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To the Graduate Council:

I am submitting herewith a dissertation written by Kathryn Erin Purple entitled "Investigation of the potential role of bird baths in the transmission of *Trichomonas gallinae* in wild birds." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Richard W. Gerhold Jr., Major Professor

We have read this dissertation and recommend its acceptance:

Debra L. Miller, Marcy J. Souza, Mary Katherine Tolbert

Accepted for the Council:

Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

Investigation of the potential role of bird baths in the transmission *Trichomonas gallinae* in wild birds

A Dissertation Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Kathryn Erin Purple

August 2018

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ABSTRACT

Understanding the role of bird baths and shared water sources in the ecology and epidemiology of the deadly protozoan parasite *Trichomonas gallinae* is crucial to identify mitigation strategies for population-limiting epidemics. We evaluated multiple factors that influence the transmission of this pathogen including characterizing persistence in simulated bird baths, evaluating potential effects persistence may have on virulence, and investigating the molecular epidemiology of a concurrent outbreak. Trichomonads were thought to be extremely labile in the environment since their discovery over 200 years ago to 5 years ago when maximum recorded persistence in water was 20 min. We show that trichomonads persist up to 48 hr in simulated bird baths at 37°C with organic material (OM). We also measured persistence in a variety of conditions including, with and without organic material (4 and 16 hr, respectively), with artificially decreased dissolved oxygen (30 hr), and with exposure to UV light (4hr). Moreover, when cytopathic effect (CPE) of post-persistence trichomonads on cultured avian cells was compared to non-persistence control isolates, virulence changed significantly by treatment or persistence time. Post-UV treated persistence broad-winged hawk isolate destroyed significantly more of the cell monolayer than those from the OM treatment illustrating that persistence type can alter virulence. Currently, live animal or cell culture infection trials are the only tool to define virulence of an isolate; however, the more we understand the phylogenetics and epidemiology of *Trichomonas* spp, the better we can contribute phenotypic differences to genetic data. Using molecular techniques, we showed that circulating genotypes in subclinical hunter-killed doves differ from birds with lesions consistent with trichomonosis. Therefore, hunter-killed doves may not be the source of deadly strains circulating in other sympatric species. Combining laboratory work with the parasite, assessment of virulence on cell culture, and molecular techniques we have shown that the role of bird baths and water sources in transmission cannot be underestimated when addressing outbreaks and that molecular information will continue to improve our effects to mitigate the disease in wild birds.

TABLE OF CONTENTS

CHAPTER I INTRODUCTION 1
ABSTRACT 2
LITERATURE REVIEW 3
CHAPTER II *TRICHOMONAS GALLINAE* PERSISTENCE IN FOUR WATER TREATMENTS 12
ABSTRACT 13
SHORT COMMUNICATION 14
Acknowledgments 19
CHAPTER III PERSISTENCE OF TWO ISOLATES OF *TRICHOMONAS GALLINAE* IN SIMULATED BIRD BATHS WITH AND WITHOUT ORGANIC MATERIAL 20
ABSTRACT 21
INTRODUCTION 22
MATERIALS AND METHODS 23
Statistical analysis 24
RESULTS 24
DISCUSSION 27
Acknowledgments 27
CHAPTER IV ARTIFICIALLY DECREASED DISSOLVED OXYGEN INCREASES THE PERSISTENCE OF *TRICHOMONAS GALLINAE I*N WATER 29
ABSTRACT 30
INTRODUCTION 31
RESULTS 32
DISCUSSION 35
MATERIALS AND METHODS 38
Measuring DO levels with added OM 38
Measuring DO with added Oxyrase® 39
Measuring persistence of trichomonads in water with decreased DO 39
Acknowledgements 40
CHAPTER V EVALUATION OF THE CYTOPATHIC EFFECT OF *TRICHOMONAS GALLINAE* BEFORE AND AFTER PERSISTENCE IN SIMULATED BIRD BATHS USING AVIAN FIBROBLASTS IN CELL CULTURE 41
ABSTRACT 42
INTRODUCTION 42
MATERIALS AND METHODS 43

Parasites 43
Persistence Experiments 44
Cell culture 45
Infection trials 46
Assessment of CPE 46
Statistical Analyses 47
RESULTS 47
DISCUSSION 52
CHAPTER VI MOLECULAR INVESTIGATION OF *TRICHOMONAS* SPP. FROM CLINICALLY AFFECTED BIRDS AND SUBCLINICAL HUNTER-KILLED COLUMBIFORMES IN CALIFORNIA 56
ABSTRACT 57
INTRODUCTION 57
MATERIALS AND METHODS 59
Culture for subclinical hunter-killed birds 59
Tissue from suspected infected birds 60
Molecular Analyses 60
RESULTS 61
Culture of hunter-killed birds 61
Molecular 61
DISCUSSION 64
CHAPTER VII CONCLUSION 77
REFERENCES 81
APPENDIX 89
SUPPLEMENTAL DATA 90
VITA 91

LIST OF TABLES

TABLE II-I. *TRICHOMONAS GALLINAE* PERSISTENCE OVER TIME IN VARIOUS WATER TREATMENTS. 17

TABLE II-II. WATER PH MEASUREMENTS. 18

TABLE III-I. *TRICHOMONAS GALLINAE* PERSISTENCE OVER TIME IN TWO DIFFERENT WATER TREATMENTS USING TWO ISOLATES.. 26

TABLE IV-I. DISSOLVED OXYGEN (DO) WITH ORGANIC MATERIAL (OM). 33

TABLE IV-II. DISSOLVED OXYGEN (DO) SATURATION AND PH (WITH STANDARD DEVIATION) WITH OXYRASE ®. DO AND PH OVER TIME AFTER THE ADDITION OF DIFFERENT CONCENTRATIONS (% VOL/VOL) OXYRASE ® TO 500 ML DISTILLED WATER IN PLASTIC CONTAINERS.. 36

TABLE V-I. PERSISTENCE RESULTS. 48

TABLE VI-I. CULTURE RESULTS. 70

TABLE VI-II. *TRICHOMONAS* SPP. ISOLATES FROM CALIFORNIA AVIAN TISSUE SAMPLES.................................... 71

TABLE VI-III. MOLECULAR RESULTS 72-77

TABLE A-I. OXYRASE AND PERSISTENCE. 90

LIST OF FIGURES

Figure III-I. Persistence of trichomonads. 25
Figure IV-I. Organic material and resulting dissolved oxygen. 34
Figure IV-II. Isolate persistence. 37
Figure V-I. Bird bath treatment differences. 50
Figure V-II. Changes to cell coverage after infection. 51
Figure VI-I. Range map. 67
Figure VI-II. Molecular Phylogenetic analysis based on ITS by Maximum Likelihood method. 68
Figure VI-III. Molecular Phylogenetic analysis based on FeHyd by Maximum Likelihood method. 69

CHAPTER I

Introduction

Abstract

Trichomonosis, also known as frounce or canker, is a historic disease that continues to have significant negative impacts on individuals and wild bird populations around the world. Since the discovery of the causative agent of trichomonosis, *Trichomonas gallinae*, in the late 1800s many characteristics of the protozoan parasite have been well established. These eukaryotic cells are approximately 5 by 10 µm, have four anterior flagella and an undulating membrane termed recurrent flagellum. Like other members of Trichomonidae, mitochondria have been replaced with hydrogenosomes, which are better suited to produce energy in microaerophilic environments in which the organisms thrive, such as the luminal spaces of hollow organs in animals. The primary host of *Trichomonas gallinae* is a Columbiform, the rock pigeon (*Columba livia*), although members of other Orders including Accipitriformes and Passeriformes may also be naturally infected. Infections range from asymptomatic cases to ingluvitis, caseous necrosis and death. Pathogenicity depends on the "inherent" virulence of the individual strain of *T. gallinae*, the avian host species and the host's previous exposure to *Trichomonas* spp. Birds are exposed to trichomonads via crop feeding from infected parents, billing during courtship and mating, contamination of shared food and water sources, and from eating infected prey. Deadly outbreaks have negatively impacted wild bird populations throughout written history. Historically columbiforms were the most commonly affected, but in recent years, beginning in the 2000s, passerines have faced large-scale population declines. From 2007-2009 the breeding population of greenfinches (*Carduelis chloris*), common garden residents in the United Kingdom, experienced a 35% decline. Similar population effects were also documented in the Canadian Maritime Provinces in the purple finch (*Haemorhous purpureus*) in 2007. These more recent outbreaks have raised questions of transmission dynamics of *T. gallinae*, most importantly, on the mechanism of spread from the typical carrier, a columbiform, to the passerine species that have become more recently affected. Many researchers have implicated contaminated bird baths as a source of trichomonads for backyard songbirds. There have been few studies on the persistence of *T. gallinae* in

the environment, which established that trichomonads can survive in water for short periods of time, i.e. 20 min, but information is lacking on the persistence capabilities of *T. gallinae* which could bolster support for, or offer an evidential challenge to, the theory that bird baths serve as a nidus for transmission.

Literature Review

A disease of trained falcons, known as canker or frounce, was described by falconers as early as the 1500 and 1600s as a potentially deadly illness “originating in the mouth” (Stabler, 1954). *Trichomonas gallinae*, the causative protozoan parasitic agent of this disease, was recovered from the upper digestive tract of a pigeon squab in Italy and first described by Rivolta in 1878. Rivolta named the parasite *Cercomonas gallinae.* Rivolta also described, and separately named, a liver form of the flagellate *Cercomonas hepaticum*, which today is known to be the same species as *Trichomonas gallinae*. In 1880 Rivolta and Delprato also described a fecal flagellate that we now believe was *Tetratrichomonas gallianarium*, a related but non-pathogenic intestinal protozoa (Stabler, 1947).

Stabler described the morphology of *Trichomonas gallinae* as a mostly pear-shaped organism with four anterior flagella arising from a basal granule, an undulating membrane of ⅔ to ¾ the length of the organism, and an axostyle that protruded posteriorly for “a short distance”. He measured *T. gallinae* between 6.2-18.9 µm by 2.3-8.5 µm, with an average size of 10.5 µm by 5.2 µm (Stabler, 1947). As newer technology became available, Scanning Electron Microscopy (SEM) revealed that *Trichomonas gallinae* are pleomorphic and can present as piriform, ovoidal or spherical. Four anterior flagella of unequal length and an undulating membrane adherent to the cell for the majority of its length were confirmed. An internal axostyle, present for structural integrity, protrudes from the organism’s posterior end for about ⅓ the length of the cell. There is also a spherical form characterized by flagellar retraction and disappearance of the undulating membrane, known as the pseudocyst. The pseudocyst is theorized to protect the trichomonad in unfavorable conditions, including desiccation,

limiting temperatures and changes in pH or oxygen content; however, this pseudocyst form may also be seen in "normal" culture conditions (Tasca and De Carli, 2003).

Trichomonads lack mitochondria, which typically produce energy in eukaryotic cells in the form of ATP (adenosine triphosphate) by utilizing oxygen as the final proton acceptor. However, in organisms that thrive in oxygen-restricted environments, such as trichomonads, mitochondria have undergone an evolution of reductive alterations of content and function. These evolutional pressures have resulted in Type 4 Mitochondrial-related organelles called hydrogensosomes, which create ATP and hydrogen gas through fermentation (Makiuchi and Nozaki, 2014). Accordingly, trichomonads are microaerophilic meaning they prefer conditions with very low oxygen concentrations (Amin et al., 2014b). Trichomonads can be cultured *in vitro* in different growth media (Ahmed, 2014; Clark and Diamond, 2002; Cover et al., 1994; Diamond, 1954; Visvesvara and Garcia, 2002), with Hollander's Fluid shown to be the best media to maximize rate and extent of growth (Amin et al., 2010; Clark and Diamond, 2002; Smith, 1983).

Birds are the only natural hosts of *Trichomonas gallinae* because they can "acquire[ ] the infection without intervention of experimental procedures, even though the acquisition was accomplished under conditions of confinement or domestication" (Stabler, 1954). In particular the rock pigeon, *Columba livia*, is the primary host of *Trichomonas gallinae* and has been credited with its dispersal around the globe. By 1954 *T. gallinae* had been found naturally in multiple columbid species, turkeys, chickens, Java sparrows, peregrine falcons and sea gulls, and experimentally in species including swallows, goldfinches and song sparrows (Stabler, 1954). Forrester, et al. compiled a comprehensive list of avian hosts, location, wild or captive status, number of individuals affected and authors of each report of *Trichomonas gallinae* (2008). Briefly, naturally occurring *T. gallinae* infection has been described in 19 columbiforms, 26 species of falconiforms, and 9 species of strigiforms. For captive

and experimental infections *T. gallinae* has been documented in psittaciforms, passeriforms, galliforms, gruiforms, and anseriforms (Forrester et al., 2008).

Young birds are exposed to *T. gallinae* through crop milk from infected parents, and adults are exposed through contaminated feed, prey, and/or water sources (Stabler, 1947). Trichomonads cause gross caseous necrosis consisting of purulent exudate containing mostly heterophils in the oral cavity, pharynx, esophagus (Amin et al., 2014b). Trichomonads are capable of penetrating the eye, head sinuses and brain. They can also cause necrosis in other organ systems including the liver (most commonly), the lung, air sacs, heart, pericardium, and pancreas, which are presumably accessed by the bloodstream (Amin et al., 2014b). Histopathologic changes include edema, congestion of blood vessels, and infiltration of the oropharyngeal mucosa with mononuclear cells (El-Khatam et al., 2016). Focal areas of necrosis, eosinophilic infiltration, and bile duct epithelial hyperplasia have been described in the liver (Anderson et al., 2010; El-Khatam et al., 2016). Clinical signs of infection include matted feathers around the head due to dysphagia and regurgitation, depression, emaciation and even death (Anderson et al., 2010). Strains of *Trichomonas* spp. are known to vary greatly in pathogenicity with a spectrum of various levels of intermediate virulence (Kreier, 1992). Virulence also varies due to host species and the host's previous exposure status to *Trichomonas* spp. Protection was experimentally conferred to pigeons by first exposing them to a moderate to highly virulent strain and subsequently challenging them with a different virulent strain, which suggests the role of an adaptive humoral antibody response (Stabler, 1951). Some strains appear "inherently avirulent" and are unable to cause pathology in any host/condition (Kreier, 1992).

A definitive diagnosis of trichomonosis cannot be made based on clinical signs alone as there are other diseases that produce similar oral lesions including salmonellosis, fungal infection (e.g. aspergillosis or candidiasis), hypervitaminosis A, or avian poxviruses. Histopathologic findings in internal organs may be confused with herpesvirus, paramyxovirus, or adenovirus or other diseases which cause granulomas including

tuberculosis, salmonellosis, mycoplasmosis, and coligranuloma (Amin et al., 2014b). Definitive diagnosis can be made through laboratory and/or molecular identification of the organism in addition to clinical signs. Wet mounts, prepared with oral cavity swabs and saline, can be viewed under light microscopy immediately; however, this method has a low sensitivity and cannot differentiate strains of *Trichomonas* spp. (Bunbury et al., 2005). Culture increases sensitivity by allowing trichomonads to replicate so that likelihood of finding motile trichomonads under direct light microscopy increases. Media can be made inexpensively in lab, including Diamond's media and Hollander fluid, or purchased in a *Trichomonas foetus* InPouch (BioMed Diagnostics, White City, OR, USA) (Amin et al., 2010; Diamond, 1954; Smith, 1983). The latter method has the benefit of being easier to store and carry into the field but is more expensive than laboratory prepared media. Molecular identification can be made from live or dead trichomonads in culture or on swabs and also from tissue samples of affected oral mucosa or affected organs. Multiple DNA targets, including the internal transcribed spacer region (ITS), 18S rRNA, and iron hydrogenase (FeHyd), have proven effective for identifying trichomonads and differentiating strain types (Felleisen, 1997; Gerhold et al., 2008; Lawson et al., 2011a).

