

Interactions of ciliates with cells and viruses of fish

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

This thesis develops and utilizes in vitro approaches to study ciliate/fish interactions. The thesis is divided into six chapters. Chapter one reviews the literature on culturing ciliates and fish cells. Chapter two develops methods for culturing the ciliate *Tetrahymena thermophila* in media developed and used for mammalian and piscine cells. Chapter three explores the interactions of *T. thermophila* with monolayers of epithelial cells from fish and mammals. Chapter four studies the interactions of *T. corlissi*, *T. thermophila*, and *T. canadensis* with monolayers of epithelial and fibroblasts from a wide range of animals. The interactions of *T. thermophila* with the fish viruses are described for the rhabdovirus, viral hemorrhagic septicemia virus (VHSV), in chapter five and for the aquareovirus, Chum salmon reovirus (CSV) in chapter six. The summaries for these six chapters are presented in the following six paragraphs.

How the ciliates of fish can be cultured and used to study ciliate/fish interactions are reviewed. The culturing of ciliates is done in media based on either freshwater, seawater, or vertebrate bodily fluids together either with bacteria, fish cells, or organic matter, which can be undefined, such as proteose peptone, or defined. Some ciliates can be pathogenic but with a variable dependency on the fish host. The most dependent and difficult to culture has been *Ichthyophthirius multifiliis*. *Cryptocaryon irritans* has been maintained successfully in co-cultures with fish cells. Pathogenic scuticociliates and tetrahymenas can be cultured axenically. Established cultures have been used to screen drugs for their potential chemotherapeutic value and to study pathogenic mechanisms. As well as being pathogens, ciliates interact with fish in other ways. Free-living forms can modulate the activities of other fish microbial pathogens and be food for fish larvae. *Tetrahymena* spp. have been shown in culture to phagocytose pathogenic bacteria and microsporidia spores. Large-scale cultures of both freshwater and marine ciliates have been achieved and could be a source of feed for fish larvae. In the future cell cultures should be invaluable in studying these and other possible relationships between fish and ciliates.

The transfer of *Tetrahymena thermophila* from normosmotic solutions (~20 to 80 mOsm/kg H₂O) to hyperosmotic solutions (> 290 mOsm/kg H₂O) was investigated. During the first 24 h of transfer from proteose-peptone yeast extract (PPYE) to either 10 mM HEPES or PPYE with added NaCl to give ~300 mOsm/kg H₂O, most ciliates died in HEPES but survived in PPYE. Supplementing hyperosmotic HEPES or PPYE with fetal bovine serum (FBS) enhanced survival. When ciliates were transferred from PPYE to a basal medium for vertebrate cells, L-15 (~320 mOsm/kg H₂O), only a few survived the first 24 h but many survived when the starting cell density at transfer was high (100,000 cells/mL) or FBS was present. These results suggest that nutrients and/or osmolytes in either PPYE or FBS helped ciliates survive the switch to hyperosmotic solutions. FBS also stimulated *T. thermophila* growth in normosmotic HEPES and PPYE and in hyperosmotic L-15. In L-15 with 10 % FBS the ciliates proliferated for several months and could undergo phagocytosis and bacterivory. These cell culture systems and results can be used to explore how some *Tetrahymena* species function in hyperosmotic hosts and act as opportunistic pathogens of vertebrates.

Although several species of *Tetrahymena* are often described as histophagous and opportunistic pathogens of fish, little is known about ciliate/fish cell interactions, but one approach for studying these is in vitro with cell lines. In this study *T. thermophila*, B1975 (wild type) and NP1 (temperature sensitive mutant for phagocytosis) were cultured on monolayers of three fish epithelial cell lines, CHSE-214, RTgill-W1, and ZEB2J, and of the rabbit kidney epithelial cell line, RK-13. Generally the ciliates flourished, whereas the monolayers died, being completely consumed over several days. The destruction of monolayers required that the ciliates be able to make contact with the animal cells through swimming, which appeared to dislodge or loosen cells so that they could concurrently be phagocytosed. The ciliates internalized into food vacuoles ZEB2J from cell monolayers as well as from cell suspensions. Phagocytosis was essential for monolayer destruction as monolayers remained intact under conditions where phagocytosis was impeded, such as 37 °C for NP1 and 4 °C for B1975. Monolayers of fish cells supported proliferation of ciliates.

These results show for the first time that *T. thermophila* can ‘eat’ animal cells or be histophagous in vitro, with the potential to be histophagous in vivo.

The activities of *T. corlissi*, *T. thermophila*, and *T. canadensis* were studied in co-culture with cell lines of insects, fish, amphibians, and mammals. These ciliates remained viable regardless of the animal cell line partner. All three species could engulf animal cells in suspension. However, if the animal cells were monolayer cultures, the monolayers were obliterated by *T. corlissi* and *T. thermophila*. Both fibroblast and epithelial monolayers were destroyed but the destruction of human cell monolayers was done more effectively by *T. thermophila*. By contrast, *T. canadensis* was unable to destroy any monolayer. At 4 °C *T. thermophila* and *T. corlissi* did not undergo phagocytosis and did not destroy monolayers, whereas *T. canadensis* was able to undergo phagocytosis but still could not destroy monolayers. Therefore, monolayer destruction appeared to require phagocytosis, but by itself this was insufficient. Additionally the ciliates expressed a unique swimming behavior. *Tetrahymena corlissi* and *T. thermophila* swam vigorously and repeatedly into the monolayer, which seemed to loosen or dislodge cells, whereas *T. canadensis* swam above the monolayer. Therefore differences in swimming behavior might explain why *T. corlissi* has been reported to be a pathogen but *T. canadensis* has not.

