

Article

Environmental Correlates of Prevalence of an Intraerythrocytic Apicomplexan Infecting Caribbean Damsel fish

Akacia K. Halliday-Isaac ¹, Jennilee B. Robinson ², Edwin Cruz-Rivera ^{1,2} , Andrew G. Campbell ³ 
and Paul C. Sikkell ^{4,5,*} 

¹ Master of Marine and Environmental Science Program, College of Science and Mathematics, University of the Virgin Islands, St. Thomas, VI 00802, USA; akacia.halliday@gmail.com (A.K.H.-I.); edwin.cruzrivera@uvi.edu (E.C.-R.)

² Department of Biological Sciences, College of Science and Mathematics, University of the Virgin Islands, St. Thomas, VI 00802, USA; jennilee.beth@gmail.com

³ Department of Molecular Microbiology & Immunology, Brown University, Providence, RI 02912, USA; Andrew_Campbell@brown.edu

⁴ Department of Biological Sciences, and Environmental Sciences Program, Arkansas State University, Jonesboro, AR 72467, USA

⁵ Water Research Group, Unit for Environmental Sciences and Management, North-West University, Potchefstroom 2520, South Africa

* Correspondence: paul.sikkell@gmail.com



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Abstract: Parasites are an integral part of coral reef ecosystems due to their influences on population dynamics, biodiversity, community structure, and food web connectivity. The Phylum Apicomplexa contains ubiquitous animal associates including the causative agents of globally important human diseases such as malaria and cryptosporidiosis. Despite their ubiquity, little is known about the biology, ecology, or distribution of these microorganisms in natural animal populations. In the US Virgin Islands, the dusky damselfish (*Stegastes adustus*) had a high but variable incidence of a *Haemohormidium*-like blood apicomplexan among 30 sites sampled. Microscopic analyses of blood smears allowed us to group these fish as infected, having low intensity infections, or uninfected. Regression analyses detected no significant differences in the condition indices (expressed as length–mass ratio). However, infection was clearly associated with potentially extremely high leukocyte counts among infected *S. adustus* that were not seen in uninfected fish. These results suggested the potential for some impact on the host. Linear mixed effects models indicated that *S. adustus* population density and meridional flow velocity were the main predictors of apicomplexan prevalence, with presence of other *Stegastes* species, population distance from watershed, zonal flow velocity, the complexity of the surrounding habitat, and season not showing any significant relationship with fish infection.

Keywords: apicomplexan; damselfish; Caribbean

1. Introduction

Parasites are diverse and abundant, reaching high biomass in natural communities [1] and comprising up to 80% of organisms in tropical coral reefs [2]. Parasites derive nutrients from their hosts and, by extension, have a potentially negative impact on infected organisms, influencing population dynamics, biodiversity, community structure, and food web connectivity (e.g., [3–5]). Generalist parasites can reduce biodiversity in habitats by mediating apparent competition [6,7], while specialist parasites can increase biodiversity by limiting the abundance of dominant species [6–9]. Parasites also mediate trophic cascades; increase food web connectivity through their interactions with their host, its predators, its prey, and other parasites; and contribute substantially to ecosystem energy budgets [10–13]. Due to the sensitivity of specific parasitic groups to certain pollutants, parasites may also be used as indicator species in aquatic systems [14–17].

Although parasites are largely defined by their negative effects on hosts such as reducing host immune response, competing with the host for nutrients, and releasing toxins that affect host fitness [18], some organisms considered parasites may cause no measurable effect or may even provide surprising benefits to the host. Some of these positive effects include supplying hosts with compounds that complement deficient diets, aiding digestion by breaking up large food pieces, or reducing pollutant levels within infected hosts in stressful environments [14,19]. As such, the definition of an organism as a parasite requires an assessment of overall costs and benefits to the host.

The Apicomplexa are a group of unicellular organisms collectively classified as parasites, known for their distinctive elongated shape and a structure for penetrating host cells called the apical complex. These protists are obligate intracellular parasites with a wide array of terrestrial and marine hosts [20,21] including important human parasites such as *Plasmodium falciparum* and *Toxoplasma gondii* [22]. Despite the documented ecological and economic importance of apicomplexan infections in mammals and birds, fish apicomplexan research is still in its infancy (e.g., [23–26]), and the taxonomy of many species is uncertain, precluding comprehensive assessments of species diversity and phylogeny. For example, Xavier and coauthors [25] noted that the genetic diversity of coccidia (a subgroup of Apicomplexa) infecting fish has been largely neglected, despite evidence for their widespread occurrence. *Haemogregarina bigemina* is reported to infect 96 species of host fishes, representing 70 genera and 34 families worldwide [27]. However, new research suggests that *H. bigemina* may be a species complex and that this parasite is not a haemogregarine [26]. Other apicomplexans such as *Haemogregarina rubrimarensis*, which has been reported from 104 fish species from the Red Sea using microscopic observations [28], could be complexes of cryptic species, as well. In addition to mode of transmission, the host physiological effects, environmental predictors, and ecological consequences of apicomplexan infections in fishes also remain largely unknown.