Molecular techniques allow improved detection of trichomonads in low amounts due to the sensitivity of polymerase chain reaction (PCR) to identify parasite DNA. Using molecular data further allows assessment of phylogenetic relationships among similar organisms to infer geographic and host origin and movement. Most investigations use ITS and 18S rRNA to discriminate trichomonad species and to separate lineages within *T. gallinae* (Ganas et al., 2014). Gene targets are chosen based on their conservation and divergence in the host. In general, non-coding regions, including ITS1 and 2, are under less conservational pressure than regions that code for proteins. As normal background mutations occur in DNA, mutations in regions that code for genes may improve or inhibit gene function. If a mutation occurs in a coding region that alters gene function that negatively affects host fitness, the mutation will not be passed to offspring nor perpetuated in the population. However, mutations in the non-coding regions are not

closely tied to function and therefore carry no advantageous or deleterious effects on the organism and can be introduced into the population genetic code. In this way, more conserved regions help identify organisms and noncoding regions allow a finer scale differentiation within species. Genes found in many organisms, such 18S, can be used for diagnosis of protozoan infections including *Toxoplasma gondii* (Cooper et al., 2016) and *T. vaginalis* (Mayta et al., 2000), and characterizing new species including *Tritrichomonas* spp. (Walden et al., 2013). However, 18S is not the preferred tool to distinguish trichomonads beyond the species level (e.g. differentiating *T. gallinae*, *T. vaginalis*, and *T. foetus*). In contrast to the above designation, the non-coding ITS region has been accepted as a proven tool to separate avian trichomonads beyond the species level into 2 major groups the *T. gallinae* clade and *T. vaginalis*-like clade based on ITS genotyping (Gerhold et al., 2008). The first genetic analyses of trichomonads targeted the 5.8S rRNA gene and the flanking internal transcribed spacer regions 1 and 2 (Felleisen, 1997). This assay described by Felleisen has also been adapted to characterize related trichomonads including the economically important cause of abortion and infertility in cattle, *Tritrichomonas foetus (Girard et al., 2014b; Grahn et al., 2005).* As another exception to the general rule, the FeHyd coding region produces finer-scale separations of isolates and improves phylogenetic relationships in amitochondrial protists (Chi et al., 2013). FeHyd is a single copy gene known as a "house-keeping" gene which means that the gene is required for basic cellular function (Ganas et al., 2014) and is useful for identifying genotypes and strains within species (Lawson et al., 2011a). Restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD), which involve digestion of an amplified DNA target by specific restriction enzymes, have been suggested to allow investigation of more than single, individual regions of DNA at a time (Sansano-Maestre et al., 2016). A recent study proposed a robust multi-locus genotyping with 16 loci, including ITS and FeHyd, which has the ability to differentiate between strains that at first appeared identical (Abdulwahed, unpublished). Molecular techniques have been able to describe more recent strains, but without archived tissues cannot be applied to historic mortality events.

Reports of trichomonosis outbreaks have been common in columbids and raptors since *Trichomonas gallinae* was first described. Mourning doves (*Zenaida macroura*) across the US have been historically plagued by epizootics (Schulz, 2005; Stabler, 1951a). Although it is difficult to estimate mortality numbers or percentages of wild populations, a 1950-51 outbreak in Alabama had estimated mortality of 4,000 individuals in one county alone and an estimated 25% decrease in population in another county (Haugen, 1952). *Trichomonas gallinae* is postulated to be a major contributing factor in the extinction of the passenger pigeon (*Ectopistes migratorius*) (Haugen, 1952) and is a leading concern in the conservation of the endangered Mauritian pink pigeon (*Nesoenas mayeri*) (Swinnerton et al., 2005). In the 1990s Cooper's hawk nestlings (*Accipiter cooperii*) in Arizona had a 41% mortality rate in urban areas. Larger populations of pigeons in urban areas are thought to drive the connection of *Trichomonas*-related deaths in young birds as the hawks' diets consist of a greater proportion of pigeons (Boal et al., 1998). Adult Cooper's hawks had a lower prevalence of trichomonosis, which could be linked to better immune defenses in adults or oral pH differences in adults versus nestlings (Urban and Mannan, 2014).

The first documented cases in wild Passeriformes began in 2002, when a small outbreak affected house finches (*Carpodacus mexicanus*) and house sparrows (*Passer domesticus*) in Kentucky, USA (Forrester et al., 2008). In 2005, two common European garden birds, greenfinches (*Carduelis chloris*) and chaffinches (*Fringilla coelebs*), experienced population declines, including an estimated 35% decrease in the breeding bird population of greenfinches (Lawson et al., 2012). The UK outbreak was attributed to a clonal strain in ITS group A (Lawson et al., 2011a). An outbreak in the Canadian Maritimes beginning in 2007 adversely affected purple finches (*Haemorhous purpureus*) and American goldfinches (*Spinus tristis*) was attributed to the same clonal UK strain subtyped as A1 with FeHyd (Forzán et al., 2010). Both the UK and Canadian outbreaks were specifically associated with backyard bird feeding stations.

Trichomonosis also continues to negatively impact columbiforms, particularly Pacific Coast band-tailed pigeons (*Patagioenas fasciata),* as evidenced by ongoing mortality events linked to shared water sources in southern California. These outbreaks have been exacerbated by drought conditions in the region which concentrate high numbers of individuals near scarce water resources (Rogers et al., 2016). A new *Trichomonas* species, *Trichomonas stableri*, (formerly ITS Group K) was described in the Pacific Coast BTPI (Girard et al., 2014a). Using molecular techniques, we investigated trichomonads cultured from subclinical hunter-killed doves and lesion tissue samples from 16 species of affected birds. This opportunity to compare and contrast isolates from overlapping temporal and spatial ranges gave us an ideal chance to compare associated strains with subclinical and clinical cases in relation to geography, avian host source, and time.

As illustrated, trichomonosis outbreaks are capable of causing significant mortality events in birds (Forzán et al., 2010; Lawson et al., 2011b; Rogers et al., 2016). Studies have implicated water troughs in domesticated birds, and bird baths and feeders as a source of infection for passerines (Anderson et al., 2009; Lawson et al., 2012; Schulz et al., 2005). However, it has not been sufficiently proven to what extent *T. gallinae* can persist in water past 20 min. Previous persistence investigations of *T. gallinae* in contaminated water and feed sources found that some trichomonad isolates could persist in distilled water combined with NaCl and in grain extracts for days and that the parasite was negatively affected by lower temperatures of 10°C and 25°C (Kocan, 1969). Gerhold, et al. (2013) reported that *T. gallinae* isolates survived up to 20 min (the final sampling point in the study) in chlorinated or distilled water with added organic material and in distilled water without organic material; however, they documented no persistence of isolates in chlorinated water without organic material.

Establishing the capability of trichomonads to persist in water longer than 20 min will shift our understanding of transmission. Instead of requiring that birds overlap temporally in their use of bird baths, birds visiting water sources could be infected

hours, or possibly days, after contamination. Further research is also needed on other factors in water such as salinity, pH, temperature and UV light exposure. Exploring these factors, and the mechanisms with which organic material increases trichomonad persistence could lead to practical information to distribute to wildlife biologists and home owners to make changes with bird baths to help limit the severity trichomonosis outbreaks.

Certain laboratory procedures have been shown to alter the virulence of isolates of *Trichomonas gallinae*. For this reason, it is necessary to assess any changes simulated environmental conditions, such as persistence in water, may have on their virulence. Persistence alone does not indicate the ability of birds to be become infected with virulent trichomonads through contaminated water. Early research on *Trichomonas gallinae* showed that the virulence of an isolate could be decreased after serial passage through abiotic media (Honigberg et al., 1970). However, when the researchers then passed those isolates back through live birds, the isolates regained their ability to cause disease. Honigberg, et al. also showed that long-term cryopreservation with DMSO preserved pathogenicity for up to 7 years and that maintenance of virulent trichomonads in avian cell culture, but not in live mice, could retain virulence (1970).

Research has failed to establish a causative relationship between molecular data and virulence of an isolate (Anderson et al., 2009; Chi et al., 2013; Ecco et al., 2012; Gerhold et al., 2009b; Grabensteiner et al., 2010) leaving the only reliable measure of virulence as the ability of that isolate to produce disease in a live bird (Honigberg et al., 1971; Stabler and Kihara, 1954; Stabler, 1948a, b, 1951; Stabler and Braun, 1975; Stabler and Engley, 1946). Researchers tried to apply methods that worked to inform virulence capability in *Trichomonas vaginalis,* including assessing hemolytic potential (Gerhold et al., 2009b) and looking for DNA-viruses (Gerhold et al., 2009a), neither of which was successful. Early evaluations of virulence used a bioassay method in mice by determining if subcutaneously injected trichomonads could produce lesions (Amin et al., 2014a) or live bird infection trials (Stabler and Engley, 1946). Due to ethical reasons,

refining live animal models by using *in vitro* methods is preferred when possible. Cell culture has been confirmed as a suitable model to assess virulence in trichomonads (Silva Filho and de Souza, 1988). Many cells lines have been used to assess the virulence of trichomonads *in vitro* including trypsin-dispersed cells (Honigburg, et al., 1964; Kulda and Honigberg, 1969) and cells grown on culture plates (Alderete and Pearlman, 1984; Amin et al., 2012a). An avian fibroblast cell line (QT35) was used as a comparative cell type to the epithelial lining of the pharynx, a cell target for *T. gallinae* (Amin et al., 2012a). Liver cells have also been used as an appropriate representative of the most affected internal origin of *T. gallinae* (Amin et al., 2012a). Using avirulent and virulent isolates purchased from a cell repository (ATCC), Amin, et al. showed the virulent isolates caused more damage than the avirulent or control isolates (2012a). Cytopathic effect has been measured both qualitatively, on a visual scale, and quantitatively by cell staining methods including crystal violet, CellTitre 96 and trypan blue. A study on the virulence of pseudocysts used fluorescent stains to detect CPE (Pereira-Neves et al., 2012) and found CPE was greater for pseudocysts than the piriform *T. vaginalis*. There has been much debate over the mechanism of pathogenicity, namely adherence-mediated versus secreted substances but evidence has been provided to support both theories (Alderete and Pearlman, 1984; Amin et al., 2012b; Gilbert et al., 2000; Gould et al., 2017).

The persistence of *Trichomonas* spp. in simulated environmental conditions remains mostly unexplored. Along with genetic characterization of circulating genotypes of *T. gallinae*, persistence, and virulence thereafter, we will give a more complete assessment of ongoing outbreaks. By filling this gap in knowledge we can suggest evidence-based strategies to decrease the spread of trichomonosis via bird baths.

CHAPTER II

Trichomonas gallinae persistence in four water treatments

A version of this chapter was previously published by Kathryn E. Purple, Jacob M. Humm, R. Brian Kirby, Christina G. Saidak and Richard Gerhold:

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Formatting changes and omission of certain sections (author affiliations, word count, and key words) have been made to the original publication.

Abstract

Trichomonas gallinae is a protozoan parasite commonly found in columbids, passerines, and several raptor species. It is suspected that *T. gallinae* is spread between individuals and across species through shared water sources. However, little research has been conducted regarding the persistence of *T. gallinae* in the environment. To determine the persistence of *T. gallinae* in various communal water sources, we inoculated 1 x 10^6 trichomonads into 500 mL samples of distilled water, quarry water, bird bath water, and rain barrel water in two replicates. Aliquots of 0.5 mL were collected from each source at -1, 0, 15, 30, and 60 min; aliquots were incubated at 37°C and examined for trichomonads by light microscopy for five consecutive days. Live trichomonads were observed in all samples and at all sampling times except prior to inoculation (-1 min). The pH of water sources ranged from an average of 5.9 to 7.4 post sampling. Our findings indicate that *T. gallinae* can persist for up to 60 min in various water treatments and thus be infectious for birds drinking *T. gallinae*-contaminated water.

Short Communication

Trichomonas gallinae is a protozoan parasite that primarily infects birds of the orders Columbiformes (Kocan, 1969; Robinson et al., 2010; Stabler, 1954) and Passeriformes (Anderson et al., 2009; Robinson et al., 2010), as well as several raptor species (Ecco et al., 2012; Gerhold et al., 2008). Avian trichomonosis, the disease caused by *T. gallinae* or *T. stableri* n. sp., usually manifests as lesions and caseous masses in the oral cavity and upper respiratory tract (Ecco et al., 2012; Girard et al., 2014a; Stabler, 1954). Clinical infection causes difficulty with respiration and feeding, emaciation, and eventual death (Ecco et al., 2012). Spread of the parasite is perpetuated by the feeding behavior of columbids, which involves adults providing nestlings with crop milk; therefore, *T. gallinae* is commonly transmitted through this route (Stabler, 1954). Other behaviors such as billing, when mates touch and clasp each other's bills, also result in transmission among adults (Gerhold et al., 2007). Trichomonosis is considered the most important disease of the mourning dove (*Zenaida macroura*), a highly popular game species in the United States (Gerhold et al., 2007).

Trichomonosis outbreaks in passerine species have been documented in Canada, Europe, and the United States, and have caused substantial declines in British finch populations since 2005 (Robinson et al., 2010). All of these outbreaks have been associated with bird feeders and artificial water sources (e.g., bird baths). However, few studies have examined the persistence of *T. gallinae* in water or food sources that may serve as a route of transmission (Gerhold et al., 2013; Kocan, 1969). Kocan (1969) used varying concentrations of NaCl in distilled water at a pH of 6.5 and found that motility and survival of *T. gallinae* were greatest and of the longest duration in concentrations greater than or equal to 0.05% NaCl. Gerhold et al. (2013) found that *T. gallinae* persists for variable periods of time (up to 20 min) in both distilled and chlorinated water that contains organic material, but it does not survive in clean, chlorinated water. Organic material may include deciduous leaf litter, soil, and other vegetation. Although the exact mechanism is unknown, it is suggested that the

presence of organic material in water sources such as bird baths or well water may provide sufficient nutrients to allow *T. gallinae* to persist in the environment (Gerhold et al., 2013). Increased persistence in the presence of organic material may also be due to change in pH, dissolved oxygen, microorganisms or a combination of factors. Quarry water, bird bath water, and rain barrel water naturally contain organic material as it may inadvertently fall into open water sources or become suspended as water runs through contaminated rain gutters (in the case of the rain barrel). Therefore, we hypothesized that *T. gallinae* would persist in the three water treatments with organic material for greater than 20 minutes. We also wanted to evaluate the differences between these three water types, which would be expected to have varying particle sizes from smaller (in quarry and rain barrel water) to larger pieces of material (in bird bath water). Although distilled water contains no organic material, Gerhold et al. (2013) showed that *T. gallinae* can persist in distilled water. Accordingly, the objective of our study was to examine the persistence of *T. gallinae* in four water treatments from various sources (i.e., distilled water, quarry [i.e. pond] water, bird bath water, and rain barrel water).

Two isolates of *T. gallinae* collected from a Cooper's hawk and a rock pigeon were used for the experiment. Individual plastic containers were filled with 500 mL of one of the four water treatments – distilled water, quarry water, bird bath water, and rain barrel water – and maintained at room temperature, approximately 23°C. Two replicates of each water treatment were completed for each isolate, for a total of 16 plastic containers. A hemocytometer was used to determine the desired inoculating concentration of trichomonads (1×10^6 trichomonads/mL), and each container was inoculated with 1×10^6 trichomonads (1 mL), which were immediately stirred using a plastic pipette. One million trichomonads has been used as the dose for experimental oral infection as well as in previous persistence trials (Conti et al., 1985; Gerhold et al., 2013).

To evaluate the persistence of the trichomonad isolates in all four water treatments, 0.5 mL aliquots were collected at four time points, including immediately following

inoculation (time 0), and at 15, 30, and 60 min post-inoculation. One sample from each container was collected prior to inoculation to serve as negative controls (time = -1 min). Rather than measure pH at every time point, pH was measured only after all samples had been taken as it was assumed that pH after the organic material had been present for over 24 hr, and after the trichomonads had been added would be more representative of pH in natural conditions. Each 0.5 mL aliquot was collected from the center of the container at a depth of approximately 3 cm, and pipetted into a pre-labeled flask containing Diamond's media (Diamond, 1983) with the following antibiotics and antifungals: 10% Penicillin/Streptomycin, 0.4% Kanamycin and Gentamycin and 1% Amphotericin B. All flasks were incubated at 37°C for five days. Flasks were examined daily during the incubation period for the presence of live trichomonads using a light microscope. As few as one motile trichomonad observed in a sample was considered a positive result.

No trichomonads were detected in any of the water treatments prior to inoculation (-1 min), illustrating that no water source initially contained any live trichomonads. Both trichomonad isolates were detected during all sampling points (0, 15, 30, and 60 min) for all four water treatments post-inoculation (Table II-I). The pH in each container varied by treatment water type: distilled water had a mean pH of 6.3, 95% CI [6.00, 6.60], quarry water, 5.9, 95% CI [5.80, 5.97], bird bath, 7.4, 95%CI [7.10, 7.69], and rain water, 7.1, 95%CI [6.94, 7.21] (Table II-II). However, there was no significant difference in persistence between water types with different isolates.