Incubating the fish pathogen VHSV with the ciliate *T. thermophila*, inactivated the virus, depending on the incubation temperature. Without the ciliates, the VHSV titre declined significantly over 72 h at 30 °C, but remained unchanged at 22 °C and 14 °C. At 30 °C, the ciliates only slightly enhanced the heat inactivation of VHSV. At 22 °C, the ciliates inactivated a substantial proportion of the VHSV by 24 h but no inactivation had occurred by 72 h at 14 °C. The ciliates vigorously phagocytosed fluorescent beads at 22 °C but not at 14 °C. When VHSV were labeled with the nucleic acid stain SYBR Gold, internalization of the virus into food vacuoles was seen at 22 °C. Thus phagocytosis was one possible mechanism for VHSV inactivation by ciliates. However, another VHSV/ciliate interaction was revealed by immunofluorescent staining and might contribute to inactivation. After being incubated for 24 h with VHSV, washed, and stained at various times afterwards for VHSV G protein,

the ciliates stained transitorily. The strongest staining was seen at approximately 30 minutes after washing and was confined largely to the cilia but after 60 minutes this staining was lost.

Tetrahymena thermophila strains B1975, wild type, and NP1, a temperature sensitive mutant, activated the fish aquareovirus CSV, depending on the temperature. CSV caused fish cells to form syncytia. This cytopathic effect (CPE) was used to titre CSV in the fish cell line, CHSE-214. The CSV titre remained stable during incubations of up to 96 h in Leibovitz's L-15 with FBS at 4, 14, 22 and 30 °C. When CSV was incubated with B1975 or NP1 at 22 °C in the same medium for between 24 and 96 h, the virus titre increased approximately 3 log. At 4 °C, the titre was unchanged by ciliates and *T. thermophila* was unable to phagocytose beads. At 30 °C, B1975 enhanced CSV infectivity and underwent phagocytosis, whereas NP1 did neither. When CSV were labeled with the nucleic acid stain SYBR Gold, internalization of the virus into B1975 food vacuoles was seen. Therefore the viral activation pathway likely involved phagocytosis. *Tetrahymena canadensis* were incubated with CSV-infected CHSE-214, washed by centrifugation through a step gradient of polysucrose, and transferred to new CHSE-214 cultures, which developed the characteristic CSV CPE. Thus as well as activating CSV, ciliates could transport CSV.

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I owe it all to you.

Dedication

To Jola.

You coughed in my ginger ale.
And I haven't regretted a second since.

To Zack.

You are my greatest teacher.

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List of Abbreviations

| | |
|-----------------|---|
| ΦX174 | bacteriophage ΦX174 |
| λ | bacteriophage λ |
| A6 | African clawed frog kidney cell line |
| ANOVA | one-way analysis of variance |
| ANCOVA | analysis of co-variance |
| AqRV | Aquareovirus |
| ATCC | American Type Culture Collection |
| B1975 | “wild type” <i>Tetrahymena thermophila</i> |
| BF-2 | bluegill fin cell line |
| BME | basal medium |
| BSA | bovine serum albumin |
| CHSE-214 | Chinook salmon embryo cell line |
| CFDA-AM | 5'-carboxyfluorescein diacetate acetoxymethyl ester |
| cox 1 | cytochrome c oxidase subunit 1 |
| CPE | cytopathic effects |
| CSV | Chum salmon reovirus |
| DAPI | 4',6-diamidino-2-phenylindole |
| dFBS | dialyzed FBS (10,000 MW cut-off) |
| DIC | differential interference contrast |
| DNA | deoxyribonucleic acid |

| | |
|------------------|--|
| dsDNA | double-stranded DNA |
| dsRNA | double-stranded RNA |
| EMEM | Eagle's minimal essential medium |
| EPC | Fathead minnow epithelioma cell line |
| FBS | fetal bovine serum |
| FCS | fetal calf serum |
| FHM | fathead minnow fathead minnow testis cell line |
| g | the force of gravity on Earth |
| gfp | green fluorescent protein |
| h | hours |
| HeLa | human cervix cell line |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| i-antigen | immobilization antigens present on the cilia of some ciliates |
| Ich | Ichthyophthiriosis, or its causative agent <i>Ichthyophthirius multifiliis</i> |
| IgG | immunoglobulin G |
| IPNV | infectious pancreatic necrosis virus |
| ISVP | infectious subviral particle |
| IU | international units |
| L-15 | Leibovitz's L-15 medium |
| L-929 | mouse connective tissue cell line |
| mAB | monoclonal antibodies |
| MEM | minimal essential medium |
| min | minutes |

| | |
|------------------|--|
| MS2 | bacteriophage MS2 |
| MW | molecular weight |
| n | sample size |
| NBF | neutral, buffered formalin |
| NP1 | temperature-condition mutant of <i>Tetrahymena thermophila</i> |
| P | P-value |
| PBS | phosphate-buffered saline |
| pen/strep | penicillin and streptomycin solution added to medium |
| p.i. | post infection |
| PPYE | proteose peptone yeast extract medium |
| PP | proteose peptone medium |
| PS | phosphatidylserine |
| RFU | relative fluorescence units |
| RK-13 | rabbit kidney epithelial cell line |
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| rRNA | ribosomal RNA |
| RTbrain | Rainbow trout brain cell line |
| RTG-2 | Rainbow trout gonad cell line |
| RTgutGC | Rainbow trout intestinal cell line |
| RTH-149 | Rainbow trout hepatoma cell line |
| RTgill-W1 | Rainbow trout gill cell line |
| RTS-11 | Rainbow trout monocyte/macrophage cell line |