Damselfishes of the family Pomacentridae are small- to medium-sized demersal fishes common in tropical and subtropical marine habitats [29,30]. They include planktivores, herbivores, and omnivores inhabiting all areas from shoreline to deep-reef structures [29,30]. While some species defend permanent multipurpose territories, in others, only the males are territorial when defending nests. Given their small size and abundance, they are a common prey source for piscivorous predators (e.g., [31–33]). Damselfishes play an important role in coral reef ecosystem dynamics as determinants of benthic structure [34–36]. In the Caribbean, pomacentrids include members of the genera *Chromis*, *Abudefduf*, *Stegastes*, and *Microspathodon*. In a recent study of Caribbean reef fishes, Cook and coauthors [23] reported that 14 species of fishes could become infected with eight distinct haemoparasites. They noted that a “*Haemohormidium*-like” infection was found in all six species of Caribbean *Stegastes* damselfishes. While it was common in the five benthophagous species (those that eat off the bottom), it was rare in the one species that was a planktivore, *S. partitus* [37]. It was also not found in any other genera of Caribbean damselfish. Analysis of phylogenetic relationships and geographic distribution of this protozoan across the Caribbean confirmed its presence in *S. adustus* as small as 2.9 cm [25,37].

In both studies, prevalence among infected species was highly variable over large spatial scales. However, finer-scale variation, the factors contributing to this variation, and the potential effects on hosts were not examined. As the next step in understanding the ecology of this apicomplexan, this study mapped fine-scale prevalence of the protozoan and drew inferences about the environmental factors influencing its prevalence in the US Virgin Islands. We also determined whether the prevalence and intensity of apicomplexan infections were related to two measures of fish health: condition factor and leucocyte count.

2. Results

Among a total of 387 *Stegastes adustus* sampled, we found apicomplexans, identical to those reported in our previous studies, inside the erythrocytes (A1) of 90.

2.1. Infection Prevalence and Host Condition

Comparing 90 infected fish and 297 uninfected fish screened, there was no significant difference in condition index when “high intensity”, “low intensity”, and “uninfected” *S. adustus* were compared ($p = 0.699$, ANCOVA; Figure 1).

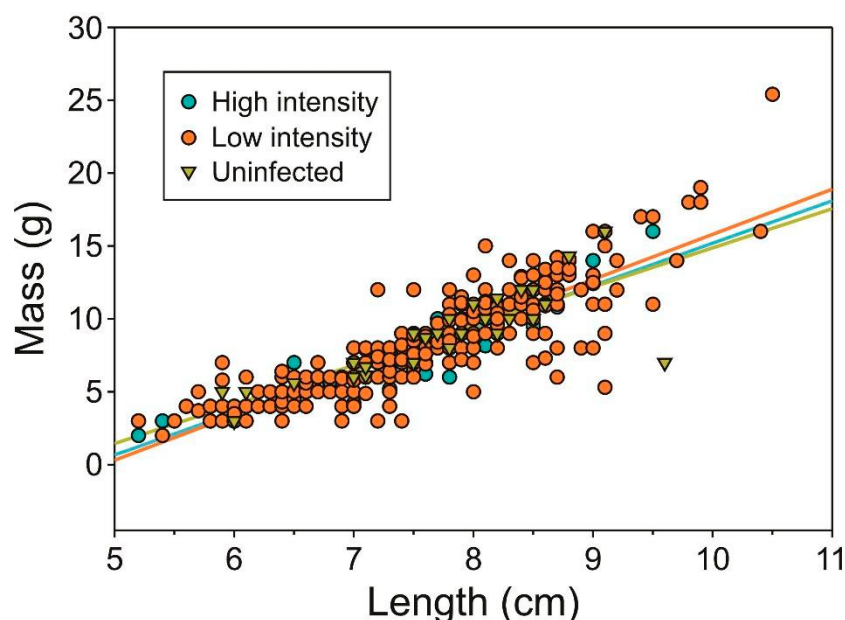


Figure 1. Length–mass relations (condition indices) for *Stegastes adustus* grouped by infection status. Regression lines for each group are presented. All 387 fish are included, but symbols overlap due to similarities in fish sizes among groups.