Gerhold et al. (2013) found that *T. gallinae* is more likely to persist in distilled water containing organic material than in distilled water containing no organic material. Additionally, in that same study, *T. gallinae* persisted in distilled and chlorinated water containing organic material at varying times up to 20 min post-inoculation, but did not survive in clean, chlorinated water (Gerhold et al., 2013). Organic material may provide a nutrient source for *T. gallinae* to persist in water for longer periods of time

Table II-I. *Trichomonas gallinae* persistence over time in various water treatments.

Detectable growth was determined by light microscopy detection of at least one motile trichomonad.

Sampling times (min)	Distilled water				Quarry water				Bird bath water				Rain barrel water			
	Isolate 1[a]		Isolate 2[b]		Isolate 1		Isolate 2		Isolate 1		Isolate 2		Isolate 1		Isolate 2	
	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
30	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
60	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] Isolate 1 = Cooper's hawk; [b] Isolate 2 = rock pigeon; [c] Rep = replication; [d] + = detectable growth; [e] - = no detectable growth.

Table II-II. Water pH measurements.

The pH of each container following final sample collection.

pH	Average	95% Confidence Interval
Distilled water	6.3	6.00, 6.60
Quarry water	5.89	5.80, 5.97
Bird bath water	7.40	7.10, 7.69
Rain barrel water	7.07	6.94, 7.21

(Gerhold et al., 2013). Collection time points for the current study were extended up to 60 min (-1, 0, 15, 30, 60 min) to further assess the duration of time that the protozoan parasite can persist in four types of water sources. We measured a positive result by the presence of as few as one motile trichomonad; therefore, it is possible that low numbers of *T. gallinae* were present in the distilled water. This result is of particular importance since it has been shown that experimental infection with one trichomonad can cause mortality in pigeons (Stabler and Kihara, 1954).

Our results support past research that *T. gallinae* can persist in water for short periods of time, however, determining the persistence of *T. gallinae* for longer periods of time, for example, 75, 90, and 120 min, would provide additional information about the persistence of *T. gallinae* in common bird water sources, and may help determine the point at which *T. gallinae* is no longer viable in the environment. Our study did not take into account variation between treatments such as salinity and dissolved oxygen, which may be important for the survival of *T. gallinae* in the environment. Therefore, future research should account for these variables when evaluating trichomonad persistence in water, including various pH levels. Research focusing on a better understanding of the lifecycle of *T. gallinae* would provide biologists and wildlife managers with valuable insight on how to manage and prevent the spread of *T. gallinae* in wild bird populations.

Acknowledgments

We thank members of the University of Tennessee Ecology and Management of Wildlife Health course (WFS 401/501) for assistance in carrying out the experiment. We received guidance and support from the Parasitology Laboratory, College of Veterinary Medicine, University of Tennessee, and funding was provided through the University of Tennessee Department of Forestry, Wildlife and Fisheries.

CHAPTER III

Persistence of two isolates of *Trichomonas gallinae* in simulated bird baths with and without organic material

A version of this chapter was published by Kathryn E. Purple and Richard Gerhold:

Purple, K.E., Gerhold, R.W., 2015. Persistence of Two Isolates of *Trichomonas gallinae* in Simulated Bird Baths With and Without Organic Material. Avian Dis 59, 472-474.

Formatting changes and omission of certain sections (author affiliations, key words, and abbreviations) have been made to the original publication.

Abstract

Trichomonas gallinae, a well-documented protozoan parasite of avian hosts, has been implicated in major passerine mortality events recently and historically throughout the literature. It has been suggested that bird baths and artificial water sources could serve as a source of infection for naïve birds, however, trichomonad persistence in water is not well understood. We measured the persistence of *T. gallinae* isolates from two avian hosts in distilled water and distilled water with the addition of organic material. We inoculated plastic containers in a laboratory setting with 1×10^6 trichomonads and then sampled 500 µl from each container at various time points post-inoculation (0-20 hr). The 500 µl aliquots were inoculated into flasks with 5 ml of modified Diamond's media at each time point. Flasks were incubated at 37 C and examined by light microscopy for 5 consecutive days for the characteristic movements of live trichomonads. The maximum persistence was 16 hr with a Cooper's hawk isolate in the organic material treatment, far longer than the 1 hr persistence previously reported. We show that *T. gallinae* isolates are capable of persisting for long periods of time in water, illustrating that bird baths may be validated as a potential source of transmission in epidemics.

Introduction

Trichomonas gallinae is a protozoan parasite of birds first described by Rivolta in 1878 (Stabler, 1947). Cases of trichomonosis range from subclinical infection to oropharyngeal ulceration, caseous necrosis, visceral organ involvement, and death (Stabler, 1947). Young birds are exposed to *T. gallinae* through crop milk from infected parents, and adults are exposed through contaminated feed, prey, and/or water sources (Stabler, 1947).

The natural host of *T. gallinae* is the rock pigeon (*Columba livia*), although other potential hosts include passerines, domestic and wild turkeys and chickens, and raptors (Lawson et al., 2011b; Stabler, 1954). An outbreak in the Canadian Maritimes beginning in 2007 adversely affected purple finches (*Haemorhous purpureus*) and American goldfinches (*Spinus tristis*); it was determined that the outbreak was associated with backyard bird feeding stations (Forzán et al., 2010). Additionally, trichomonosis caused large-scale mortality in Great Britain from 2007–2009 in finches, which resulted in a 35% reduction of breeding greenfinches (*Chloris chloris*) (Lawson et al., 2012). These recent outbreaks have reemphasized the importance of fully understanding *T. gallinae*, especially its mode of spread to naïve, susceptible host species. Previous persistence investigations of *T. gallinae* in contaminated water and feed sources found that some trichomonad isolates could persist in distilled water combined with NaCl and in grain extracts for days and that the parasite was negatively affected by lower temperatures of 10°C and 25°C (Kocan, 1969). Gerhold, et al. reported that *T. gallinae* isolates survived up to 20 min (the final sampling point in the study) in chlorinated or distilled water with added organic material and in distilled water without organic material; however, they documented no persistence of isolates in chlorinated water without organic material (Gerhold et al., 2013). We further established an increased persistence of up to 1 hr post-inoculation with 2 isolates in distilled, rain barrel, residential bird bath, and quarry (natural fresh water source) waters (Purple et al., 2015).

More research is needed to elucidate the extent of persistence and the ecology of *Trichomonas* in common backyard bird baths. This report describes the persistence of 2 isolates of *T. gallinae* in distilled water, with and without organic material, to simulate bird baths at various levels of sanitation and examines if these anthropogenic water sources may be a source of *T. gallinae* transmission.

Materials and Methods

Previously described *T. gallinae* isolates from two avian hosts included Cooper's hawk (*Accipiter cooperii*) 4 from Arizona (COHA) and broad-winged hawk (*Buteo platypterus*) 1 from Florida (BWHA) (Gerhold et al., 2008). Two treatments included (1) distilled water, and (2) distilled water with the addition of 15 g organic material. Organic material, including soil, deciduous leaf litter, and ground vegetation, was collected at a local natural area (latitude, 35.957369000; longitude, −83.869332000; elevation, 274 m) in Knoxville, TN.

Rigid plastic containers were filled with 500 ml commercially obtained distilled water. The organic material containers received 15 g of organic material approximately 24 hr before inoculation. Isolates were stored in liquid nitrogen before use and cultured in trypticase-yeast extract-maltose (TYM) media as previously described (Diamond, 1983), with the following additions: 10% heat-inactivated horse serum, 10% penicillin/ streptomycin, 0.4% kanamycin and gentamicin, and 1% amphotericin B. Isolates were quantified with a hemocytometer and adjusted to a concentration of 1×10^6 trichomonads/ml inoculating stock. Using the protocol from Gerhold et al. (Gerhold et al., 2013), each experimental container was then inoculated, within 2 hr of quantification, with 1×10^6 trichomonads from one of the respective isolates. Each combination of isolate and treatment was replicated in triplicate. The water was stirred immediately upon inoculation, and aliquots of 500 ml were collected from each container at six time points, ranging from 0 to 20 hr post-inoculation, and placed into 50-ml-capacity sample flasks (Corning Inc., Corning, NY) containing 5 ml TYM media, modified as above. After the 20-hr sampling time point, flasks were placed into an

incubator at 37°C and read for five consecutive days by light microscopy. Flasks were examined for approximately 1 min (six reading frames) once a day. Negative control containers consisted of distilled water and organic material from the same sources as the experimental containers that were not inoculated with *T. gallinae*. Positive control flasks, to ensure the media would support growth of trichomonads if present, were obtained for each isolate by inoculating the flask with 500 ml of inoculating stock at time point 0 hr. All flasks were read until they became positive or, if they remained negative, until day 5.

Statistical analysis

ANOVA was conducted with SAS (Glimmix procedure, SAS Institute, Cary, NC, v. 9.4), and least squares means were compared with Tukey test to compare mean persistence by treatment and by isolate. An alpha level of ≤0.05 was used to detect significant differences.

Results

Isolates consistently had a higher mean persistence in the organic material treatment than in distilled water, but mean persistence in the two treatments was not significantly different for either isolate (Fig. III-I). The mean (±SD) persistence of the COHA isolate was 5.33 ± 1.15 hr in the distilled water and 9.33 ± 6.11 hr in the organic material treatment. The BWHA isolate persisted 1.5 ± 0.87 hr in the distilled water and 2.67 ± 1.15 hr in the organic material treatment (Table III-I). COHA mean persistence was higher than the BWHA in both treatments. In distilled water the COHA persistence was significantly higher than the BWHA ($F = 14.4$, $p = 0.02$); however, this relationship was not significant in the organic material treatment ($F = 3.5$, $p = 0.14$). Flasks from negative control containers remained negative for 5 days, while positive control flasks became positive on day 1.

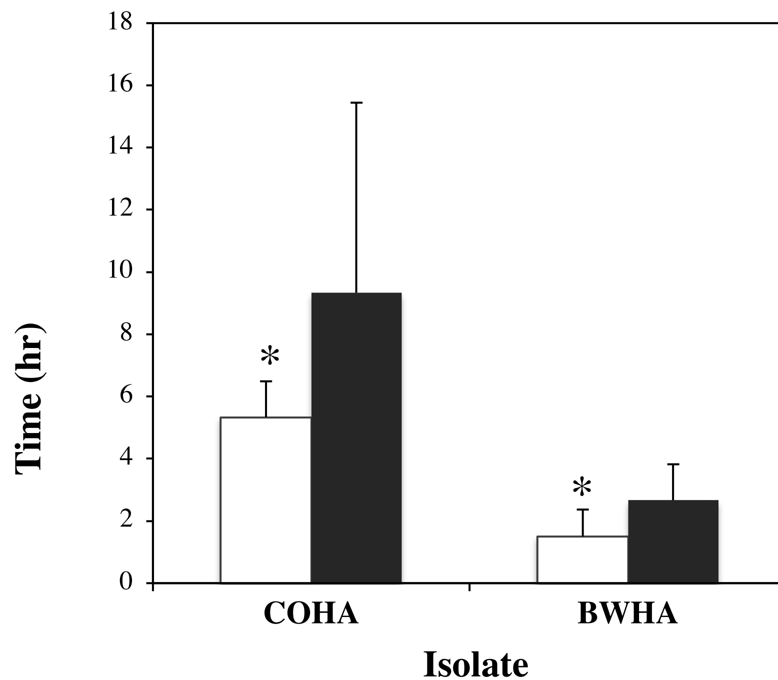

Figure III-I. Persistence of trichomonads.

Persistence of *Trichomonas gallinae* isolates in simulated bird baths. Mean persistence of a *T. gallinae* isolate from a COHA and a BWHA was measured in either 500 mL distilled water (white bars) or 500 mL distilled water with 15 g of organic material (grey bars). Negative controls remained negative for 5 days and positive controls became positive on day 1. Error bars indicate standard deviation among 3 replications. *Indicates a significant difference in mean persistence (p=0.02).

Table III-I. *Trichomonas gallinae* persistence over time in two different water treatments using two isolates.

	Water treatments													
	Distilled							Organic material						
		COHA			BWHA				COHA			BWHA		
Sampling time (hr)	Neg control	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Neg control	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0	-	+	+	+	+	+	+	-	+	+	+	+	+	+
0.5	-	+	+	+	+	+	+	-	+	+	+	+	+	+
2	-	+	+	+	+	+	-	-	+	+	+	+	+	+
4	-	+	+	+	-	-	-	-	+	+	+	+	-	-
6	-	+	+	-	-	-	-	-	+	-	-	-	-	-
8	-	-	-	-	-	-	-	-	+	+	-	-	-	-
16	-	-	-	-	-	-	-	-	-	+	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-

One isolate from a Cooper's hawk (COHA) and one from a Broad-winged hawk (BWHA) with gross lesions consistent with trichomonosis. Detectable growth, determined by light microscopy detection of at least one motile trichomonad. + = detectable growth; − = no detectable growth. Positive controls, to ensure media would support growth of trichomonads, were positive by day 1 for both isolates in each water treatment. Negative (neg) controls, to detect contamination in water treatments, were negative for all 5 days.

Discussion

Trichomonosis outbreaks are capable of causing significant mortality events in birds (Forzán et al., 2010; Lawson et al., 2012). Studies have implicated bird baths and feeders as a source of infection for passerines (Anderson et al., 2009; Lawson et al., 2012; Schulz et al., 2005), although it has not been sufficiently proven to what extent *T. gallinae* can persist in water past 1 hr. We demonstrated the trend for the addition of organic material to increase the persistence of *T. gallinae*, suggesting that contaminated bird baths may be a contributing factor in the transmission of *T. gallinae* during outbreaks. We established a new persistence endpoint of between 16 and 18 hr, which is substantially increased compared with previously demonstrated persistence of *T. gallinae* of 1 hr (Purple et al., 2015). We also noted a significant difference in persistence between two isolates in distilled water; however, the causes for interspecific differences are unknown. Investigators have discovered various genotypes of *T. gallinae* (Gerhold et al., 2008), and further work is needed to determine whether specific genotypes are associated with enhanced persistence. Both isolates in this experiment were from the *T. gallinae* clade; COHA4 has been further classified into the ITS sequence group A, and BWHA1 into group B (Gerhold et al., 2008).

In this study both isolates were from raptors and from closely related internal transcribed spacer sequence groups. Future studies should include isolates from columbids and passerines as well as isolates from different related trichomonad species. Studies on the effect of temperature and ultraviolet light on *T. gallinae* will expand our knowledge of naturally occurring conditions in bird baths and how these factors may influence *T. gallinae* persistence.

Acknowledgments

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CHAPTER IV

Artificially decreased dissolved oxygen increases the persistence of *Trichomonas gallinae* in water

A version of this chapter has been submitted for publication by Kathryn E. Purple, Todd Amacker, Chauntelle Williams, and Richard Gerhold to the American Society of Microbiologists Journal of Applied and Environmental Microbiology.

Some formatting changes and omission of certain sections (author affiliations, key words, and abbreviations) have been made to the original submission.

Abstract

Water containing organic material (OM) has been shown to increase the persistence of the avian pathogenic protozoa, *Trichomonas gallinae*. We hypothesized that the decrease in dissolved oxygen (DO) due to the microbes in the OM could increase persistence of these microaerophilic trichomonads. Using simulated birdbaths, we determined 1) the levels of DO in distilled water with various amounts of OM, 2) the concentration of the oxygen scavenging enzyme, Oxyrase®, needed to achieve the DO levels obtained in OM-contaminated water, and finally, 3) the persistence of two *T. gallinae* isolates in Oxyrase®-supplemented water. An average 9.6% DO was obtained with 15 g OM in 500 ml of distilled water; whereas OM-free water had 86.2% DO. The addition of 0.5% and 1.0% Oxyrase® to OM-free water yielded DO of 18.6% and 6.9%, respectively. Using 0.5% and 1.0% concentrations of Oxyrase®, we evaluated the persistence of two trichomonad isolates by inoculating ~$1x10^6$ trichomonads into 500 ml distilled water in triplicate. At various time-points, 0.5 ml aliquots of trichomonad-inoculated water were transferred into Hollander Fluid media, incubated at 37°C, and read by light microscopy every other day for 5 days. Utilizing 1% Oxyrase®, both of our isolates persisted to our final sampling time point (30 hr) which is a substantial increase from the previously reported persistence of 4 and 16hrs for the BWHA and COHA isolates, respectively. These results indicate the mechanism of OM-mediated trichomonad persistence is associated with decreased DO and further emphasize the importance of keeping birdbaths free of OM to discourage trichomonad persistence.