| | |
|--------------------------|---|
| RVI | regulatory volume increase |
| SB255 | mucocyst-reduced mutant of <i>Tetrahymena thermophila</i> |
| Sf9 | Fall armyworm ovarian cell line |
| SD | standard deviation |
| ssDNA | single-stranded DNA |
| SE | standard error |
| sp. | unclassified member of a genus |
| spp. | multiple species of a genus |
| ssRNA | single-stranded RNA |
| syn. | synonymous species used in the literature |
| T4 | bacteriophage T4 |
| T5 | bacteriophage T5 |
| TCID₅₀ | tissue culture infectious dose |
| TSA | trypticase soy agar |
| UV | ultraviolet radiation |
| v/v | volume to volume |
| VHSV | viral hemorrhagic septicemia virus |
| w/v | mass to volume |
| YE | yeast extract medium |
| ZEB2J | zebrafish embryo cell line |

Chapter 1

Use of cell cultures to study the interactions of ciliates with fish

1.1 Introduction

Ciliates are single-celled organisms of the kingdom Protista. They are placed in the protozoan subdivision and belong to the phylum Cilophora (Lynn, 2008). Most ciliates are heterotrophs and can be broadly considered as either free-living or symbiotic. The boundary between free-living and symbiotic forms can be hard to draw (Finlay et al., 1996), especially so, if consideration is given to the ciliates that retain functional prey chloroplasts or contain photosynthetic symbionts (Sanders, 2011). Here the definitions will hinge simply on the ciliate's relationship to metazoans. Free-living ciliates survive independently of metazoans. Symbiotic ciliates live closely with a metazoan either in mutual, commensal or parasitic relationships. As heterotrophs, ciliates depend on an external source of organic compounds.

Free-living ciliates and symbiotic ciliates obtain organic compounds by similar processes, phagotrophy and osmotrophy. Most ciliates have “mouths” and acquire nutrients by engulfing solid organic matter. This is phagotrophy and has been referred to as holozoic nutrition (Jones, 1974). Free-living ciliates usually get nutrients by eating microbes, such as bacteria, algae, fungi, or other protozoa. Symbiotic ciliates eat microbes associated with metazoans, such as the rumen bacteria of cattle (Jarvis and Hungate, 1968), or organic matter arising from the metazoans themselves. The organic matter can be solid, such as dead tissue or living cells. The eating of living metazoan cells is referred to as histophagy or ‘tissue eating’ (Struder-Kypke et al., 2001). Some ciliates can also utilize organic compounds in solution. This is osmotrophy and has been referred to as saprozoic nutrition (Jones, 1974). Osmotrophy is found among both symbiotic and free-living ciliates (Basson and Van As, 2006; Jones, 1974). The free-living ciliate, *Tetrahymena pyriformis* Ehrenberg, 1830 can simultaneously eat bacteria and consume dissolved organic matter (Glaser, 1988). The

relative importance of phagotrophy and osmotrophy for a particular species is largely unknown. A few mutants of *T. thermophila* Nanney and McCoy, 1976 appear capable of relying only on osmotrophy (Orias and Rasmussen, 1976).

Many ciliates are found in water and ciliate/fish interactions have long been of interest (Basson and Van As, 2006; Corliss, 1960; Hoffman et al., 1975; Lom and Corliss, 1968) but increasingly they are becoming of economic importance as well (Iglesias et al., 2001; Imai et al., 2000; Kim et al., 2004ab; Pimenta Leibowitz et al. 2005; Rhodes and Phelps, 2008). Usually the focus has been on symbiotic relationships but here a more conservative approach is taken. For the purpose of this chapter, any of four types of interactions with fish will define a ciliate as a fish ciliate, and these interactions can be seen as a continuum from complex and dependent to simple and independent. Firstly, the ciliates that are pathogens of fish will be considered fish ciliates. These ciliates could be in opportunistic, mutualistic, commensal or parasitic relationships. A second group would be ciliates that feed on detritus from living, dying and dead fish. They could be ectocommensals or free-living. A third group would be ciliates that either inactivate or protect viral and microbial pathogens of fish. These could be free-living or symbiotic ciliates. A fourth group would be ciliates that can serve as food for the early life stages of fish. These would be free-living ciliates.

One way to study some of these interactions is through the use of cell cultures. Cell cultures can be defined as the maintenance of cells in engineered structures. They allow engineers and scientists the ability to control either an industrial process or a research experiment. For industry, the yield can be either the cells or cell products; for science, the output is information. The histories for culturing single cell eukaryotic organisms and cells of multicellular animals are long, but they have only infrequently crossed paths. Yet ciliates and fish do cross paths in numerous ways. The goals of this chapter are to provide a quick overview of ciliate/fish interactions, to review efforts to culture ciliates alone or with fish cells, and to comprehensively review the use of ciliate cultures in fish biology.

1.2 Ciliates of fish

The four ciliate/fish interactions defined in the Introduction are reviewed briefly in the following sections. All the ciliates mentioned in the chapter are listed in Table 1.1 along with a single word or phrase describing their possible relationship with fish and the attempts to culture them.

