because some studies used more than one methodology to analyze prevalence data.

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