Introduction

The avian protozoan parasite *Trichomonas gallinae* has been, and continues to be, an important cause of mortality in many bird species (Amin et al., 2014a; Stabler, 1947) and is typically transmitted from rock pigeons (*Columba livia*), to other Columbiformes (Bunbury et al., 2007; Girard et al., 2014a), raptors (Boal et al., 1998; Martínez-Díaz et al., 2015), and passerines (Forzán et al., 2010; Neimanis et al., 2010; Robinson et al., 2010). Transmission of trichomonads from Columbids to passerine species has been linked to backyard birdbaths and other contaminated water sources (Anderson et al., 2009; Forrester et al., 2008; Ganas et al., 2014; Stabler, 1954). Trichomonads were traditionally believed to be labile in the environment due to a lack of a true cyst stage (Kocan, 1969); however, recent research has shown that trichomonads persist in various simulated ambient conditions in water (Gerhold et al., 2013; Purple and Gerhold, 2015; Purple et al., 2015a) and on moist bird seed (McBurney et al., 2017). The addition of organic material (OM), including leaves, soil, and other detritus, has consistently resulted in increased persistence of *T. gallinae* in water (Gerhold et al., 2013; Purple and Gerhold, 2015; Purple et al., 2015a), although the mechanism remains unknown. The added organic components may include a variety of environmental microorganisms. The relationships among *Trichomonas* spp., environmental bacteria, and other soil-dwelling protists provided by OM, is suspected to play a role in the increased persistence of trichomonads in distilled water with the addition of OM.

Most trichomonad species adapted to anaerobic conditions in the host gastrointestinal tract by replacing mitochondria with hydrogenosomes, evolutionary mitochondrial replacement organelles (Makiuchi and Nozaki, 2014). This adaptation allows *Trichomonas gallinae* to thrive in the microaerophilic microclimate in the oral cavity created by the destruction of the esophageal lining, and the resulting caseous lesions. We hypothesized that environmental microorganisms within OM may consume oxygen

and create microaerophilic microclimates thus providing a mechanism by which OM increases the persistence of *T. galline* in water.

To determine the relationship between DO and trichomonad persistence (in the absence of the confounding properties of OM including provision of nutrients, alterations in pH, or other unkown factors) we recorded 1) the levels of DO in water due to the addition of various amounts of OM, 2) the concentration of the oxygen scavenging enzyme, Oxyrase®, needed to achieve the DO levels in OM-contaminated water, and finally, 3) the persistence of two *T. gallinae* isolates in Oxyrase®-supplemented water containing DO concentrations found in OM-contaminated water. Oxyrase® is a commercially available enzyme system that decreases DO in water (Oxyrase®, Inc., Mansfield, OH). It is effective over a wide temperature range (5-65°C) and a wide pH range (6.8-9.4) (Oyrase® white paper).

Results

Measuring DO levels with added OM

The DO saturation with 15g OM, at 24hr, was 10.3% (+/-4.43) (Table IV-I). The DO in the control containers (0g OM) at 24hr were 86.6% (+/- 2.12). Dissolved oxygen decreased proportionally as the amount of OM added to the simulated birdbaths increased (Fig IV-I). At all time-points (0.5, 24, 48, 72hr), excluding time 0hr, DO was highest in the control container and was lower in the remaining treatments. Containers with OM had an average pH of 7.0 (+/- 0.07) at 24hr (Table IV-I), similar to 7.02, the pH in trichomonad-specific growth media. Containers without OM had a pH of 5.84 at 24hr.

Measuring DO with added Oxyrase®

Oxyrase® added in 0.5% and 1.0% vol/vol resulted in DO saturations of 19.0% and 6.9%, respectively, between the DO saturation range that results from the addition of 15g OM, the previous amount used to measure persistence (Table IV-II). The 0% Oxyrase® control container averaged 92% DO, similar to the 85.6% average for the 0g

Table IV-I. Dissolved oxygen (DO) with organic material (OM).

DO saturation and pH (with standard deviation) over time after the addition of different amounts of organic material (OM) in 500 mL distilled water in plastic containers.

	Hours post-addition of OM (hours)							
	0	0.5	24		48		72	
Organic Material (g)	**DO (mean +/- SD)**	**DO (mean +/- SD)**	**DO (mean +/- SD)**	**pH (mean +/- SD)**	**DO (mean +/- SD)**	**pH (mean +/- SD)**	**DO (mean +/- SD)**	**pH (mean +/- SD)**
0	88.42 +/- 1.57	88.94 +/ - 1.42	86.6 +/- 2.12	5.84 +/- 0.10	84.74 +/- 0.97	5.68 +/- 0.0.22	87.14 +/- 1.79	5.64 +/- 0.27
1	84.05 +/- 2.34	86.44 +/ - 0.73	73.83 +/ - 0.23	6.97 +/- 0.08	69.27 +/- 0.72	7.11 +/- 0.11	73.32 +/- 1.24	7.23 +/- 0.14
5	90.0 +/- 3.68	79.32 +/- 4.45	30.37 +/- 14.29	6.98 +/- 0.07	29.09 +/- 14.40	7.19 +/- 0.05	33.29 +/- 15.61	7.28 +/- 0.07
10	83.3 +/- 1.31	75.53 +/ - 1.08	14.65 +/- 0.44	7.02 +/- 0.03	24.37 +/- 3.65	7.20 +/- 0.06	15.22 +/- 3.35	7.25 +/- 0.02
15	85.1 +/ -5.48	65.09 +/- 2.68	10.31 +/- 4.43	6.88 +/- 0.07	8.35 +/- 4.76	7.12 +/- 0.16	10.19 +/- 0.88	7.10 +/- 0.08

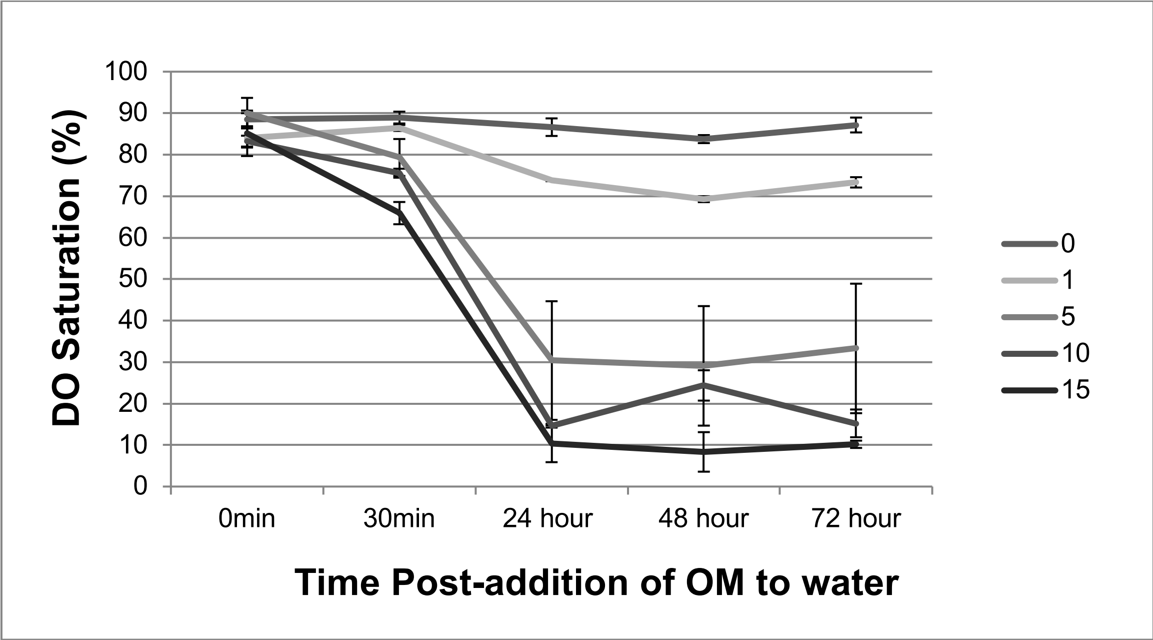

Figure IV-I. Organic material and resulting dissolved oxygen.
Dissolved oxygen (DO) saturation over time after the addition of different amounts of organic material (OM) to 500 mL distilled water in plastic containers. Error bars= standard deviation from 3 replicates. Legend title: OM (in grams).

OM control in the preceding experiment. The 0% Oxyrase® control container had a pH of 5.1 averaged from time-points 24, 48 and 72 hr, whereas the 0.5% and 1.0% treatments had average pHs of 7.13, and 6.88 over the same time-points (Table IV-II).

Measuring persistence of trichomonads in water with decreased DO

Persistence in containers with 0.5% and 1% Oxyrase® was greater than previously

reported persistence in water with OM for both the COHA and BWHA isolates (Figure IV-II). The experimental 0.5% and 1% Oxyrase® treatments had 43.9% and 16.1% DO, respectively (Appendix 1: Supplemental data). In the present study, the maximum persistence occurred in the 1% Oxyrase® treatments, where both the COHA and BWHA persisted to our final sampling time point of 30 hr. At time-point 0 hr the 0.5% and 1.0% Oxyrase® had an average pH of 7.13 and 7.24 for the COHA, and 7.10 and 7.24 for the BWHA, respectively.

Discussion

We determined that artificially decreased DO, using Oxyrase®, increased the maximum trichomonad persistence to 30 hr in the COHA and 30hr in the BWHA. This increase is substantial compared to previous published studies using 15 g of OM in which COHA isolate persisted 16 hr and BWHA isolate persisted 4 hr.

We report maximum persistence because both isolates persisted until our final sampling time-point (30 hr) in at least 1 replicate (1/3 for the COHA; 2/3 for the BWHA). Maximum persistence indicates the isolates persisted to the final sampling time-point; however, the isolates may have persisted even longer. Future persistence trials should extend past 30 hr to determine the potential persistence end points.

Amoeba-bacteria and protozoan-bacteria relationships provide examples of one microorganism benefiting from the reduction in DO created by another. *Acanthamoeba castellanii,* a common environmental protist, has been shown to increase the survival of

Table IV-II. Dissolved oxygen (DO) saturation and pH (with standard deviation) with Oxyrase ®. DO and pH over time after the addition of different concentrations (% vol/vol) Oxyrase ® to 500 mL distilled water in plastic containers.

	Hours post-addition of Oxyrase ® (hours)					
	24		48		72	
Oxyrase ® (vol/vol)	**DO**	**pH**	**DO**	**pH**	**DO**	**pH**
0%	89.50 +/- 0.57	5.07 +/- 0.25	92.59 +/- 0.23	5.05 +/- 0.20	94.02 +/- 0.21	5.18 +/- 0.31
0.50%	17.52 +/- 3.32	6.89 +/- 0.01	13.37 +/- 12.19	6.99 +/- 0.02	25.15 +/- 9.26	7.52 +/- 0.04
1%	6.00 +/- 0.97	7.10 +/- 0.02	5.30 +/- 0.80	6.68 +/- 0.21	9.35 +/- 4.29	6.85 +/- 0.08

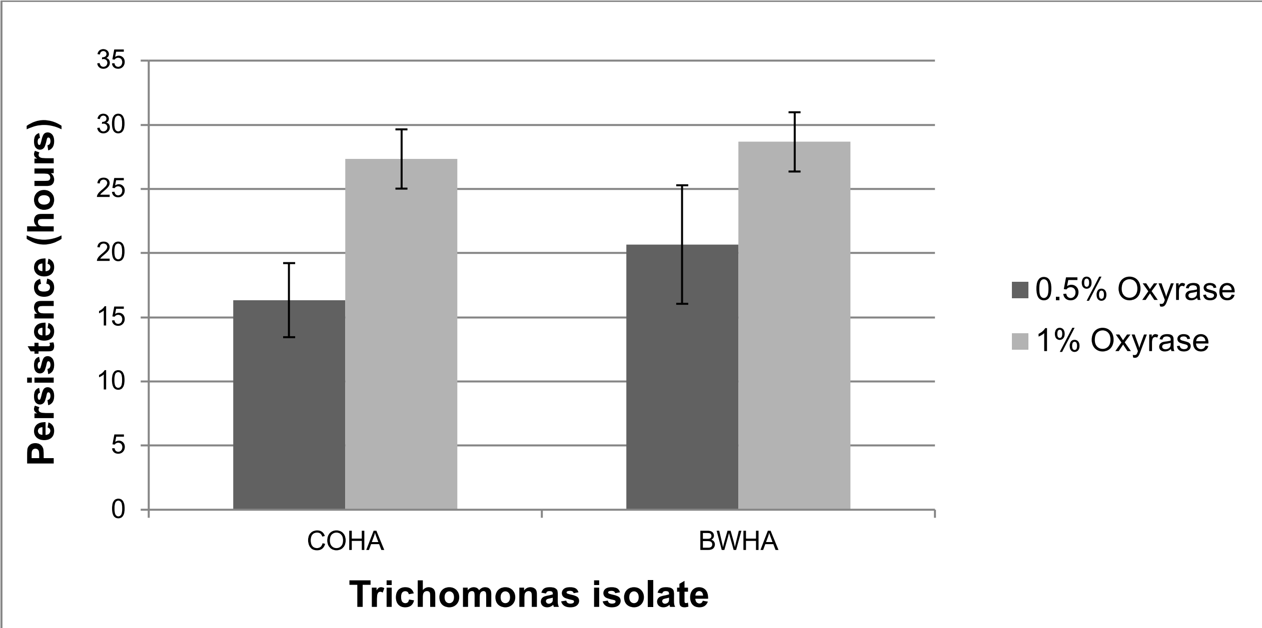

Figure IV-II. Isolate persistence.

Persistence of two *Trichomonas* isolates (COHA, BWHA) in 500 mL distilled water in plastic containers with different concentrations (vol/vol) of Oxyrase®. Error bars= standard deviation from 3 replicates. Legend title: Concentration of Oxyrase® (vol/vol).

the microaerophilic bacterium, and medically important pathogen, *Campylobacter jejuni,* by decreasing DO in a shared liquid medium (Bui et al., 2012). The same authors found a similar trend with the addition of the protozoa *Tetrahymena pyriformis,* another oxygen consumer, suggesting this phenomenon is not unique to *A. castellanii*.

We conclude that DO is an important factor that leads to increased persistence of trichomonad isolates when OM is added. This information will be helpful to the public and to biologists that provide supplemental water (i.e. birdbaths) for wildlife. Prompt removal of OM from these outdoor sources and mechanical aeration of the water could benefit birds that visit these places by increasing DO and making artificial waterers less suitable to *Trichomonas* spp. Chemical treatment of water for bacterial or protist contamination is precluded by an inability to calculate and create disinfectant levels safe for bird and wildlife consumption, in a volume that is ever-changing (due to refilling, rain, splashing, and evaporation). Aerators, like those used in fish tanks, would add DO to the water and could be explored as an option, although issues with power and function in the outdoors would have to be investigated.

Materials and Methods

Measuring DO levels with added OM

Organic material, including deciduous leaf litter, soil, and other detritus, was collected from the same local natural area as in previous persistence studies (Purple and Gerhold, 2015). Organic material in the amounts of 1, 5, 10, and 15 grams (with 0 g as a negative control) were added to 500 mL of distilled water in plastic containers in 3 replicates. Dissolved oxygen and temperature were measured with a Sper Scientific DO Meter (Scottsdale, AZ) at 5 time-points after the OM was added to the water (0min, 30min, 24hr, 48hr, 72 hr). pH was measured at 24 hr, 48 hr, and 72 hr with a Denver Instrument UltraBASIC pH/mV meter (Arvada, CO). Both meters were calibrated per manufacturer’s instructions before each time point.

Measuring DO with added Oxyrase®

To establish the amount of Oxyrase® needed to decrease DO to levels achieved with the addition of OM found in the preceding experiment, we added 2 amounts of Oxyrase® (2.5, and 5 mL) to 500 mL of distilled water (0.5%, and 1% vol/vol, respectively). Dissolved oxygen, temperature, and pH were measured as above at time points 24, 48, and 72 hr.

Measuring persistence of trichomonads in water with decreased DO

Using the results from above, we re-created the levels of DO found in OM-supplemented distilled water by adding 0.5% and 1% Oxyrase® (vol/vol) to plastic containers with 500 mL of distilled water. We evaluated two trichomonad isolates, one from Cooper's hawk 4 (COHA) and one from broad-winged hawk 1 (BWHA), which were used in previous persistence trials (Gerhold et al., 2013; Purple and Gerhold, 2015; Purple et al., 2015a). Both of these hawks showed pathologic lesions consistent with trichomonosis characterized by fulminate oral necrosis. *Trichomonas* was collected at necropsy, cultured in Diamond's media (Diamond, 1957) with antibiotics until cultures were axenic, and cryopreserved in liquid Nitrogen. These isolates were analyzed with PCR to amplify the ITS region and sequences were compared with other *Trichomonas* isolates (Gerhold et al., 2008). The COHA sequence was in ITS group A, and the BWHA in group B.