1.2.1 Ciliates as fish pathogens

As pathogens of fish, ciliates show a continuum of host dependencies that make interactions difficult to define formally in certain cases. Some ciliates are completely dependent on fish to complete their life cycle and act internally so are defined as obligate parasites or obligate endoparasites. This is the case for *Ichthyophthirius multifiliis* Fouquet, 1876, which causes “ich”, and *Cryptocaryon irritans* Brown, 1951, which causes “marine ich”. A second group acts internally but opportunistically and so are termed facultative parasites or facultative endoparasites. Belonging in this category are some members of the order Scuticociliatida Small, 1967 that cause the disease scuticociliatosis, and some species of *Tetrahymena* Furgason, 1940 that cause the disease tetrahymenosis. A third group is found on the outer surfaces of living fish without harming them but under some conditions can be harmful and so can be considered facultative parasites. On the skin and gills are *Chilodonella piscicola* Zacharias, 1894 and *Brooklynella hostilis* Lom and Nigrelli, 1970 that can damage fish by feeding directly on epithelial cells and cause respectively the diseases, chilodonellosis and brooklynellosis. In the intestinal lumen are species of *Balantidium* Claparède and Lachmann, 1858, which can cause balantidiasis. A final group feeds on bacteria and mucus on the surface of living fish but under some conditions can damage fish by irritating skin and gill epithelium and so go from being ectocommensals to facultative ectoparasites. This is the case for species of *Trichodina* Ehrenberg, 1838, which cause trichodiniasis.

1.2.1.1 Ich and marine ich

The most complex and destructive of the fish ciliate pathogens are the obligate parasites, *I. multifiliis* and *C. irritans*. Although belonging to different classes, oligohymenophorea for *I. multifiliis* and prostomeata for *C. irritans* (Lynn, 2008), they have similar polymorphic life

cycles (Colorni, 1985; Buchmann et al., 2001). Prey-seeking theronts swim in search of moribund fish host, and although they have fully formed oral features, do not feed. Once a host is found, ciliates may enter the body cavity through the skin or attack the gill epithelium, and upon invasion theronts increase greatly in size forming trophonts. Trophonts begin feeding on tissue and will eventually exit the host and form a divisional cyst, which will ultimately release between 50 and several thousand, for *I. multifiliis*, daughter cells, or tomites. Tomites are a small life stage that transform soon after release to theronts (Buchmann et al., 2001). *Ichthyophthirius multifiliis* causes ichthyophthiriosis and has a near universal host range on freshwater fish (Dickerson and Dawe, 2006; Mathews, 2005; Noga, 2010). Ich causes massive economic losses to the aquarium and aquaculture industries. *Cryptocaryon irritans* causes the marine equivalent of ich and has a more restrictive host range (Colorni and Burgess, 1997). The species is of a concern in the warm water food fish aquaculture (Tookwinas, 1990; Montero et al., 2007).

1.2.1.2 Scuticociliatosis and Tetrahymenosis

Scuticociliatosis is caused by ciliates belonging to the subclass Scuticociliatia Small, 1967 and since the 1990s has been a problem around the world for marine fish aquaculture (Noga, 2010). Impacted industries include Asian Japanese flounder (Yoshinaga and Nakazoe, 1993; Kim et al., 2004a,b), Mediterranean turbot and European seabass (Iglesias et al., 2001), and Australian bluefin tuna (Munday et al., 1997). Scuticociliatosis is caused by a number of species including *Uronema marinum* Dujardin, 1841, *Miamiensis avidus* Thompson and Moewus, 1964 (senior syn. of *Philasterides dicentrarchi* Dragesco et al., 1995), and *Parauronema virginianum* Thompson, 1967 (Lynn, 2008). Characteristically marine organisms, recently *Pseudocohnilembus persalinus* Evans and Thompson, 1964 was isolated from the ovarian fluid of freshwater rainbow trout spawning in Idaho, suggesting a wider range of osmotic tolerance than otherwise expected for scuticociliates (Jones et al., 2010). Early infections with scuticociliates may appear as lesions on skin or gill tissue, but the ciliates can quickly infest deeper tissues and organs, including muscle, peritoneal cavity, kidney, pancreas, liver, urinary bladder, spinal cord, and brain (Noga, 2010). Treatment and control of scuticociliatosis is especially difficult not only because of invasion of the fish host

tissues, but also due to their success as free-living bacterivores in the environment (Jee et al., 2001).

Tetrahymenosis is the freshwater counterpart to scuticociliate-borne infestations. Members of the genus *Tetrahymena* have long been noted to harm fish (Speide, 1954; Nigrelli et al., 1956; Corliss, 1960; Stolk, 1959), although the ciliates are able to grow independently of fish or any other animal and so are considered free-living but facultative endoparasites. In the production of tropical fish for the aquarium trade, tetrahymenosis has caused considerable economic losses (Pimenta Leibowitz et al., 2005). Infected fish have necrotic skin lesions that extend into the musculature, and the pathogen can reach the circulatory system and destroy internal organs. The most frequent reports of infection have been in the guppy, *Poecilia reticulata* (Imai et al., 2000) but infections have also been noted in cichlids, black mollies and tetras (Hoffman et al., 1975; Johnson, 1978). As well, infections have been discovered in fish being farmed for human consumption. Tetrahymenosis appeared to be the cause of rapid perch die-offs in two pond facilities in Australia (Callinan et al., 1994; Herbert and Graham, 2008) and *Tetrahymena* were found in cranial ulcerations in farmed Atlantic salmon in Canada (Ferguson et al., 1987). Ciliates have also been isolated from diseased common carp, catfish, and rainbow trout (Shulman and Jankovski, 1984). Usually the causative agent has been identified simply as belonging to genus *Tetrahymena* (*Tetrahymena* sp.). However, in the case of the Atlantic salmon the ciliate was thought to be *T. corlissi* Thompson, 1955 or possibly *T. rostrata* Kahl, 1926 (Ferguson et al., 1987). Experimentally, *T. pyriformis* was found to infect several species of ornamental fish if the fish had received a deep skin wound (Ponpornpisit et al., 2000).