Infection burden (% of infected cells) was low, with 0–0.5% of erythrocytes from 50 slide images of fish blood per fish deemed infected after 40 min of screening. Although ANCOVA revealed overall differences in leukocyte number among the three groups ($F = 3.25$, $df = 2$, $p = 0.043$), there was no relationship between length or mass and leukocyte count (all $p > 0.779$). Levene’s test revealed significant differences in within-group variation among the three infection groups ($F = 5.13$, $df = 2$, $p = 0.008$). This was attributable to the low variation within the uninfected group, as post hoc comparison revealed nearly identical, high variation between “high intensity” and “low intensity” infection groups ($p = 0.999$). Bootstrap calculations revealed (mean (95% CI)) values in “high intensity” [54.93 (38.17, 71.58)] and “low intensity” [54.54 (30.42, 78.37)] groups that were nearly identical to one another, but more than double those of “uninfected” [24.96 (21.53, 28.4)] fish. Thus, leukocyte counts tended to be higher and more variable in infected versus uninfected fish (Figure 2). In particular, uninfected fish had no extreme high counts, whereas many infected fish did. When examining the relationship between infection level and leukocyte count within the “high intensity” group, there was a tendency for fish with more infected erythrocytes to have more leukocytes ($t = -1.8438$, $df = 38$, $p = 0.073$).

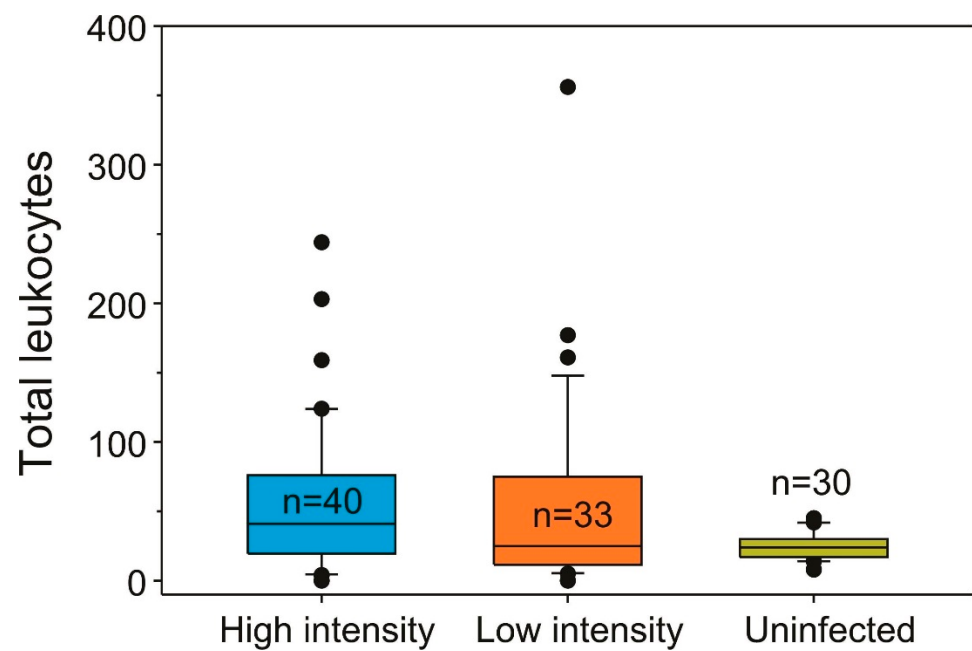


Figure 2. Comparison of leukocyte counts in relation to *S. adustus* infection status. Outliers are indicated by the black dots.

2.2. Factors That Predict Prevalence

Infection prevalence differed significantly among sites ($p < 0.001$, chi-square; Figures 3 and A2 in Appendix A), ranging from 0 to 87%. The highest frequencies of infected fish were observed in the two Fredericksted sites and Rock Beach in St. Croix (>50% infection, Figure A2). Black Point and Brewers Bay East in St. Thomas and Chenay Bay West in St. Croix had infection rates at, or above, 40%.