Before *Trichomonas* inoculation into our simulated birdbaths, isolates were revived from cryopreservation in Hollander media (Smith, 1983) with supplemental fetal bovine serum, antibiotics, and antifungals as previously reported (Purple and Gerhold, 2015). After logarithmic growth was achieved, trichomonads were counted using a hemocytometer, and cultures were adjusted to a concentration of $1x10^6$ trichomonads/mL of media. The inoculating dose of $1x10^6$ trichomonads was used to allow comparison with live animal infection studies using $1x10^5$-$5x10^5$ (Kocan and

Knisley, 1970), $2x10^4$ organisms to test in vitro drug efficacy (Franssen and Lumeij, 1992), and $1x10^6$ as in our previous persistence work.

For the persistence experiment, containers were filled with 500 mL of bottled, distilled water. Oxyrase® was pipetted into the water in the amounts of 0.5 and 2.5 mL. At time point 0 min, 1 mL of each trichomonad culture was added to individual simulated birdbaths. The water was immediately stirred with the pipette tip and the 0 min sample was taken. Samples, aliquots of 0.5 mL from the baths taken at 4, 8, 13, 18, 26, and 30 hr, were introduced into Falcon® 25 mL plug-sealed tissue culture flasks (Corning Inc., Corning, NY) with 5 mL Hollander fluid media. Flasks were incubated at 37°C for 5 days. On days 1, 3, and 5 post-inoculation, flasks were examined by light microscopy to detect the characteristic movement of live trichomonads. Positive flasks, containing at least one live trichomonad, were recorded and negative flasks were read until day 5. Research, and personal experience of the authors, show that samples still negative at day 5 are unlikely to become positive (Bunbury et al., 2005; Cover et al., 1994). Dissolved oxygen and temperature were measured as above at time-point 0 (after Oxyrase®, but before trichomonads were added), 8, and 30 hr. pH was measured at 0 and 30 hr.

The accession number for the COHA-4 is EU215369 and for the BWHA-1 is EU215368.

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CHAPTER V

Evaluation of the cytopathic effect of *Trichomonas gallinae* before and after persistence in simulated bird baths using avian fibroblasts in cell culture

Abstract

The transmission potential of *Trichomonas gallinae* at bird baths will improve our knowledge of the role of shared water sources in outbreaks of the deadly avian disease, trichomonosis. We have established that trichomonads can persist in simulated bird baths when different environmental conditions are recreated. Although persistence is established, the effect of persistence on parasite virulence has not been investigated. We aimed to determine any effects persistence trials may have on trichomonad virulence to assess whether persistent trichomonads retained the capability of trichomonads to cause disease and death in birds. We used cell culture to measure cytopathic effect (CPE) of trichomonads maintained in parasite-specific culture versus parasites that persisted after different time points in simulated environmental conditions. We documented a broad-winged hawk (BWHA) trichomonad isolate exposed to UV light for 2 and 4 hours in clean, distilled bird baths produced increased CPE than media cultured BWHA (or non-persistence (NP)). In an organic material bird bath BWHA and Cooper's hawk (COHA) persistence isolates did not show statistically different changes in CPE than NP isolates indicating less effect of these conditions on trichomonad virulence. We have established that trichomonad virulence can be affected by persistence in bird baths and this discovery can change our understanding about the role of bird baths and shared water sources in the transmission of *T. gallinae*.

Introduction

Trichomonas gallinae, the causative protozoan parasite of a trichomonosis, is responsible for widespread mortality events and decreased recruitment in doves, pigeons, raptors and song birds (Boal et al., 1998; Bunbury et al., 2007; Gerhold et al., 2007; Haugen, 1952; Lawson et al., 2006). Within the last decade, studies in the United Kingdom and the Canadian maritime provinces have associated mortality events with backyard bird feeders and bird-baths (Forzán et al., 2010; Robinson et al., 2010). Ongoing epidemics in California have coincided with drought conditions and

subsequently decreased fresh water access for band-tailed pigeons, which has been suggested to increase the transmission of trichomonads via shared water sources (Girard et al., 2014b; Rogers et al., 2016). This perceived connection of trichomonosis to water has been investigated in laboratory conditions and the persistence of *T. gallinae* in simulated bird-baths up to 16 hr has been established (Purple and Gerhold, 2015). The addition of organic material to water, to recreate neglected backyard bird-baths, has consistently increased persistence (Gerhold et al., 2013; Purple and Gerhold, 2015; Purple et al., 2015).

Certain laboratory practices, including passage of trichomonads through abiotic culture media, have been shown to decrease the virulence of some strains of *T. gallinae* (Honigberg and Goldman, 1968; Honigberg et al., 1970). Trichomonads can regain their former virulence, however, when passed through a series of live birds (Stabler et al., 1964). This ability to alter phenotypic behavior suggests that the virulence of certain strains of trichomonads, like those used in our previous persistence experiments, could be altered by the process of persisting in water baths, which are less hospitable to trichomonads than either abiotic culture media or live birds. We aimed to document any changes in virulence that may occur after persistence in different conditions in simulated bird-baths. Cell culture has been validated as an adequate and appropriate bioassay to assess virulence of trichomonads (Alderete and Pearlman, 1984; Amin et al., 2012). We elected to use cell culture to assess changes in virulence as a precursor to possible live animal infection studies because of the proven effectiveness of cell culture models, and in accordance with the institutional animal care and use committee guidelines to replace live animals with non-animal systems when possible.

Materials and Methods

Parasites

Two isolates, one from a Cooper's hawk 4 (COHA) and one from a broad-winged hawk 1 (BWHA), were previously described and persistence was characterized (Gerhold et

al., 2008; Purple and Gerhold, 2015). Isolates were cryopreserved and stored in liquid Nitrogen before use. To standardize parasites for experiments, cultures were grown to maximum capacity and cryopreserved in large batches to achieve parasites from the same passage. Cryovials were revived in Hollander Fluid medium with serum and antibiotics (HF). Logarithmic growth was reached by 24 hr at which time cell viability approached 100% as determined by trypan blue staining. Trichomonads were passed no more than 10 times prior to use in these experiments. Trichomonad culture was adjusted to a concentration of [$1x10^6$ parasites/ml]. From each isolate culture, trichomonads were assigned to two groups- “non-persistence” (NP) (maintained in HF) or “persistence.” “Persistence” trichomonads were then inoculated into simulated bird-baths as previously described (Gerhold et al., 2013; Purple and Gerhold, 2015), sampled at certain time points into HF. For the duration of the persistence study, parallel, NP cultures for each isolate were maintained in HF at 37°C. Before infection of cell culture, all trichomonad cultures were resuspended in cell culture media, “non-serum” Dulbecco’s Minimum Essential Medium (DMEM) with 1% Streptomycin/Penicillin (hereafter referred to as “NS DMEM”). Prior to the persistence experiments, motility and survival of trichomonads in cell media were confirmed by light microscopy for 72 hr to ensure NS DMEM could support both cells and parasites for co-culture experiments.

Persistence Experiments

Simulated bird-baths were set up as in previous research (Gerhold et al., 2013; Purple and Gerhold, 2015; Purple et al., 2015). Briefly, 500 ml of distilled water in plastic tupperware containers were used to simulate bird baths. The “OM” treatment had 15 g of organic material (Purple and Gerhold, 2015) and was maintained at 37°C. The “UV” treatment containers contained 500 ml of distilled water and were exposed to a 5.0 UVB 18 watt ReptiSun UV light (Zoo Med Laboratories Inc., CA), at a distance of 12 in from the top of each container at room temperature. Twenty-four hours after bird baths treatments were applied, 1 ml of trichomonads at [$1x10^6$ /ml] were added and aliquots were taken at regular intervals as before (Gerhold et al., 2013). The UV treatment baths

were sampled at 2, 4, and 6 hr post-inoculation and the OM treatment baths at 2, 6, 24, and 48 hr. Sample flasks were incubated at 37°C until high densities were reached (between 24-48 hr). Persistence sample cultures were then quantified and adjusted, centrifuged (1,000 rpm) for 5 min, and the resulting pellet was resuspended in 3 mL NS DMEM to yield [$1x10^6$/mL]. NP controls for each isolate were maintained in HF until needed, adjusted and pelleted as above, and resuspended in NS DMEM before infection of cell culture. Aliquots of 250 µL of the post-persistence and NP parasite suspension were used to infect cell culture.

Cell culture

Virulence of trichomonads was assessed on spontaneously transformed avian fibroblasts cells (DF-1; ATCC® UMNSAH/DF-1) grown at 37°C with 5% CO_2. Cells were cultured in 5 ml of DMEM with 10% fetal bovine serum (FBS) and 1% Streptomycin/Penicillin (hereafter noted as "DMEM") in 25 cm^2 cell culture flasks until confluence was reached (generally in 96 hr) then trypsinized and passed into 75 cm^2 flasks with 15 ml DMEM. After cells reached 80-90% coverage they were incubated with trypsin until dislodged and DMEM was added to stop the action of the trypsin. Cells were pelleted, washed with phosphate-buffered saline (PBS), pelleted again and resuspended with 1 µL /$1x10^6$ cells Vybrant™ DiI (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate ('DiI'; $DiIC_{18}(3)$)) cell-labeling solution (Molecular Probes Inc., Invitrogen) suspended in 1 mL sterile PBS. DiI remains in the cell cytoplasm until the cell membrane is compromised during cell death and then quickly disperses into the media. Cells were protected from light as much as possible after the DiI fluorescent dye was added. Cells were incubated with the stain at 37°C for 15 min. Cells were then washed with PBS, pelleted and resuspended in DMEM before seeding onto 48-well tissue culture plates and grown until confluent before inoculation (~72 hr). One T-75 yielded $4x10^6$ cells once confluent; each flask yielded four 48-well plates seeded with 50 µL of $4x10^5$/mL cells suspended in 10 mL DMEM.

Infection trials

Parasite concentrations were determined in a pilot study by adding [$1x10^4$], [$1x10^5$] and [$1x10^6$] to determine cytopathic effect (CPE) of NP isolates. A concentration of [$1x10^6$/ml] of trichomonads produced CPE between 24 and 48 hr and was chosen for infection trials. Cell cultures were infected with 250 µL of the parasite cultures at [$1x10^6$/ml] for both the NP and the post-persistence trichomonads, while 250 µL of DMEM was the negative control. As the experimental parasite cultures reached appropriate densities, four 48-well plates were infected. The initial set of two 48-well plates included 6 treatments (BWHA OM 2 and 6 hours, and COHA OM 2, 6, and 24 hours) and 3 controls (DMEM, NP BWHA, and NP COHA). The second set of two 48-well plates were infected with (BWHA UV 2 and 4 hours, BWHA OM 24 and 48 hours, and COHA OM 48 hours) and controls were repeated. All persistence treatments were repeated in 10-12 well-replicates and the NP and DMEM controls at 21 well-replicates.

Assessment of CPE

DiI-red fluorescence was observed with an 8-bit standard rhodamine filter set (excitation BP 534- 558 nm, emission LP 590 nm) on a Zeiss microscope and imaged with NIS Elements Imaging Software (BR 4.13.05). Trichomonads showed autofluorescence with a standard fluorescein filter set (excitation BP 450-490 nm, emission LP 520nm). Red fluorescent images were before infection, 24, 48 and 72 hr post-infection. Green fluorescent images were taken with every red fluorescent photograph, without moving the plate, to allow superimposition of two photos per well, and to document any background green fluorescence. After taking the 72 hr images, the plates were washed twice with PBS to remove unadhered trichomonads, 500 µL non-serum DMEM was added, and wells were imaged again with both red and green fluorescent filters for “post-wash” images (hereafter referred to as “post-wash images”). All images were centered in each well of the 48-well plates and taken with the same exposure time (100 ms) and magnification (10x). Images were analyzed using ImageJ free software (NIH). Percent area coverage (% cover) was measured for every red fluorescent photograph

after subtracting background with rolling ball set at 60 and threshold adjusted to 25-255. For green fluorescent photos rolling ball was set 20 and threshold to 11-255.

Statistical Analyses

Percent cover with cells at 0 hr and post-wash were compared. Differences between treatments were compared with a nested completely randomized design (CRD). Differences among cells infected with treated trichomonads, NP trichomonads, and the DMEM control wells were analyzed in a split plot ANOVA with repeated measures and a square root transformation. A Bonferroni correction was applied. All statistical assumptions were met.

Results

For the OM treatment, both BWHA and COHA were positive at every sampling point (2, 4, 6, 24 and 48 hr) (Table V-I). For the UV experiment the BWHA was positive at 2 and 4 hr and negative at 6 hr, while the COHA did not persist at any sampling time point (2, 4, or 6 hr).

During incubation of the infected cell culture, some wells experienced bacterial/fungal overgrowth making wells opaque and prohibiting inclusion in the trial (Table V-I). The contamination was limited to specific parasite-treatment combinations; COHA OM 6 hr and BWHA OM 24 hr were contaminated in every well indicating contamination presence in the persistence sample. BWHA OM 6 hr had 3 contaminated wells leaving 7 well-replicates for analysis and BW OM 48 hr lost all but 1 well to contamination. There was no contamination in any control wells.

Percent cover by red fluorescence was skewed by trichomonads that incorporated red stain and resulted in cells that were indistinguishable from trichomonads cells in images. Trichomonads could be detected when in motion at a live feed for the microscope, but this movement was not uniform among all trichomonads and could not be captured in still images. Therefore, images taken at intermediate time points (24, 48, and 72 hr)

Table V-I. Persistence results.

Isolates: BWHA = broad-winged hawk, COHA = Cooper's hawk. Bird bath treatments: UV = clean, distilled water at room temperature with UV light exposure, OM = distilled water with the addition of 15 g organic material (OM) at 37°C. Culture results: + = positive for live trichomonads, - = negative for live trichomonads. After infection of cell culture: * = all replicates lost due to contamination, ° = some replicates lost to contamination.

			Isolate	
		Sampling time point (hour)	BHWA	COHA
Bird Bath Treatment	UV	2	+	-
		4	+	-
		6	-	-
	OM	2	+	+
		6	+°	+*
		24	+*	+
		48	+°	+

were not informative. Red fluorescent images of cells before infection and after post-wash were not confounded by the unintended uptake of DiI stain by trichomonads and were compared to assess CPE of the trichomonads.

We measured the percent cover of cells in each well (with ImageJ “% area cover” function) to evaluate changes and quantify CPE measured before infection and post-wash. Comparing BWHA “early hours” persistence trichomonads from OM (2 and 6 hr) and UV (2 and 4 hr) treatments we found a significant difference in percent cover by treatment type ($p<0.0001$) (Figure V-I). Cells infected with OM treatment trichomonads had an average of 10.4% cover (SD +/-0.84) while UV treated trichomonads had 4.1% cover (SD +/- 0.73). Both hours for UV (2 and 4 hr) were significantly different from both hours of OM (2 and 6 hr) ($p<0.005$). For the BWHA isolate, parasites from the UV treatment caused more cell destruction by area than trichomonads in the OM treatment. COHA did not persist in any UV treatment.

Differences between cells with treated trichomonads, NP trichomonads, and the DMEM control wells were statistically significant for BWHA UV 2 and 4 hr ($p<0.0001$), BWHA OM 2, 6, and 48 hr ($p<0.0001$), and COHA OM 2, 24, and 48 hr ($p<0.0001$). For the BWHA UV treatment, time in bath (2 or 6 hr), picture time (0 hr or post-wash), and the interaction between the two factors were all statistically different ($p<0.0001$) (Figure V-IIa). Cells in DMEM control wells did not change significantly from 0 to 72 hr (2.95 +/-0.17 SD to 2.97 +/-0.17 SD) as expected. Cells treated with NP, 2 hr, and 4 hr UV BWHA significantly decreased from 0 hr to post-wash ($p<0.0001$; $p<0.0002$; $p<0.0001$ respectively). With the greatest change in the UV treatment at 4 hr (2.36 +/-0.34 SD).