1.2.1.3 Chilodonellosis and Brooklynellosis

Two species of *Chilodonella* Strand, 1928, *C. hexasticha* Kiernik, 1909 and *C. piscicola* cause chilodonellosis in fresh- and brackish water fish, and was at one time the most significant single disease to the commercial tropical fish industry (Leibovitz, 1980). However, the broad host range is believed to include a universal range of freshwater teleosts (Shulman and Jankovski, 1984; Noga, 2010). Mass mortalities of feral fish have also been

reported resulting from *Chilodonella hexasticha* infection (Langdon et al., 1985). These organisms are able to survive by feeding on bacteria when apart from the host, but are facultative ectoparasites, feeding directly on epithelial or gill cells with a penetrating cytostome (Wiles et al., 1985). In marine fish aquaculture, a similar disease, brooklynellosis, is caused by *Brooklynella hostilis* and may cause recurrent mass mortalities (Lom and Dykova, 1992). Aquarium-raised teleosts are affected similarly to those parasitized with *Chilodonella* sp., however *B. hostilis* is restricted to attacking gill tissue where epithelial and blood cells are fed upon resulting in serious lesions (Lom and Nigrelli, 1970). Currently no cases of brooklynellosis have been identified in feral fish, and the ability of *B. hostilis* to survive free of the host is unknown, however a recently identified addition to the genus, *B. sinensis* Gong and Song, 2006, is free-living (Gong and Song, 2006).

1.2.1.4 Balantidiasis

Balantidiasis has been reported in the intestinal lumen of several fish species and occasionally has been associated with enteric diseases (Basson and van As, 2006). For example, in the grass carp *Balantidium ctenopharyngodonis* Chen, 1955 appeared to be the cause of intestinal lesions in older fish (Molnar and Reinhardt, 1978). However, the ciliates usually are harmless.

1.2.1.5 Trichodiniasis

The ectocommensal trichodinids are commonly found gliding along and loosely adhering to fish skin and gills, where they feed on waterborne particulates, bacteria, and fish detritus. Seven genera within the family are known to interact with freshwater or marine fish, the most notable being species of *Trichodina* (reviewed in Basson and Van As, 2006), and they are characterized by the presence of an adoral adhesive disk incorporating blade-like denticles. Fish that are stressed due to changing or poor environmental conditions, or juvenile fish, are susceptible to infestation of excessive numbers of trichodinids on the skin or gill surface. Trichodiniasis results from the ciliates repeatedly detaching and reattaching from the host, which causes irritation and damage to epithelial cells (Lom, 1973; Noga, 2010). At this point

the ciliates may feed on disrupted cells, and subsequent bacterial population, and may be considered facultative ectoparasites.

1.2.2 Ciliates as fish passengers and carcass consumers

The sessiline peritrich ciliates (order Peritrichia Stein, 1933) of fish might be considered as passengers or more formally as ectocommensals (Basson and Van As, 2006). They are motile with specialized features for transient adherence to fish surfaces (reviewed in Basson and Van As, 2006; Lynn, 2008). Among the sessilids found associated with fish, notable species are included within the families Epistylididae Kahl, 1933; Scyphidiidae Kahl, 1933; Operculariidae Fauré-Fremiet in Corliss, 1979; and Ellobiophryidae Chatton and Lwoff, 1929. Attachment to the host skin or gill surface occurs at the scopula – a thigmotactic structure at the posterior end of the ciliate – either directly, or through a secreted stalk, and ciliates may occur singly or as macroscopic colonies. These ciliates use their hosts as a platform from which they feed on organic debris and bacteria. Generally their numbers are low and they do not damage their host (Lom and Corliss, 1968).

Ciliates might also have an ecologically important interaction after the death of fish, by whatever cause. Ciliates would be expected to feed on fish carcasses because they have been observed around moribund fish (Beck et al., 2006) and are known to feed on dead material (Jones, 1974). However, as a process this activity appears unexplored but could be ecologically important as part of carcass flocculation. Flocculation is the aggregation of fine organic and /or inorganic particulates into larger fragile structures, floc, and comes about as a result of biotic and abiotic processes. Floc is part of industrial systems, such as wastewater treatment, and ecological systems, such as rivers and lakes (Liss et al., 1996). Ciliates make a significant contribution to floc formation in sewage treatment plants (Curds, 1963). In fish biology, the post-spawning die-off of Pacific salmon is an interesting example of floc formation, with fish flocs delivering salmon nutrients to stream beds and freshwater food chains (Petticrew et al., 2011).

1.2.3 Ciliates interacting with fish microbial and viral pathogens

Increasingly the interaction between ciliates and microbial/viral pathogens of mammals is being studied, and although early work was done with fish pathogens, fish generally have received less attention. The overarching question is whether the transmission of the disease can be potentially modulated by ciliates, and although no definitive answers have been possible to date, several fascinating interactions have been revealed. The ciliate that has been most often used in these studies has been *T. pyriformis*, which itself can be pathogenic to fish (Ponpornpisit et al., 2000). Overall, two contrasting themes have emerged: the ciliates either destroy or protect the pathogen.