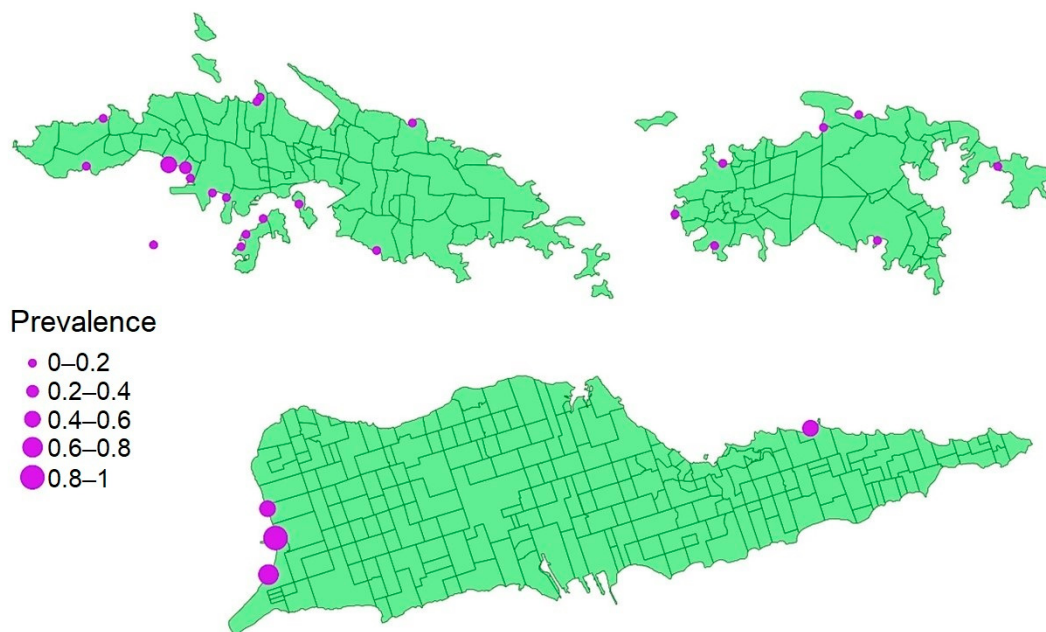


Figure 3. Map of the prevalence of the *Haemohormidium*-like apicomplexan protozoan in *S. adustus* populations from the US Virgin Islands. The size of circles indicates the proportion of infected fish in the sampled population. An interactive version of this map is available online at <https://arcg.is/0nra4f> (accessed on 24 April 2021).

The sites where *S. adustus* populations were sampled were composed primarily of hard bottoms and sand (Supplementary Figure S1). Coral was present in approximately 67% of the sites and seagrass, and sponges were present in approximately 33% of the sites. Rubble was present in <25% of the sites. To elucidate potential environmental drivers of apicomplexan infection in the fish, a linear mixed effects model was used. Stepwise procedures (Supplementary Table S1) determined that apicomplexan prevalence in *S. adustus* correlated with total conspecific population density ($p = 0.048$) and average meridional velocity (a measurement of horizontal northward or southward flow) ($p < 0.003$). Although AIC results also showed that distance of the *S. adustus* population from the nearest watershed increased model predictive value, the contribution of this variable was negligible ($p = 0.165$; Table 1).

Table 1. Results from the linear mixed effects model (left) and the selected best fit model (right). Both models are based on $n = 30$ observations.

Model	Linear Mixed Effects R ² /R ² Adjusted = 0.499/0.236 Prevalence				Selected Best Fit R ² /R ² Adjusted = 0.399/0.329 Prevalence		
	Predictors	Estimates	CI	<i>p</i>	Estimates	CI	<i>p</i>
(Intercept)		0.97	−0.71–2.65	0.241	−0.03	−0.30–0.24	0.835
<i>S. adustus</i> population density		0.01	−0.08–0.09	0.864	0.01	0.00–0.03	0.048
<i>Stegastes</i> spp. population density		0	−0.03–0.02	0.833			
Habitat complexity		−0.43	−2.01–1.16	0.580			
Average zonal velocity		8.46	−8.33–25.25	0.305			
Average meridional velocity		−10.49	−34.97–13.98	0.381	3.36	1.24–5.49	0.003
Distance from nearest watershed		−15.93	−44.65–12.79	0.260	−8.12	−19.78–3.55	0.165
Average zonal velocity × average meridional velocity		−172.65	−502.87–157.57	0.288			
Average meridional velocity × distance from nearest watershed		89.50	−298.56–477.56	0.635			
<i>S. adustus</i> population density × <i>Stegastes</i> spp. population density		0	−0.00–0.00	0.918			
<i>S. adustus</i> population density × habitat complexity		0.02	−0.08–0.11	0.711			

3. Discussion

In this study, the prevalence of a *Haemohormidium*-like blood apicomplexan in the Caribbean damselfish *S. adustus* differed among sites and was correlated with *S. adustus* population density and the average meridional flow velocity of the area where the population was located. There was clearly no relationship between one measure of fish health, condition factor, and infection. However, for the second, leucocyte count, there was the potential for extreme high counts for infected but not uninfected fish.