For BWHA in the OM treatment, the DMEM control and 2 hr trichomonads did not cause a statistically significant decrease in percent cover, however they were both statistically different from NP and the 48 hr ($p<0.05$), which caused significant decreases from 0 hr to post-wash ($p<0.05$) (Figure V-IIb). OM treatments, 2 hr, 6 hr, NP, and DMEM were not statistically different from each other at post-wash.

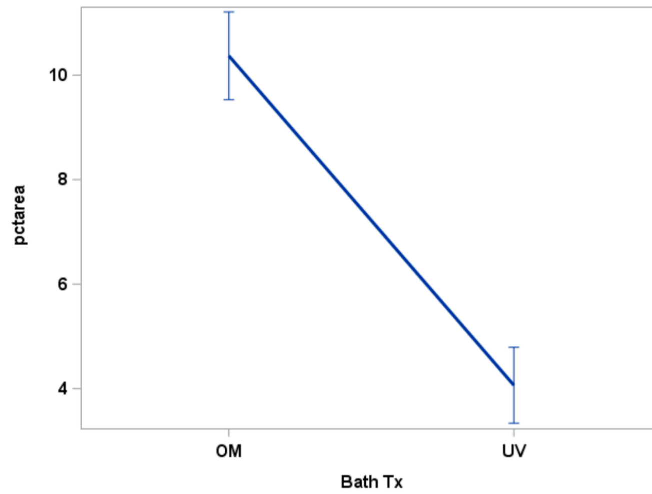
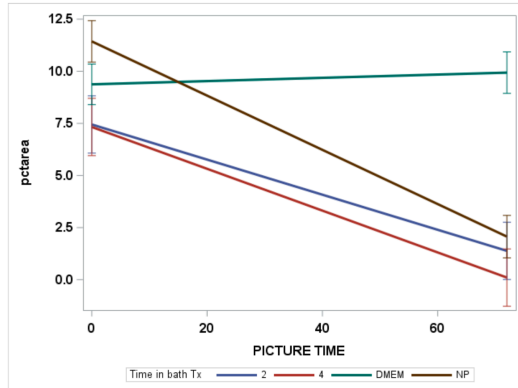
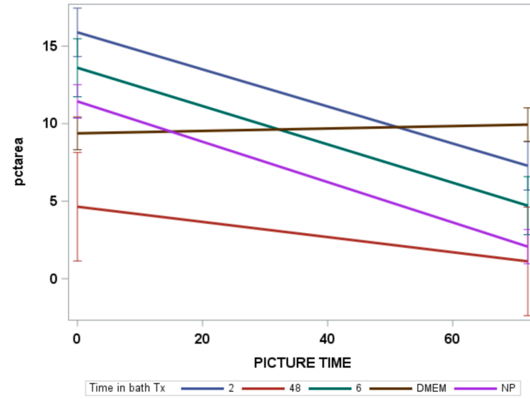


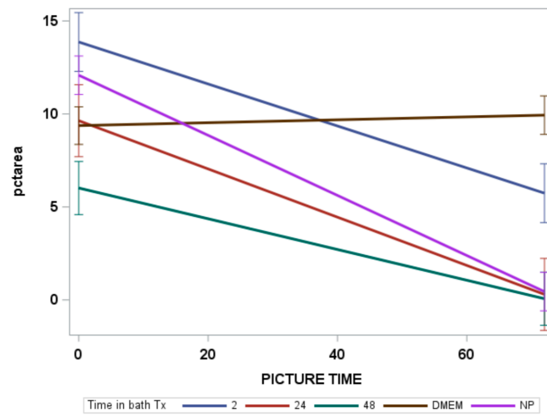
Figure V-I. Bird bath treatment differences.

Percent area of wells covered with cells was lower when infected with UV (ultraviolet light) treated trichomonads versus OM (organic material) treated. Error bars represent standard deviation (SD) of the mean. Pctarea = area of cell coverage.





(a) **(b)**



(c)

Figure V-II. Changes to cell coverage after infection.

Percent area of wells covered with cells changes among (a) UV (ultraviolet light) treatments for BWHA (broad-winged hawk). Blue = 2 hours, red = 4 hours, gold = non-persistence (NP), and green = DMEM (negative control), (b) OM (organic material) treatments for BWHA. Blue = 2 hours, red = 48 hours, green = 6 hours, brown =, DMEM, and purple = NP, (c) OM treatments for COHA (Cooper's hawk). Blue = 2 hours, red = 24 hours, green = 48 hours, brown = DMEM, and purple = NP. Error bars represent standard deviation (SD) of the mean. Pctarea = area of cell coverage.

For the COHA OM, there were significant differences in CPE between treatments (2, 24, 48 hr, DMEM and NP) ($p<0.0001$). The 2 hr OM treated cells were not significantly different from the DMEM control, however, the longer persistence in the OM treatment (24 and 48 hr) and NP treatments were all statistically different from the control ($p<0.05$) (Figure V-IIc). Interestingly, the 2 hr OM treatment produced significantly less destruction than the NP for both isolates (BWHA $p<0.004$; COHA $p<0.0001$). The 2 hr BWHA UV was lower, but not statistically different from the NP, but the 4 hr was significantly lower ($p<0.05$) than NP indicating an increase in CPE.

Discussion

We used fluorescent imaging as a quantitative alternative to visual scoring. We discovered that trichomonads incorporated the DiI stain in their cytoplasm as it was released by the dying avian cells. This precluded our ability to image avian cells alone over time. Trichomonads were stained with CellTrace™ CFSE Cell Proliferation Kit in a pilot study (data not shown), but we later noticed significant green autofluorescence under the FITC filter without staining, so we omitted dying trichomonads for this study. The autofluoresence of the trichomonads allowed us to image trichomonads at multiple time points without destructive sampling, however, this flouresence was not uniform and could not be relied upon to quantify trichomonads. If an accurate measure of trichomonads at each imaging time-point were available, we could have monitored changes in trichomonad density and changes in parasite morphology. While these changes could help our understanding of trichomonad activity in cell culture, the number and morphology of trichomonads is not correlated with CPE in cell culture. For example, a virulent isolate may not require high numbers to destroy a monolayer while a large number of avirulent parasites may cause little effect on co-cultured cells. Trichomonads incorporated the red DiI stain during co-culture, which confounded using red fluorescence as a measure of cell density and coverage. Because red fluorescence of trichomonads occurred, and autofluorescence was inconsistent, we could not use red and green fluorescence to reliably distinguish between cells and parasites. However,

the pre-infection (0 hr) had no parasites yet, and the post-wash images had minimal trichomonads after being washed twice with PBS. Therefore, we used 0 hr and post-wash images to compare treatments.

We documented significant differences in CPE produced between the non-persistence and the persistence treatments indicating a change in virulence due to persistence trials. Interestingly, the persistence effect for the earlier sampling points for BWHA showed that UV exposure to the bird baths produced trichomonads that were more virulent than non-persistence treatments. We theorize that by sampling trichomonads that persisted in this inhospitable environment we selected the most robust, and likely more virulent, trichomonads. Another influence could be the stress of the persistence conditions, which could induce a higher number of pseudocyt formation. Pseudocysts of *T. foetus* have been shown to be the more cytotoxic than the pyriform cells (Pereira-Neves et al., 2012). The change in virulence may have resulted from a higher number of trichomonads becoming pseudocysts in the simulated bird baths. Trichomonads reverted to the pyriform morphology in our sample cultures, but the transition through the pseudocyst form may have affected the cytotoxicity of the persistence trichomonads. The increased virulence of the UV trichomonads compared to their OM and NP counterparts could indicate that the higher stress environment favored the hardier, more virulent trichomonads For the longer time points in the OM treatment, the differences between NP and 24 and 48 hr post-persistence trichomonads were not statistically different suggesting the persistence neither increased nor decreased the virulence of the trichomonads.

We saw differences in CPE produced between the BWHA and COHA isolates. This is not surprising as we documented differences in isolate persistence in earlier trials, and other authors have decribed differences in isolate behavior in cell culture (Amin et al., 2012), suggesting that even among virulent isolates behavior and virulence can vary. Isolate differences in each treatment could not be appropriately compared due to missing data at some levels. For example, no COHA persisted in the UV treatment, but

2 and 4 hr were available for BWHA. Also, the BWHA later hours from the OM treatment (24 and 48 hr) were lost as a result of contamination. Using the nested statisical design we made comparisons where the data allowed.

Our treatment combinations, OM at 37°C and clean distilled water with UV light exposure, were chosen to mimic common environmental factors including environmental temperature changes and exposure to UV radiation from sunlight. Based on trial persistence experiments (data not shown), we chose conditions we expected to be ideal for parasite survival outside of the host, increased temperature and OM, and the conditions that would decrease the persistence of *T. gallinae* the most, clean, distilled water with UV light exposure. With these extremes we hoped to measure any changes to virulence that may occur. Now that we have shown that CPE differences exist after persistence trials, subsequent studies can isolate individual factors, or expand on combinations of factors, that may affect the cytotoxicity of trichomonads.

In addition to documenting destruction of the monolayer we also saw noticed that cells lost their original, i.e. healthy, stellate appearance. Cells experienced shrinkage, tended to cluster, and stain intensity increased. Our findings support an Amin et. al study that showed trichomonads tended to make holes in the monolayer that expanded before complete detachment (2012). This behavior resulted in a pattern of remaining adhered cells into a “spider web” appearance (Pindak et al., 1986). Another study also documented cellular response to damage by trichomonads and found “progressive degeneration of the cells, including shrinkage” (Abraham and Honigberg, 1965), but did not use fluorescent staining. The changes they saw were also with *T. gallinae* and avian fibroblasts. The effects of cellular damage on DF-1 cells is not well described, but the changes we saw with trichomonads in this study clearly were different to non-serum controls and consistently followed the same changes to detachment.

After careful consideration we did not eliminate bacterial/fungal contamination from our post-persistence trichomonad cultures. Birds in the wild are not expected to acquire clonal, axenic parasites, therefore microorganisms other than trichomonads will always

be a part of the equation during true natural infection. The bacteria in our cultures came from environmental sources, e.g. the OM we used, and would not be expected to be particular pathogens of birds. The effect of antibiotics in media to diminish virulence is documented, but requires many passages in antibiotic medium (~100) to have such an effect. Our sample aliquots in HF from each bird bath contained antibiotics and our cultures were passed in this media 1-2 times. We felt that this level of treatment would decrease bacterial contamination without affecting the virulence of the trichomonads, but chose not to pass any longer for unknown influence of antibiotics on the virulence of these post-persistence trichomonads. Further, both the DMEM and NS DMEM contained the same amount of antibiotics as the HF so all parasites and cells had the protective effect of antbiotics throughout co-culture. Two treatments (COHA OM 6 hr and BWHA OM 24 hr) were completely excluded from the study due to bacterial or fungal overgrowth in the cell culture, which also caused a third treatment (BWHA OM 48 hr) to have only one uncontaminated replicate. In the future eliminating bacteria from post-persistence trichomonad culture and/or infecting cells with bacteria from OM treatments as a control will improve the conclusions we can draw from this type on infection trial. In this trial we were limited by the unknown effect on cell culture of bacterial and fungal contamination that may have been introduced with persistence trichomonads from the simulated bird baths.

In conclusion, we documented changes in virulence after trichomonads persisted in simulated bird baths with different environmental conditions. Previously we documented persistence of trichomonads in simulate bird baths, however, it was unknown whether these trichomonads were still capable of causing disease. We used cell culture in this study as a measure of virulence and suggest more trials to characterize different conditions. After in vitro methods have characterized CPE with a range of conditions, live animal infections can be used for an *in vivo* assessment. Improving our knowledge of the relationship of trichomonad virulence and bird baths we can suggest strategies to help mitigate outbreaks and decrease transmission.

Chapter VI

Molecular investigation of *Trichomonas* spp. from clinically affected birds and subclinical hunter-killed Columbiformes in California

Abstract

Avian trichomonosis is a deadly disease in wild birds caused by flagellated protozoan parasites of the *Trichomonas* genus. We investigated whether *Trichomonas* spp. isolated from subclinical, hunter-killed Columbiformes belonged to the same genetic groups, based on ITS1-5.8S-ITS2 region (ITS) and iron hydrogenase gene (FeHyd), as isolates we collected from carcasses with lesions consistent with trichomonosis. We then compared the genotypes of the aforementioned isolates to those responsible for a spatially and temporally overlapping outbreak in Pacific Coast band-tailed pigeons (*Patagioenas fasciata monilis*) (BTPI) in San Diego County, CA. Hunter-killed doves were sampled for *Trichomonas* spp. via the InPouch™ TF culture system (BioMed Diagnostics) in Imperial County, CA during September, 2015. Tissue samples of trichomonas lesions were collected at the Wildlife Diseases Investigations Laboratory (Rancho Cordova, CA) from 16 species from 2013-2018. We documented a prevalence of *Trichomonas* spp. in 53% of hunter-killed birds (17/32) by culture and 70% of clinically affected birds (77/110) using PCR. We found ITS group genotypes I and L in hunter-killed cultures and A, D, and I in tissue samples; FeHyd subtyping revealed subtypes E1 and K1 in hunter-killed samples, and A1 and A2 in tissue samples. Phylogenetic analyses indicate that hunter-killed isolates cluster together while tissue lesion genotypes form a separate group. A single hunter-killed isolate grouped with the tissue lesion genotypes. These data suggest the isolates responsible for clinical disease are not primarily circulating in the subclinical columbids we sampled. Ongoing investigations of avian host species, trichomonad genotype, and geographic associations among California wild birds is needed to improve our understanding of the ecology and epidemiology of avian trichomonosis.

Introduction

Trichomonas gallinae, the pathogenic protozoan parasite of birds, is responsible for negative population-level effects. Trichomonosis has historically been reported in

Columbiformes and raptors, and more recently described in songbirds (Boal et al., 1998; Forzán et al., 2010; Haugen, 1952; Robinson et al., 2010; Stabler, 1954). The often asymptomatic natural host, the rock pigeon (*Columba livia*), has been implicated in the worldwide distribution of the parasite (Stabler, 1954). Other columbid species including mourning doves, white-winged doves, Eurasian collared doves, and band-tailed pigeons can harbor trichomonads subclinically, and be a source of infection for naïve birds (Conti and Forrester, 1981; Conti et al., 1985), or they may develop clinical disease when infected with virulent isolates. The Pacific Coast Band-tailed pigeon (*Patagioenas fasciata monilis*) is currently experiencing marked increase in mortality in Southern California leading to population declines. Periodic outbreaks of trichomonosis have plagued Pacific Coast Band-tailed pigeons (BTPI) since the 1940s (Rogers et al., 2016). Drought conditions have reportedly exacerbated the outbreak as birds congregate around limited water sources and are more likely to acquire trichomonads from shared food and water sources (Bunbury et al., 2007; Kocan, 1969; McBurney et al., 2017; Purple and Gerhold, 2015).

Molecular analyses of trichomonad strains associated with outbreaks in a variety of species has temporally and spatially described the relationships among strains (Ganas et al., 2014; Lawson et al., 2011b). The internal transcribed spacer (ITS) region (5.8S rDNA and flanking ITS regions, ITS1 and ITS2) and the hydrogenosomal Fe-hydrogenase (Fe-Hyd) gene are widely used for genotyping trichomonad isolates (Lawson et al., 2011a; McBurney et al., 2015). A clonal strain belonging to ITS group A (Gerhold et al., 2008) and FeHyd subtype 1 was shown to be responsible for outbreaks in the U.K. (Lawson et al., 2011a), Spain (Sansano-Maestre et al., 2009), Austria (Grabensteiner et al., 2010), Mauritius (Gaspar da Silva et al., 2007), and Brazil (Kleina et al., 2004) based on typing at the ITS and FeHyd loci (Ganas et al., 2014; Lawson et al., 2011a; McBurney et al., 2015). The FeHyd target, a house-keeping gene previously identified in *T. vaginalis*, allowed further subtyping to separate avian trichomonad strains that are indistinguishable with ITS analysis alone (Sansano-Maestre et al., 2016). Applying these molecular techniques, we aimed to characterize *Trichomonas*

spp. isolates from non-clinical hunter-killed columbids. In addition, we analyzed tissue samples, from multiple avian species, with lesions consistent with trichomonosis and compared the results to those of the hunter-killed columbids. The collection of the aforementioned avian samples spatially and temporally overlapped in southern California from 2013-2015. In particular, we were interested to determine if any of the hunter-killed or tissue samples aligned with previously determined ITS groups, especially L and K (now *T. stableri*), that have been associated with trichomonas mortality events in Pacific Coast BTPI in California (Gerhold et al., 2008; Girard et al., 2014a).