Ciliates can eat and inactivate bacteria. Often this has been studied in order to develop predator-prey models for describing sewage treatment plants and aquatic microbial ecosystems (Fredrickson, 1991; Habte and Alexander, 1978; Taylor, 1978; Vayenas and Pavlou, 1999). The most common combination has been either *T. thermophila* or *T. pyriformis* with *Escherichia coli*. *Escherichia coli* is not pathogenic to fish, although the species is found in the fish intestine (Hansen et al., 2008), but some *E. coli* strains are pathogenic to humans (Chaudhuri and Henderson, 2012). In common co-culture, *T. pyriformis* greatly reduced but never completely eliminated *E. coli* populations. A small, stable bacterial population persisted along with the ciliates and might have been supported by products of ciliate lysis and/or metabolism (Sambanis and Fredrickson, 1987; Sambanis and Fredrickson, 1988). Interestingly, Matsui et al. (2000) noted that when bacteria density was low, the growth of *E. coli* was stimulated by *T. thermophila*. One explanation advanced for this surprising result was that *T. thermophila* metabolites detoxified metabolites of *E. coli*, which normally inhibited the growth of the bacteria (Matsui et al., 2000).

As well as potentially destroying pathogens, ciliates can enhance the survival of bacterial pathogens in the environment through several mechanisms. This has been demonstrated with several species of *Tetrahymena* and several human pathogens, including *Campylobacter jejuni*, *Salmonella enterica*, and *Legionella pneumophila*. None of these would be considered fish pathogens, although *L. pneumophila* is found in freshwater and can kill fish in the laboratory (Weeks et al., 1988). However, the mechanisms by which the ciliates act likely

apply to fish pathogens as well. To date, the mechanisms all require initial internalization by the ciliates. Once engulfed by *T. pyriformis*, bacterial pathogens, like *C. jejuni*, remained viable inside and were better able to resist chlorine residuals (King et al., 1988). Other protective mechanisms involve the subsequent externalization of bacteria by the ciliates either inside vesicles (Brandl et al., 2005; Ghaffari et al., 2008) or in pellets (Berk et al., 2008). *Salmonella enterica* released in vesicles were viable, less susceptible to disinfection treatments than free bacteria (Brandl et al., 2005), and had increased acid resistance, which could contribute to the bacteria surviving in humans (Rehfuss et al., 2011). *Legionella pneumophila* were released in pellets as mature intracellular forms (MIFs) and these were more resistant to several stresses than stationary-phase forms (SPFs) (Koubar et al., 2011). Thus, ciliates could be constantly creating a protected reservoir of pathogenic bacteria.

Some ciliates appear to help bacteria maintain their virulence to animals when the pathogens are external to the host (Pushkareva and Ermolaeva, 2010). In fact, exotoxins might have evolved as a bacterial antipredator defense and their actions on vertebrates incidental to their original purpose (Lainhart et al., 2009). Survival within ciliates can correlate with expression of virulence genes, as illustrated with *T. thermophila* and two strains of the fish pathogen, *Aeromonas hydrophila*, virulent (J-1) and avirulent (NJ-4) (Li et al., 2011). Inside the ciliates, J-1 up-regulated genes for bacterial virulence factors and survived, whereas NJ-4 did not and was digested. Ciliates and other protozoa have been considered as training grounds for intracellular bacterial pathogens (Molmeret et al., 2005). Therefore, ciliates can serve as experimental surrogates for phagocytic cells of vertebrates and ciliate cultures can be used to explore the cellular basis of bacterial pathogenesis in animals (Benghezal et al., 2007).

Ciliates have been investigated sporadically over the last 70 years for possible associations with viruses of humans, and less frequently with viruses of fish, with intriguing but not always definitive results. Perhaps, the first example of this was a study on Stockholm sewage for the possibility that ciliates harbored poliovirus (Kling et al., 1942). Subsequently, laboratory investigations were done, commonly with *Tetrahymena*. The viruses included influenza virus (Groupe and Pugh, 1952), encephalomyocarditis (EMC) virus (Kovacs et al.,

1966), measles virus (Kovacs et al., 1966), vaccinia virus (Jareno, 1987), coxsackie B-5 virus (Teras et al., 1988), adenovirus 3 (Sepp et al., 1992), poliovirus (Kim and Unno, 1996), and Simian rotavirus SA11 (Benyahya et al., 1997). Usually the ciliates were either assumed or demonstrated not to support production of these mammalian viruses (Sepp et al., 1992; Benyahya et al., 1997) but sometimes the results were equivocal (Kovacs et al., 1966; Teras et al., 1988). What has been clear was that ciliates destroyed or inactivated some viruses of mammals. This was shown for influenza virus by *T. geleii* Furgason, 1940 (syn. *T. pyriformis*) and *T. pyriformis* (Groupe and Pugh, 1952; Groupe et al., 1955), adenovirus by *T. pyriformis* (Sepp et al., 1992), and poliovirus by *T. pyriformis* (Kim and Unno, 1996). For fish, connections between infectious pancreatic necrosis virus (IPNV), the ciliate, *Miamiensis avidus*, and the seahorse were explored in the laboratory (Moewus-Kobb, 1965). IPNV did not replicate in the ciliates but could be picked up by the *M. avidus* and transmitted to the seahorse. Thus, fish provide perhaps the best example of a potential role for ciliates in viral disease transmission.

1.2.4 Ciliates as fish food: protozooplankton-ichthyoplankton link

Ciliates are among the protozooplankton that can serve as food for ichthyoplankton. Ichthyoplankton is a metazoan grouping of plankton, consisting of fish larvae, although fish eggs and embryos are often put into this grouping as well. In aquatic food chains, the protozooplankton are the link between bacteria/phytoplankton and metazoan consumers. By consuming bacteria/phytoplankton, planktonic protozoa package the nutrients into larger particles that can be eaten directly by metazoans (Gifford, 1991). Usually the metazoans are considered to be invertebrates such as copepods. However, the protozooplankton can also be eaten by ichthyoplankton. This is the protozooplankton-ichthyoplankton link (reviewed in Montagnes et al., 2010).