The means by which Caribbean *Stegastes* damselfish acquire this blood-borne apicomplexan remains uncertain. Both leeches and blood-feeding gnathiid isopods have been implicated in the transmission of some blood-borne apicomplexans in marine fishes [38–40]. At our study sites, leeches are rare and we have yet to find them on *Stegastes* we have collected. Similar apicomplexan blood parasites found in blennies have also been found in parasitic feeding stages of gnathiids [23,41]. These gnathiids commonly infest *Stegastes* as well [42–44]. However, whether gnathiids are involved in transmission in *Stegastes* has not been experimentally verified. Given that *Stegastes* live in “colonies” and physically contact each other during mating or territorial behavior, it is possible that transmission occurs directly. Transmission could also occur through an oral–fecal route. Coprophagy is

common among reef fishes [45], and all but one Caribbean *Stegastes* are benthophagous and so could ingest infective stages incidental to feeding. At least some species of Caribbean *Stegastes*, including *S. adustus*, have been shown to intentionally defecate at the margins of their territorial boundaries [46], facilitating the transfer of fecal matter to neighboring conspecifics. Because higher population densities would facilitate transfer of infective stages through any of these mechanisms, the relation between infection prevalence and population density of hosts is not surprising. This density-dependent transmission has been studied using epidemiological models [47–52] and has been documented broadly in terrestrial and aquatic organisms for both unicellular and metazoan parasites [53–60].

Although the apicomplexan occurs in other *Stegastes* species, density of congeners that overlapped in distribution with *S. adustus* did not appear to affect infection prevalence for this host. These other species are infected less often than *S. adustus*, which could explain this finding [37]. However, simple population overlap of *Stegastes* spp. as a function of total individuals may obscure important behavioral subtleties that also affect infection. How *S. adustus* interacts over time with other *Stegastes* spp. in our field sites is not well known (e.g., [61]), but work on *S. adustus* territoriality elsewhere shows that intruder identity, size, and number interact to modulate aggressive encounters [62,63]. Social interactions and their seasonality can also modify disease transmission risk in animals [58–60]. Seasonality, however, was not a significant factor in our model. Work on parasite–host networks has highlighted strong context dependence in shared-parasite transmission: the same host species may be a strong driver for parasite spread at some sites but not others, depending on community structure [64,65]. While molecular data show the apicomplexan studied here infects almost all Caribbean *Stegastes* [25,37], no information on species-specific susceptibility or cross-species transmissibility of these blood microbes exists.

Average meridional current velocity (a proxy for northward or southward flow) was also positively correlated with prevalence. This suggests that infection probability is modified by shallow currents. Some modeling studies have concluded that high current velocity may reduce parasite–host encounter rate and contact time between infected and non-infected conspecifics within populations, but faster flows will also disperse water-borne parasites faster and more broadly, increasing risk to populations distant from a pathogen source [66,67]. Flow may also affect parasitism at microhabitat scales. Some fish parasites are less successful at attaching to fast swimming hosts compared to hosts generating slower flow fields around their bodies [68]. In contrast, flow increase within certain thresholds can enhance infection risk by triggering specific swimming behaviors in infective parasite larvae [69]. Current velocity also modifies the effects of pollutants on aquatic organisms [70,71], which can potentially result in complex host dynamics with parasites [14–16,72]. Interestingly, zonal flow (eastward or westward) was not correlated with infection. The difference in apparent effects between meridional versus zonal flow may in part be attributable to the orientation and topography of the islands and the distribution of our study sites. For all islands included in our study, the east–west axis was longer than the north–south, and most of our sites were located in coves or embayments along the north or south shore of the island. Thus, they were largely protected from currents that run parallel to shore but not currents that run perpendicular to shore.