Molecular analysis of BTPI in California has implicated *Trichomonas gallinae* ITS group L, and has described a new species, formerly ITS group K, *Trichomonas stableri* (Gerhold et al., 2008; Girard et al., 2014a). We sought to determine if hunter-killed columbids were carrying the same strains responsible for the Pacific Coast Band-tailed pigeon outbreak and the mortality of a wide variety of birds that presented to the California Department of Fish and Wildlife, Wildlife Investigations Laboratory.

Materials and Methods

Culture for subclinical hunter-killed birds

Sterile cotton-tipped applicators were used to swab the oral cavity of 32 hunter-killed doves on September 1st, 2015 in Imperial County, California. InPouch™ TF pouches (BioMed Diagnostics, White City, OR) were inoculated with sample swabs and mailed overnight at ambient temperature to the University of Tennessee Molecular Parasitology Laboratory in Knoxville, Tennessee. Samples were incubated at 37°C upon arrival (September 2nd, 2015) and inspected by light microscopy every other day for 7 days for motile trichomonads. Culture-positive samples were incubated until trichomonads reached logarithmic growth and were frozen in -20°C for molecular analyses.

Tissue from suspected infected birds

Oral cavity tissue samples were obtained from birds with suspected trichomonosis during necropsies performed at the Wildlife Investigations Laboratory, California Department of Fish and Wildlife (Rancho Cordova, CA) and were frozen and shipped to the University of Tennessee (Knoxville, TN) for DNA extraction. A total of 110 samples from 16 different species were examined (Table VI-II).

Molecular Analyses

DNA extraction was performed on concentrated trichomonads from culture or on tissues with Qiagen DNeasy kit according to manufacturer's instruction with the following modifications: samples were incubated at room temperature for 15-20 minutes with the microcentrifuge lids open to ensure ethanol evaporation before elution; buffer AE was incubated for 30 minutes instead of 1 minute to optimize DNA elution. Polymerase-chain reaction (PCR) reactions were performed to amplify two different targets. For each ITS 1, 5.8S, ITS 2 region PCR reaction we used a total volume of 25 uL containing 1 uL ITSF and 1 uL ITSR primers (Cepicka et al., 2005), 12.5 uL DreamTaq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA), 8.5 uL nuclease-free water (NFW), and 2 uL DNA template. Our thermocycler program was as follows: 94°C for 15 minutes, then 39 cycles of 94°C for 1 minute, 52°C for 30 seconds, and 72°C for 1 minute, followed by 72°C for 10 minutes. The FeHyd reactions were performed as above with the primers TrichhydFOR (5'-GTTTGGGATGGCCTCAGAAT-3') and TrichhydREV (5'-AGCCGAAGATGTTGTCGAAT-3') (Lawson et al., 2011a) and the same thermocycler program. A known *Trichomonas* PCR-positive rock pigeon sample, "R20" (Gerhold et al., 2008), was used as a positive control and NFW was included as negative control for every PCR reaction. Seven microliters of PCR products were run using gel electrophoresis on a 2% agarose gel with 1x TAE buffer and 2 uL ethidium bromide for ~1.5 hours at 90v. UV light enabled viewing of DNA as "bands" at ~375bp for ITS and ~900bp for FeHyd. All controls yielded appropriate results.

The remaining PCR products, 18 uL, from each PCR reaction were purified using the QIAquick® PCR purification kit (Qiagen, Valencia, CA). Purified DNA was sent to the University of Tennessee Genomics Core Facility (Knoxville, TN) for Sanger Sequencing. Sequences were automatically aligned with Sequencher™ 5.0.1 DNA Analysis software and verified manually by the authors. Sequences were compared to published sequences from NCBI GenBank® via BLAST® analysis.

For both targets, phylogenetic analyses were conducted using MEGA version 7 (Kumar et al., 2016). A maximum likelihood (ML) dendrogram was constructed to scale with evolutionary distances computed using the Kimura 2-parameter method (Figure VI-II). Bootstrapping was performed with 500 replicates.

Results

Culture of hunter-killed birds

Of the cultures, 53.1% (17/32) were positive for trichomonad growth, all detectable by day 5. Forty-three percent of mourning doves were culture positive (10/23), 100% of white-winged doves (6/6), and 33.3% of Eurasian collared doves (1/3) (Table VI-I).

Molecular

ITS Region

For the positive hunter-killed bird cultures, 100% (17/17) were PCR-positive at the target level (~300bp) of ITS. The Eurasian collared dove (*Streptopelia decaocto*) (EUCD) positive sample (HK_17) did not form a consensus when analyzed in Sequencher™. Consensus sequences, and the unidirectional strands from the EUCD, were matched with previously published ITS-group designated letters (Gerhold et al., 2008). Consensus sequences most closely matched the ITS groups previously reported in the *Trichomonas vaginalis*-like clade by Gerhold et al. (Gerhold et al., 2008). Both unidirectional strains from HK_17 most closely matched ITS group C. Non-overlapping

sequences belonging to different groups could indicate a dual or multiple strain infection, which has been described by other authors (Robinson et al., 2010; Sansano-Maestre et al., 2016)

Out of the 110 tissue samples, 103 were from birds suspected of having trichomonosis and 7 were from birds with unknown history or had no lesions consistent with trichomonosis (Table VI-II). Overall, 24.5% of samples were PCR-negative (27/110). The remaining samples were PCR-positive (75.5%; 83/110) at the expected ITS target size (~300bp), including 1 sample that sequenced as bird DNA, 5 samples that had additional bands at non-target sizes, and 1 sample which could not form a consensus but had forward and reverse strands with 100% identity to ITS group K and L, respectively. This left 76 positive samples out of 110 (69.1%) with single bands for our analyses.

For the ITS phylogenetic analysis, we combined the HK and KR sequences into one ITS dendrogram to elucidate the phylogenetic relationship among both subclinical and clinical isolates (Figure VI-II). We had 76 tissue samples (KR) and 16 hunter-killed (HK) isolates that had consensus sequences (Table VI-III). For the tissue samples, KR_44 and KR_86 had fewer base pairs and less overlap, and so were removed to maintain sequence length available to analyze. To eliminate identical sequences from interfering with the phylogenetic algorithm, we used our unique sequences (KR_25, 62, 73, 87 and HK_2, 6 and 16), ITS Groups that had 100% identity with some of our sequences (A, D, I, and L), and ITS Groups B, F, and J for their close matches to our sequences (but not identical). *Trichomonas vaginalis* (AY957955) and *T. stableri* (KC215390) were used as comparison sequences from GenBank due to their close relationship with the outbreak. The tree was rooted with *Trichomonas nonconforma* (AY886845) as the outgroup.

The phylogenetic relationships disclosed that all the tissue samples as well as ITS Groups A, B, and D clustered together (bootstrap value 88). The tissue samples all came from birds with lesions consistent with trichomonosis. Published ITS Groups did not correspond to presence or absence of clinical disease. The hunter-killed samples

could not be resolved, but were more closely grouped with *T. vaginalis* and ITS Groups I, J, and L, which are in the *T. vaginalis*-like group (Gerhold et al., 2008).

FeHyd Gene

PCR was positive at the FeHyd loci for all 17 (100%) of the culture positive hunter-killed samples. Of these, 13 samples formed consensus sequences, of which 3 samples aligned most closely with *T. gallinae*, 4 sequences with *Trichomonas* sp., and 6 sequences with *T. vaginalis*. A consensus sequence for the remaining 4 sequences was not achieved, however, two isolates had one directional match with *Trichomonas* sp. and the other with *T. vaginalis*. Of the final 2 isolates, each had only one successfully sequenced strand; one matched most closely with *T. vaginalis* and the other with *Trichomonas* sp.

Of the 76 PCR-positive tissue samples that had consensus sequences based on ITS, 28.9% (22/76) were PCR-positive for FeHyd. When comparing our FeHyd sequences to those in GenBank, 14.5% (11/76) most closely aligned with KX514380.1, 6.6% (5/76) with HG008115, 3.9% (3/76) with KP900029, and 3.9% (3/76) with KP900030.

For the FeHyd phylogenetic analysis, we had 22 tissue samples (KR) and 13 hunter-killed (HK) isolates that had consensus sequences (Figure VI-III). For the tissue samples, KR_46 had fewer base pairs and less overlap, and so was removed to maintain length available of all other sequences to analyze. There was one unique sequence, KR_97, and 2 groups based on 100% shared identity at the FeHyd locus. Group 1 contained KR_80 and 44, and Group 2 included the remaining 18 FeHyd sequences: KR_100, 36, 38, 40, 47, 49, 65, 67, 68, 82, 85, 86, 90, 93, 96, 98, 99, and 101. For the hunter-killed samples, HK_16 and HK_7 were removed to maintain length for the group. The remaining HK samples were all unique (HK_1, 5, 8-15, and 17).

We combined our HK and KR sequences with FeHyd subtyped sequences generously shared with us by Drs. Kevin Tyler and Fahad Alrefaei Abdulwahed (unpublished) into one FeHyd dendrogram to elucidate the phylogenetic relationship among our subclinical

and clinical isolates and the described subtypes. To eliminate identical sequences from interfering with the phylogenetic algorithm, we checked all sequences (521 bases once trimmed) for 100% identity. Our KR_100 group was identical with A1, A1.1, and A1.3; KR_44 and KR_80 were identical to A2; HK_1 was identical to E1; and K1.1 was identical to K1. In our final tree, we used our unique sequences (KR_97 and HK_1, 5, 8-15), with unique subtyped FeHyd sequences (A1.1, A1.2, C1, C2, C4, D1, G1, H1, K2, L1, L2, L3, L4, and L5), subtypes that were identical to some of our isolates (A1, A2, E1, and K1), and *T. stableri* (KC215390) (Table VI-III). The tree was rooted with *Trichomonas vaginalis* (AY957955) as the outgroup (there were no FeHyd sequences available on GenBank for *Trichomonas nonconforma* at the time of writing).

The FeHyd tree (Figure VI-III) shows the tissue samples, the A, C, D, E, G, and H subtypes, and the HK_17 from the EUCD at a basal location on the tree to the root, *T. vaginalis*, while the other hunter-killed isolates, the L3 and L4 subtypes, and *T. stableri* show more evolutionary divergence to the rest of the tree. Strong bootstrap values (>/= 98%) support separate branches for K subtypes, L1 and L2 subtypes, and the most divergent branch containing most of the hunter-killed isolates.

Discussion

We showed a prevalence of *Trichomonas* spp. in 53.1% (17/32) in subclinical hunter-killed columbids by culture, which was higher than the prevalence detected in hunter-killed BTPI sampled in 2011 in northern California (11.1%; 6/54) (Girard et al., 2014b) and hunter-killed mourning doves in Missouri (5.6%; 226/4052) (Schulz et al., 2005). Reported prevalence for wild-caught birds can be much higher than that recorded for hunter-killed birds, for example, wild-caught European turtle doves had a prevalence of 85.7% (12/14) (Lennon et al., 2013). Wild pigeons in eastern Spain had a higher prevalence of *Trichomonas* spp. (41%; 180/439) than birds of prey in the same study (19.6%; 20/102) (Sansano-Maestre et al., 2009). Reported prevalence can vary widely based on host species, method of capture, presence or absence of lesions, geographic location of sampling, etc. Traditionally, sampling of wildlife species often presents

similar challenges. Monitoring the prevalence of *Trichomonas* spp. in any population, identifying circulating genotypes, and publishing findings to make the data widely available, will continue to improve our understanding of the epidemiology of *Trichomonas* spp.

All of our positive cultures (100%; 17/17) were also PCR-positive at ITS. The strains from these species (MODO, WWDO and EUCD) grouped with ITS groups I, J and L as well as *Trichomonas vaginalis*. Of the tissue samples, we found *Trichomonas* spp. by ITS PCR in 70.0% of birds tested (77/110), most of which (97.4% (74/76)) had clinical evidence of trichomonosis. Tissue sample sequences formed a separate group than the hunter-killed bird isolates. These phylogenetic differences suggest that these hunter-killed doves were not carrying the strain responsible for the clinical disease in birds that presented to the Wildlife Investigations Laboratory.

Phylogenetic grouping based on the ITS locus show that hunter-killed strains group most closely with other hunter-killed isolates, ITS Groups I and J, and *T. vaginalis*, but could not be resolved any further (Figure VI-II). These sequences had no strong relationship (bootstrap values under 50) to either *T. vaginalis* or *T. nonconforma* (the outgroup). Tissue samples from the unique KR_25, 62, 73 and 87 grouped most closely with ITS Group A (identical to 67 of the KR isolates), Group D (identical to two KR isolates) and Group B.

At the FeHyd locus all hunter-killed isolates grouped more closely to one another and genotype L and *T. stableri* except for the HK_17 EUCD isolate which was identical to E1. E1 was more closely related to genotypes C and D, and the tissue samples. This suggests that the EUCD isolate could be more similar to the clinical strains and more sampling of EUCD could improve our understanding of the phylogenetic relationships. It is possible that the subclinical columbids we have shown to harbor strains related to *T. stableri* could spread this and other virulent strains to susceptible birds. It is also possible that the virulent *T. stableri* in BTPI is the result of a divergence from this group of isolates recovered from subclinical doves. The phylogenetic relationship among

FeHyd type C1, C2, D1, and E1 (HK_17) was unresolved, but diverged earlier than the hunter-killed isolate group (evolutionary distance scale <0.02) and was closer to the tissue samples within A1 and A2. The majority of hunter-killed isolates grouped more closely to one another and with genotype L and *T. stableri.* Molecular analyses will be greatly improved with the increasing accessibility and decreasing cost of whole genome sequencing.

The effects of climate change on wildlife and impacts of the subsequent emerging diseases are extensively documented and have no evidence of slowing down. Drought conditions in California are increasing in frequency and severity, which influences availability of water for wildlife both in number and size of water sources. As birds congregate around diminishing resources pathogens are transmitted in new patterns. Trichomonosis outbreaks have been linked to drought conditions in California (Rogers et al., 2016) and backyard bird feed and water stations (Forzán et al., 2010; McBurney et al., 2017). Climate change may increase the reliance of wildlife on anthropogenic sources of food and water. The prevalence of *Trichomonas* spp. in doves in Spain was associated with supplementary feeding sites for gamebirds (Lennon et al., 2013). More research is needed to identify strategies to mitigate deleterious anthropogenic effects on wildlife populations.

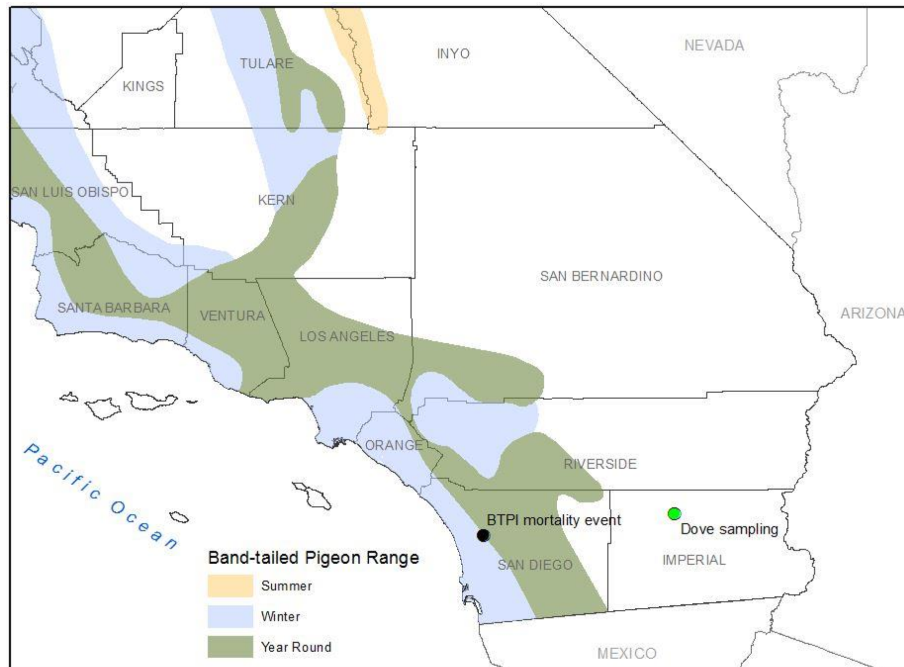


Figure VI-I. Range map.

Geographic representation of band-tailed pigeon range in Southern California relative to the location of band-tailed pigeon mortality event between January and March 2015 (solid black circle) and the sampling of the 2015 hunter-killed Columbiformes (green circle).