Field studies have demonstrated that ciliates can be food for the larvae of marine fish (Fukami et al., 1999; Nagano et al., 2001; Montagnes et al., 2010). This demonstration requires examining larval gut contents, which is a difficult task. Usually marine ciliates are

grouped into loricate (shell-like outer coverings) and aloricate (naked) forms, with naked forms being more abundant in ocean samples. By contrast, in the gut, the hard parts of the loricate ciliates; such as members of family *Tintinnidae* Claparède and Lachmann, 1858; are indigestible, making them easier to see and the first ones to be considered as food for larval fish (Last, 1978ab). Yet, naked ciliate genera have been found in the guts of most fish taxa that have been examined carefully, such as Ammodytidae, Gadidae and Gobiidae (Figueiredo et al., 2007). Ciliates include *Myrionecta* Jankowski, 1976; *Tontonia* Fauré-Fremiet, 1914; *Leegaardiella* Lynn and Montagnes, 1988; *Laboea* Lohmann; and *Strombidium* Claparède and Lachmann, 1859 (Figueiredo et al., 2007). Thus ciliates are prey items for ichthyoplankton, but the ecological importance of this requires more study (Montagnes et al., 2010).

Laboratory studies support the general conclusion of field observations: fish larvae can eat ciliates. Studies have been done with the larvae of Northern anchovy, *Engraulis mordax*, surgeonfish, *Paracanthurus hepatus*, Atlantic cod, *Gadus morhua*, and red snapper, *Lutjanus campechanus*. When presented as a first food, species of *Strombidium* Claparède and Lachmann, 1859, were detected immunochemically in larval guts of Northern anchovy (Ohman et al., 1991). Larval surgeonfish were fed species of *Euplotes* Ehrenberg, 1830 that had been first allowed to engulf on fluorescent beads (1 μm) (Nagano et al., 2000). This allowed *Euplotes* sp. to be easily visualized inside the gut of larvae. Video microscopy of first-feeding larvae of Atlantic cod showed them eating the ciliate *Balanion* sp. Wulff, 1919, and preferring them over other feed items (Hunt von Herbring and Gallager, 2000). The marine ciliate, *Fabrea salina* Powers and Mitchell, 1910, was used to successfully feed red snapper larvae (Rhodes and Phelps, 2008).

1.3 Culturing ciliates associated with fish

The culturing of fish ciliates has been done in many ways but collectively media have been developed along three themes. Media can be seen as modifications of either freshwater for freshwater ciliates, seawater for marine ciliates, or vertebrate bodily fluids for ciliates

capable of growing inside teleosts. The approximate osmolalities of freshwater, fish blood, and seawater are 50, 300 and 1000 mOsm/kg H₂O, respectively. Some ciliates from one environment are able to grow to varying extents in solutions of other osmolalities (Hanna and Lily, 1974; St. Denis et al., 2010). To feed the ciliates and complete the medium, three general types of additions have been made. These are either bacteria, complex but undefined organic supplements, or a set of completely defined organic compounds.

1.3.1 Media based on freshwater

Freshwater ciliates have perhaps the longest history of laboratory culture of any eukaryotic cell. Culturing began over 100 years ago with organic matter decaying in water. In early reports the water was often described as “tap” water but sometimes salts were added to make balanced and buffered salt solutions (Johnson, 1941). The ciliates grew along with other microbes, especially bacteria, and these were often referred to as “infusoria”. They might also be described as agnotobiotic cultures, meaning that the identity of the microbes was unknown or poorly defined. Research on culturing was most intensive with primarily just one ciliate genus, *Tetrahymena*, with species such as *T. pyriformis* and *T. thermophila* often being used to optimize procedures. Subsequently *Tetrahymena* were grown with known bacterial species, gnotobiotic cultures, and then without any other micorganisms, axenic cultures. For axenic cultures, the nutrients were either complex mixtures of organic molecules or completely defined. These successes led to efforts to scale up the production of *Tetrahymena*.

1.3.1.1 Culturing freshwater ciliates with bacteria

A single bacterial species or a combination of several known species has been found to support the growth of *Tetrahymena* and a few other freshwater, free-living ciliates. In nature, these ciliates feed on a variety of bacteria or other microbes in streams, ponds and lakes, but in cultures the identity of the microbes can be defined. Specific bacteria can be added to crude suspensions of organic matter that alone allow bacteria but not ciliate growth. Examples include sterile 0.10 % hay fusion (Hargitt and Fray, 1917) and 0.15% cerophyl (Taylor, 1978). The ciliates feed on the growing bacteria population and proliferate. Under

some conditions, *T. pyriformis* simultaneously consumed bacteria and dissolved organic matter (Glaser, 1988). Growth on bacteria alone was demonstrated more conclusively by using medium with no energy source and by starting the co-cultures with high densities of bacteria (Matsui et al., 2000; Watson et al., 1981). Greater than 10^6 *E. coli* cells/ml was required to support growth (Matsui et al., 2000). Dead as well as live bacteria could serve as food (Ducoff et al., 1964; Sambanis and Duke, 1993). Perhaps, the first report of a ciliate being cultured on a single bacteria species was by Hargitt and Fray (1917) who grew *Paramecium* Müller, 1773, on only *Bacillus subtilis*. Many early studies noted that for a given ciliate some species of bacteria were suitable food organisms while other were not (Johnson, 1941). Some bacteria in fact were toxic to ciliates (Kidder and Stuart, 1939; Burbanck, 1942).