Other fish stressors, such as terrestrial runoff, may also have an impact on infection prevalence in nearshore reef ecosystems. For example, terrestrial runoff may decrease recovery rates of infected individuals by introducing pollutants and affecting osmoregulation, thus suppressing immune responses to parasites [73]. For aquatic species, this phenomenon has been studied with particular interest in fisheries and aquaculture, with models aiming to detect host density thresholds that could result in disease outbreaks [53,74,75]. We used population distance from closest watershed as a proxy for this possibility in our model. While this variable enhanced the overall accuracy of our model, it had little explanatory power on infection patterns measured.

Parasites can affect growth and host energy allocation to different metabolic processes. However, a key limitation of correlative studies is that the most susceptible and heavily impacted hosts may die soon after infection and therefore be lost from the sampling population. The most resistant may, in contrast, show only a weak response to infection and/or be able to tolerate a heavy infection level, giving the false impression that the parasite has little impact on hosts [76]. Thus, given that there were multiple factors that could influence our measures of fish health, and that infection burden (% of infected cells) was also very low (0–0.5% of cells present), it was not surprising that there was no relationship between condition factor, a commonly used proxy for fish health [77–80], and infection level. However, the fact that infection was clearly associated with potentially extremely high leukocyte counts suggested some impact of this apicomplexan on hosts.

Though commonly regarded as parasitic, apicomplexans such as *Sarcocystis* are considered commensal or mutualistic in their terrestrial hosts [81], while others such as *Nephromyces* can be beneficial symbionts in marine animals by breaking down metabolic waste products inside the host [82]. Recently, the discovery of a symbiotic apicomplexan of corals has promoted a reassessment of the coral–zooxanthellae symbiosis [83,84]. It is important to note here that the *Haemohormidium*-like apicomplexan could affect other life history or ecological processes not quantified in this study. Damselfish compete strongly for space, and the outcome of these territorial interactions could be influenced by the presence of the blood protozoan (i.e., apparent competition; see [85]). Additionally, survivorship, growth rates, gonadosomatic index, or egg hatching success could not be quantified directly in this field-based study, all of which would provide more comprehensive assessments of fitness than condition indices based on length–mass ratios. Nonetheless, the label of “parasite” is automatically assumed for blood-inhabiting protozoans with often little ecological justification. The argument of costs and benefits in classifying symbiont host interactions goes beyond parasitism and has led to reassessment of other seemingly well-documented marine symbiotic associations [86,87]. Further research in this area will be needed to determine any long-term host effects the apicomplexan may have.

4. Materials and Methods

Sampling for this study was conducted from April 2017 to March 2019 in the U.S. Virgin Islands (18.3358° N, 64.8963° W). *Stegastes adustus* damselfish were collected at each of 30 sites across the three main islands, St. Thomas, St. John, and St. Croix, and two minor islands outlying St. Thomas (Water Island and Hassel Island). Thirteen populations were assessed in St. Thomas, seven in St. John, five in St. Croix, four in Water Island, and one in Hassel Island for the presence of *Haemohormidium*-like blood protozoans.

At each collection site, the GPS coordinates of the sampled population were recorded. To estimate population density at each site, a 25 × 2 m² belt transect survey was performed by swimming the length of the transect and recording the number of all *Stegastes* spp. of damselfishes. Every 5 m along the transect, a 1 m² quadrat was placed alongside the transect tape, and the approximate percent cover of different benthic habitat components within each quadrat (hard bottom, sand, coral, seagrass, rubble, and sponges) was recorded. At each site, adult *S. adustus* were caught at depths between 1–2 m. Sample sizes at each site varied due to population density and catching difficulty. Fish were caught using modified cast nets as described by Sikkel and coauthors [43]. After collection, fish were held in a bucket containing fresh seawater and an aerator. A blood sample was taken within 24 h of capture. Captured fish were anesthetized by placing them individually in a 3 L seawater bath, containing 1 mL of a clove oil solution made of 9 parts 94% ethanol and 1 part clove oil, for 2–5 min. Anesthetized fish were then placed on a soft dry cloth, and fork length and mass were recorded. Blood was drawn from the sinus venosus following the methods described by Sikkel and coauthors [37], and a drop was immediately smeared onto each of two replicate labeled glass microscope slides. Blood smears were fixed with 100% methanol and stained for 45 min using a 3% dilution of Giemsa stain (Sigma-Aldrich, St. Louis, MO, USA) and water buffered to pH 7.2 with monopotassium phosphate and disodium