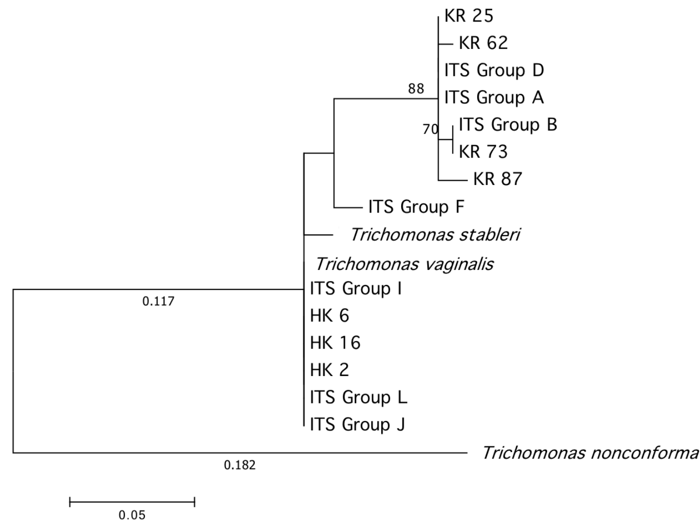


Figure VI-II. Molecular Phylogenetic analysis based on ITS by Maximum Likelihood method.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-478.55) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches). The analysis involved 17 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 175 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

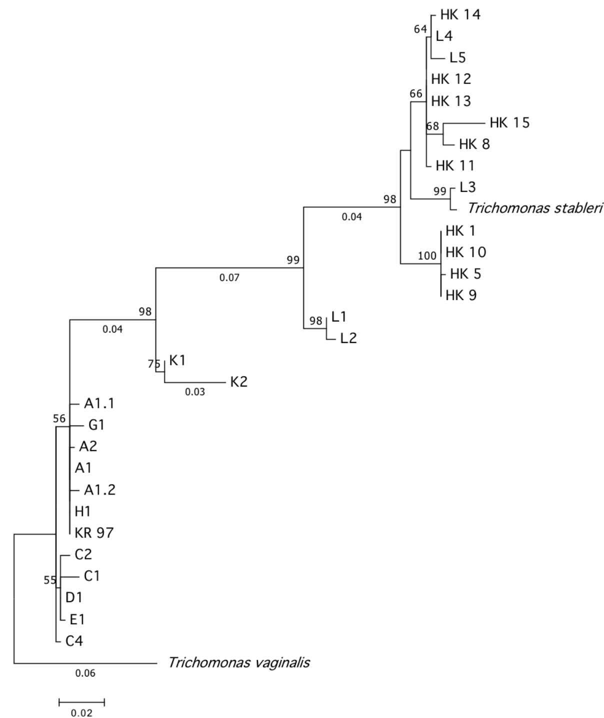


Figure VI-III. Molecular Phylogenetic analysis based on FeHyd by Maximum Likelihood method.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-1768.58) is shown. The percentage of trees in which the associated taxa clustered together is shown above the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (below the branches). The analysis involved 31 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 495 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Table VI-I. Culture results.

Culture results from hunter-killed doves from Imperial County, California in September, 2015. MODO = mourning dove; WWDO = white-winged dove; EUCD = Eurasian collared-dove.

Species	Species abbreviation	Positive/Total
Mourning dove	MODO	10/21
White-winged dove	WWDO	6/8
Eurasian dove	EUCD	1/3

Table VI-II. *Trichomonas* spp. isolates from California avian tissue samples.

Species	Species abbreviation	Total Samples (Year)
Mourning dove	MODO	5 (2013); 2 (2014); 10 (2015); 40 (2016)
Barn owl	BANO	3 (2014); 3 (2015); 2 (2016)
Red-tailed hawk	RTHA	5 (2015); 2 (2016)
Cooper's hawk	COHA	2 (2015); 4 (2016)
Rock pigeon	ROPI	3 (2014); 3 (2016)
American crow	AMCR	3 (2013); 1 (2014); 1 (2016)
Great-horned owl	GHOW	1 (2013); 2 (2015)
Pine Siskin	PISI	3 (2016)
American kestrel	AMKE	1 (2014); 1 (2016)
Red-shouldered hawk	RSHA	1 (2014); 1 (2016)
Lesser goldfinch	LEGO	1 (2016)
Steller's jay	STJA	1 (2016)
Band-tailed pigeon	BTPI	1 (2015)
Peregrine falcon	PEFA	1 (2015)
White-tailed kite	WIKI	1 (2015)
Bald eagle	BAEA	1 (2014)
Burrowing owl	BUOW	1 (2014)
Eurasian collared-dove	EUCD	1 (2014)
Northern mockingbird	NOMO	1 (2014)
Golden eagle	GOEA	1 (2013)

Table VI-III. Molecular Results

Molecular results for hunter-killed (HK) and tissue samples (KR) and ITS Groups and FeHyd subtypes for phylogenetic analyses. ITS Group Letters L, I, A, and D (Gerhold et al., 2008) aligned in column to identical to Study ID sequences in rows. FeHyd column with subtypes (Sansano-Maestre et al., 2016) E1, A1, and A2 aligned with identical study IDs in rows. Unique ITS and FeHyd labels indicate study IDs that had no identical matches to published ITS or FeHyd Groups. * = Birds with lesions consistent with trichomonosis. Host species codes: MODO = mourning dove, WWDO = white-winged dove, BANO = Barn owl, RTHA = Red-tailed hawk, ROPI = Rock pigeon, COHO = Cooper's hawk, AMCR = American crow, GHOW = Great-horned owl, PISI = Pine Siskin, AMKE = American kestrel, RSHA = Red-shouldered hawk, BTPI = Band-tailed pigeon, PEFA = Peregrine falcon, WIKI = White-tailed kite, LEGO = Lesser goldfinch, STJA = Steller's jay, GOEA = Golden eagle, EUCD = Eurasian collared-dove, BUOW = Burrowing owl, NOMO = Northern mockingbird, BAEA = Bald eagle. Gray shading = no consensus sequence for isolate and corresponding column target.

Study ID	Host Species	ITS Group	FeHyd Group
HK_1	MODO	L	Unique
HK_4	MODO		
HK_5	MODO		
HK_6	MODO		
HK_9	MODO		
HK_10	MODO		
HK_11	WWDO		
HK_12	WWDO		
HK_13	WWDO		
HK_14	WWDO		

Table VI-III continued. Molecular Results

<table>
<tr><th>Study ID</th><th>Host Species</th><th>ITS Group</th><th>FeHyd Group</th></tr>
<tr><td>HK_7</td><td>MODO</td><td></td><td rowspan="2"></td></tr>
<tr><td>HK_3</td><td>MODO</td><td rowspan="3">I</td></tr>
<tr><td>HK_8</td><td>MODO</td><td rowspan="2">Unique</td></tr>
<tr><td>HK_15</td><td>WWDO</td></tr>
<tr><td>HK_2</td><td>MODO</td><td rowspan="6">Unique</td><td rowspan="6"></td></tr>
<tr><td>HK_16</td><td>WWDO</td></tr>
<tr><td>KR_25</td><td>MODO*</td></tr>
<tr><td>KR_62</td><td>BANO*</td></tr>
<tr><td>KR_73</td><td>PEFA*</td></tr>
<tr><td>KR_87</td><td>MODO*</td></tr>
<tr><td>HK_17</td><td>EUCD</td><td rowspan="3"></td><td>E1</td></tr>
<tr><td>KR_44</td><td>ROPI*</td><td>A2</td></tr>
<tr><td>KR_86</td><td>MODO*</td><td>A1</td></tr>
<tr><td>KR_80</td><td>RSHA*</td><td rowspan="13">A</td><td>A2</td></tr>
<tr><td>KR_36</td><td>MODO*</td><td rowspan="12">A1</td></tr>
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<tr><td>KR_49</td><td>AMCR*</td></tr>
<tr><td>KR_65</td><td>BUOW*</td></tr>
<tr><td>KR_67</td><td>COHA*</td></tr>
<tr><td>KR_68</td><td>COHA*</td></tr>
<tr><td>KR_82</td><td>RSHA*</td></tr>
<tr><td>KR_85</td><td>MODO*</td></tr>
<tr><td>KR_90</td><td>MODO*</td></tr>
<tr><td>KR_93</td><td>MODO*</td></tr>
</table>

Table VI-III continued. Molecular Results

Study ID	Host Species	ITS Group	FeHyd Group
KR_96	MODO*	A	A1
KR_98	MODO*		
KR_99	MODO*		
KR_100	MODO*		
KR_101	MODO*		
KR_1	BTPI*		
KR_2	EUCD*		
KR_4	MODO*		
KR_5	MODO*		
KR_6	MODO*		
KR_7	MODO*		
KR_11	MODO*		
KR_14	MODO*		
KR_15	MODO*		
KR_16	MODO*		
KR_18	MODO*		
KR_19	MODO*		
KR_20	MODO*		
KR_21	MODO*		
KR_22	MODO*		
KR_23	MODO*		
KR_27	MODO*		
KR_29	MODO*		
KR_31	MODO*		
KR_33	MODO*		

Table VI-III continued. Molecular Results

Study ID	Host Species	ITS Group	FeHyd Group
KR_34	MODO*	A	
KR_35	MODO*		
KR_37	MODO*		
KR_39	MODO*		
KR_43	ROPI*^		
KR_45	AMCR		
KR_46	AMCR*		
KR_48	AMCR*		
KR_57	BANO*		
KR_58	BANO*		
KR_59	BANO*		
KR_60	BANO*		
KR_61	BANO*		
KR_63	BANO*		
KR_71	GHOW*		
KR_75	RSHA*		
KR_76	RSHA*		
KR_77	RSHA*		
KR_79	RSHA*		
KR_83	WTKI*		
KR_84	MODO*		
KR_88	MODO*		
KR_95	MODO*		
KR_102	MODO*		
KR_103	MODO*		
KR_105	AMKE*		

Table VI-III continued. Molecular Results

<table>
<tr><th>Study ID</th><th>Host Species</th><th>ITS Group</th><th>FeHyd Group</th></tr>
<tr><td>KR_106</td><td>COHA*</td><td rowspan="4">A</td><td rowspan="6"></td></tr>
<tr><td>KR_109</td><td>STJA*</td></tr>
<tr><td>KR_110</td><td>STJA*</td></tr>
<tr><td>KR_87</td><td>MODO*</td></tr>
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Chapter VII

Conclusion

We established that trichomonads can survive in water with added organic material for at least 48 hr. Because *Trichomonas gallinae* does not have a true cyst phase previous knowledge suggested a poor ability of trichomonads to persist in the environment. Early research that subjected trichomonads to different *in vitro* conditions including saline water and aqueous grain extracts found trichomonads were capable of persisting, and even suggested they could reproduce, in some of the conditions tested (Kocan, 1969). We continued to explore the unanswered question of whether trichomonads could persist in conditions closer to those found in the environment by using different water types found in nature (ie. bird bath, rain barrel, etc) and adding organic material to water to simulate backyard bird baths of varying levels of sanitation. For the OM and water types experiments we recorded a maximum persistence documented at 16 hr that far exceeded the 20 min persistence established in similar conditions (i.e. distilled water with added organic material). The extended persistence we demonstrated would allow more than just horizontal transfer between two birds feeding/drinking next to one another to birds that visit a water source at different times.

To further understand persistence, we isolated dissolved oxygen (DO) as a possible mechanism to explain increased persistence of trichomonads in the presence of OM. We showed that with artificially lower DO, trichomonads persisted up to 30 hr. We added more treatments and assessed the virulence of two isolates, BWHA and COHA, before and after persistence treatments. In simulated bird baths with OM at a temperature of 37°C, we documented a persistence of 48 hr for both isolates. This illustrates trichomonads are capable of long periods of persistence in certain environmental conditions. We also chose a less hospitable environment, distilled water with UV light exposure, and saw persistence of up to 4 hr with the BWHA. These findings confirm that trichomonads are not the fragile organisms as previously suggested. Our results clearly show that organic material increases persistence in water. Evaluating other methods that increase or decrease persistence will further our understanding on transmission conditions and enable us to take corrective actions to decrease mortality in outbreaks.

The research described above establishes the persistence of trichomonads in water environments. A major unanswered question remains: do these trichomonads retain their virulence? Pathogenicity of isolates is known to decline in certain *in vitro* conditions, especially over time. Amin, et al. reported that cell culture is a valuable tool to evaluate pathogenicity characteristics of trichomonad isolates (2014). To address this question, we evaluated the cytopathic effect of trichomonads on cell culture before and after persistence experiments. We found that trichomonads that persisted 4 hours in the UV treatment produced more monolayer destruction than the non-persistence trichomonads indicating increased virulence. This study confirms that persistence can alter virulence. We also found a significant treatment difference between OM and UV treatments after 2-6 hr. The microenvironment of the bird bath and other water sources can affect the length of persistence and the virulence of isolates and will be a critical target for intervention of transmission during outbreaks.

Understanding trichomonad ecology in water and the virulence of isolates has given us an understanding of the transmission on a small scale. We wanted to expand our understanding of how isolates are transmitted through populations and how virulence between strains vary. To do this we applied molecular techniques to document genotypes of *Trichomonas* spp. circulating in a current outbreak in California. Climate change is increasing occurrence and severity of extreme weather patterns. California experiences one impact of climate change in the form of severe droughts. The current outbreak and Pacific Coast band-tailed pigeons has been associated with drought (Rogers et al., 2016). As water becomes scarce wildlife congregate in larger groups around fewer, smaller resources, including water pathogens can spread more quickly and to novel hosts. We found that subclinical hunter-killed birds and a variety of clinically affected birds tend to have separate groups of primarily circulating genotypes. Our understanding of phylogenetic relationships among wild populations will be improved with the increasing accessibility of molecular techniques including whole genome sequencing. With complete genomes we will no longer be limited by single targets or even multi-locus sequencing and RAPD (random amplified polymorphic DNA)

techniques. We anticipate these and other improved molecular techniques to improve our understanding of the role of genes in virulence of trichomonads, the evolutionary changes in various strains, and also connections between strains and geography or host species.

The combination of these findings- persistence in water in a variety of conditions by a variety of different genotypes, and the effect of persistence on virulence will provide the scientific foundations for other studies. We have shown that trichomonads are not fragile in the environment and that virulence can increase after short periods (4 hr) in simulated environmental conditions. Using this information on the behavior of trichomonads on the individual level can help us understand the larger scale of genotypes circulating around the world. We have improved the understanding of transmission dynamics and can provide targets for wildlife biologists and backyard bird enthusiasts to mitigate outbreaks, namely to keep bird bath clean from organic debris.

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APPENDIX

Supplemental Data

Table A-I. Oxyrase and persistence.
Persistence of two trichomonad isolates with two different concentrations of Oxyrase® in 3 replicates with the recorded dissolved oxygen (DO) saturation for each time point. pH was also recorded at hour 30 and is shown with standard deviation. COHA= Cooper's hawk; BWHA= broad-winged hawk; STDEV= standard deviation

		Oxyrase ® (vol/vol)	
		0.5%	1.0%
	Replicate	Minimum persistence (hours)	Minimum persistence (hours)
COHA	1	13	26
	2	18	26
	3	18	30
	Mean +/- SD	16.33 +/- 2.89	27.33 +/- 2.31
BWHA	1	18	26
	2	18	30
	3	26	30
	Mean +/- SD	20.67 +/- 4.62	28.67 +/- 2.31

VITA

Kathryn (Kate) Erin Purple grew up in Wilmington, Delaware and wanted to become a veterinarian in wildlife conservation from a very young age. She received her Bachelor of Science degree with honors in 2006 from the University of Illinois with a Fish and Wildlife Conservation major and a minor in Chemistry. Her involvement in research started early in her education and ranged from studying antimicrobial susceptibility patterns of organisms cultured from the wounds of wildlife as a Howard Hughes Undergraduate Research Fellow, to documenting parasitic species collected from wildlife in the Knoxville area and addressing potential for zoonotic concerns. Kate earned her Doctor of Veterinary Medicine degree from University of Tennessee College of Veterinary Medicine in 2012. Her main research interest is infectious disease in wildlife. She aspires to teach and conduct research at a college of veterinary medicine.

During her doctoral program, Kate and her husband, Matt Murphy, welcomed a happy baby boy, Jonathan (Jack) George Murphy. Kate, Matt, and Jack live in Knoxville with their two adopted dogs, Hallie and Parker. Kate credits her family for helping her preserve to achieve her second doctoral degree.