phosphate. As in previous studies [23,25,37], these smears were screened for the presence of cells containing any apicomplexan stages using light microscopy. The protozoan found in *S. adustus* blood in this study matched the morphology of the “*Haemohormidium*-like” “haemoparasite” described by Cook et al. and Sikkal et al. [23,37] (Figure A1). A fish was considered uninfected if no infected cells were found after 40 min of screening. The use of blood smears rather than organ samples allowed for non-lethal sampling. Light microscopy was deemed more efficient for this study than the use of molecular probes and has been shown to yield similar results [88]. Sampled damselfish were allowed to recover in aerated buckets with fresh seawater and returned to their site of origin within 24 h. ArcGIS was used to map sampling locations and infection prevalence among islands (see below). Contingency table analysis (chi-square) was used to compare percentages of infected fish among sites.

A subset of the infected and uninfected fish ($n = 30\text{--}40$) were further analyzed for infection level and possible immune response. These fish were chosen randomly. Fifty representative slide images were taken from blood smears for each fish, starting from the top right corner of the slide and continuing in a grid pattern. The total number of erythrocytes (red blood cells), the number of apicomplexan-infected erythrocytes, and the total number of leukocytes (white blood cells) present were counted using a cell counting macro in Image J (version 152.a). The number of infected erythrocytes divided by the total number of erythrocytes was used to calculate the intensity of infection within the fish (% of infected cells). A portion of the fish previously determined to be infected during initial, timed screening (which screened a higher proportion of the blood smear than the quantitative screening) had no infected cells detected during this further screening of the 50 images, resulting in a category called “low intensity”. These were interpreted as having very low numbers of the blood apicomplexan. In total we analyzed 40 “high intensity”, 30 “uninfected”, and 33 “low intensity” fish.

To assess the potential impact of the microbe on fish hosts, we compared fish condition index and leukocyte counts from blood smears among the three infection-level categories (“high intensity”, “low intensity”, and “uninfected”). Condition index was assessed as the ratio of mass to fork length [78,79,89]. We performed an analysis of covariance (ANCOVA) to compare condition indices for the three infection-level groups. We also conducted an ANCOVA to determine whether body size was correlated with leukocyte count among the three groups. Based on the results of that analysis (see Results), we excluded measures of body size from further comparison of leukocyte counts. Initial examination of the data revealed consistently lower and more uniform (less variable) leukocyte counts among uninfected fish and more variable counts among infected fish. We therefore conducted three subsequent analyses to help understand the relationship between infection and leukocyte count. These included a Levene’s test of variance, comparison of Bootstrap means and 95% CI (5000 iterations), and correlational comparison of level of infection and leukocyte count within the “high intensity” group.

The prevalence of *Haemohormidium*-like protozoans in all 30 *S. adustus* populations along with key biotic and abiotic variables were mapped using ArcGIS 10.1. A prevalence map was created utilizing a base map (at the scale 1:25,000; source: USVI Estate Boundaries by FEMA) and a hydrology layer (at the scale 1:25,000; source: Coastal Zone Management, USVI Department of Planning and Natural Resources). To determine the role of population and environmental variables on damselfish infection, apicomplexan prevalence was modeled against *S. adustus* population density, total *Stegastes* spp. population density, predominant benthic habitat at each site (see above), distance from a watershed (calculated using the near analysis tool in ArcGIS), and average zonal (east- or westward) and meridional (north- or southward) flow velocities (provided for 2016 by the UVI Oceanographic Research Laboratory for each GPS point using an ocean velocity model). Due to the time length of the sampling, our model also included time of year (“season”) as a categorical variable (Spring: March–May, Summer: June–August, Fall: September–November, and Winter: December–February). A stepwise linear mixed effects

model was chosen to evaluate the influence of all these factors on apicomplexan prevalence among damselfish populations. Season was considered a random effect, while all other variables were treated as fixed effects. Similar modeling approaches have proven useful for understanding disease risk in other fish taxa [90–92]. To determine the relevance of individual and interacting variables in predicting apicomplexan infection, stepwise model iterations were evaluated using the Akaike information criterion (AIC) (Supplementary Table S1). All statistical analyses were carried out in R version 3.5.0.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/parasitologia1020009/s1>, Figure S1: Habitat composition of each of 30 sampled sites in the US Virgin Islands from which *Stegastes adustus* were collected, Table S1: Model selection table.

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Institutional Review Board Statement: The study was approved by the Institutional Animal Care and Use Committee of the University of the Virgin Islands (IACUC 1063996-1 07-17-2017).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are openly available in GitHub at <https://github.com/akaciah/Environmental-correlates-of-prevalence-of-an-intraerythrocytic-apicomplexan> (accessed on 18 April 2021).

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Appendix A

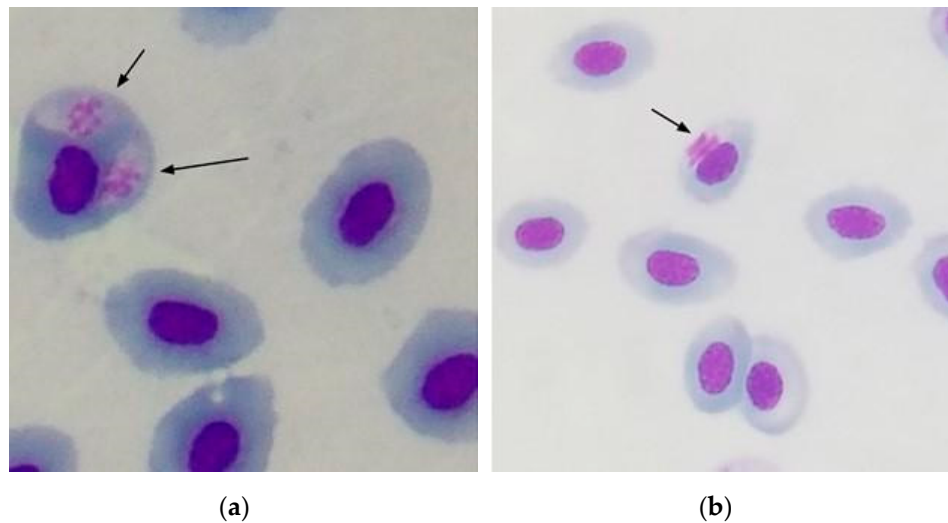


Figure A1. Images of Giemsa-stained erythrocytes cells taken from *Stegastes adustus* caught during this study at 1000 $\times$. (a) The morphology of pale purple areas of loose chromatin contained within opaque white cytoplasm as described in Cook et al. (2015). Arrows indicate infected erythrocyte. (b) The morphology of slender rods contained within an opaque white cytoplasm as described in Cook et al. (2015). Arrow indicates infected erythrocyte.

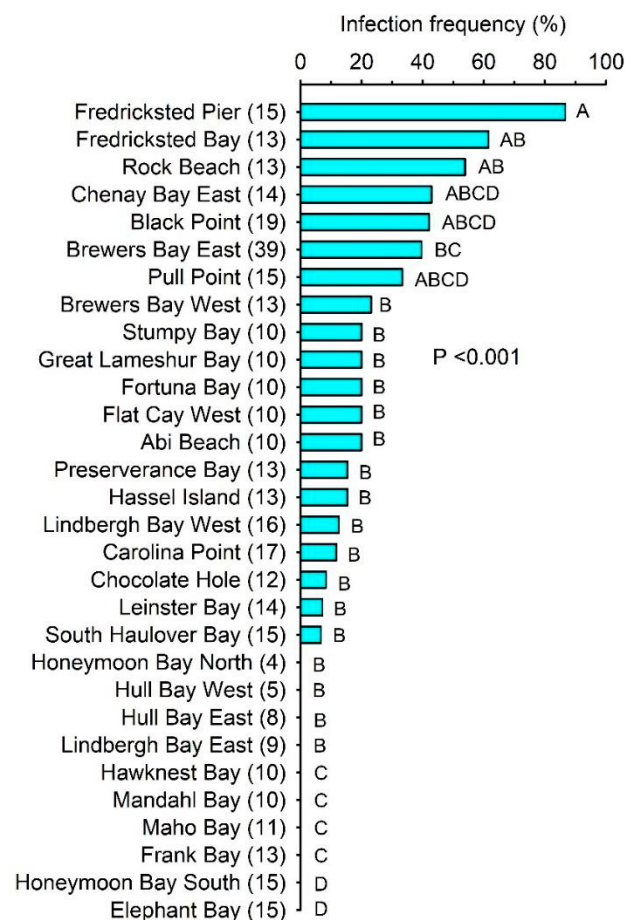


Figure A2. Infection prevalence of *Haemohormidium*-like apicomplexa in the damselfish *S. adustus* in 30 sampled populations from the US Virgin Islands. Numbers in parentheses after location names indicate sample sizes. Analysis was performed using chi-square corrected for the number of comparisons. Bars sharing same letters are not significantly different.

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