1.3.1.2 Culturing freshwater ciliates axenically

Growing ciliates axenically began in the 1920s (Lwoff, 1923). Since then, two research themes have emerged with respect to culturing. One is the simple routine maintenance of *Tetrahymena* for a wide variety of purposes through the use of complex but undefined supplements in water or a buffered salt solution. This has been referred to as rich axenic nutrient media (Orias et al., 2000). The second is growth in completely defined medium for specific experimental goals, such as studying cellular nutrition. For axenic cultures, starting solutions and components are sterilized through autoclaving and filtration.

1.3.1.2.1 Complex undefined supplements

For the routine maintenance of *Tetrahymena*, the most common nutrient source is a proteose peptone (PP). PPs are enzymatic digests of protein. A peptic digest of beef extract at approximately 2 % in water of high purity is a frequent usage (Orias et al., 2000). The PP is often supplemented with one or more of yeast extract, liver extract, glucose and iron (Orias et al., 2000). The ciliates grow vigorously in these simple to prepare and inexpensive media. In fact, this consideration was probably critical in making these ciliates a popular laboratory organism, especially *T. thermophila* (Asai and Forney, 1999). However defined media also have roles.

1.3.1.2.2 Defined media

A defined medium is one in which all the components are known chemically and in which the cells grow. Such media were developed for *Tetrahymena* in the early 1950s (Kidder and Dewey, 1951), making ciliates one of the first groups of non-photosynthetic eukaryotic cells to be grown in a defined medium. Yet, for *Tetrahymena* these media have not received wide usage, in part due to the perceived difficulties in preparing them (Szablewski et al., 1991). Several research groups have worked to improve the original medium (Holz et al., 1962; Sripati, 1987; Szablewski et al., 1991; Hagemester et al., 1999; Hellung-Larsen, 2005). The essence of these media is 19 L-amino acids, four ribonucleosides, B-vitamins, salts, trace metals, and glucose. A minor problem has been the observation of “interface-mediated death” in cultures at a low cell density, which results in non-programmed cell death due to lysis at the point of contact between the medium and air above (Hagemester et al., 1999). This has been overcome by making several different kinds of additions (Christensen and Rasmussen, 1992; Kristiansen et al., 1996), the simplest being a surfactant like Pluronic F68 (Hellung-Larsen, 2005). Defined media offer researchers the most control and can be used for many experimental purposes, especially for nutritional and genetic studies (Sanford and Orias, 1981), and will likely be used more in the future. One interesting future question is how similar the nutritional requirements for *Tetrahymena* are for the approximately 4,000 other free-living ciliates.

1.3.2 Media based on seawater

Source seawater, instant seawater, and artificial seawater have been used to prepare media to culture marine ciliates (Soldo and Merlin, 1972; Henglong et al., 2011). The salinity of seawater is approximately 35 ‰ but varying salinities have been used. In general terms the media have been completed by the addition of either bacteria, complex undefined organic supplements, or chemically defined components. Culturing of marine ciliates began later, in the 1960s, and has been less intensive than the culturing of freshwater ciliates.

1.3.2.1 Culturing with bacteria

The first attempts at marine ciliate culturing focused on the marine benthic ciliate *Uronema marinum* and used bacteria as food (Hamilton and Preslan, 1969). Hamilton and Preslan (1969) found that proliferation of this ciliate was supported by *Serratia marinorubra* but only by 2 of 10 unidentified marine bacterial isolates. Subsequently *U. marinum* was grown monoxenically on *Pseudomonas* sp. (Lee et al., 1971) and on *Vibrio* spp. (Parker, 1976). In seawater with peptone, *Vibrio natriegens* also supported *U. marinum* growth (Perez-Uz, 1995; Crosbie and Munday, 1999). A *Uronema* sp. from an infected fish, the silver pomfret (*Pampus argenteus*), was cultured on brain heart infusion broth in which a *Vibrio* sp. was growing (Al-Marzouk and Azad, 2007). Also natural bacterial assemblages from sediment sustained *U. marinum* (First and Hollibaugh, 2009).

Other marine ciliates have been cultured on bacteria. A mixed bacterial flora supported the growth of several microaerobic ciliates, including species of *Paranophrys* Thompson and Berger, 1965, and *Strombidium* (Bernard and Fenchel, 1996). The scutiociliate *M. avidus* was maintained on autoclaved *Vibrio anguillarum* (Iglesias et al., 2002).

1.3.2.2 Culturing axenically

Several marine ciliates have been grown axenically. The seawater has been supplemented with either complex biological extracts or sets of chemically defined compounds.

1.3.2.2.1 Complex undefined supplements

Several kinds of undefined supplements have been used, often as combinations, to support marine ciliate growth. Cerophyl, proteose peptone, trypticase and yeast nucleic acids were used to grow *M. avidus*, *P. virginianum*, and *U. marinum* (Soldo and Merlin, 1972). The same species were grown in seawater supplemented with fish tissue homogenates (Salinas et al., 2011). For these and other marine ciliates, Nerad and Daggett (1992) gave directions for the preparation of media that use several different extracts. These include powdered cereal grass leaves, brown rice, yeast extract and dried seaweed. The authors note that Cerophyl is no longer available from the original manufacturer and was a mixture of powdered wheat,

rye, oat and barley leaves. Some limited success has been achieved in culturing a marine peritrich ciliate using cerophyl supplements (Clamp and Coats, 2000).

1.3.2.3 Defined medium

A defined medium has been developed for at least one marine ciliate. *U. marinum* grew in a medium that had 17 amino acids, 4 nucleotides, 5 fatty acids, stigmasterol, and 8 vitamins (Hanna and Lilly, 1974). Growth occurred in complete seawater but was optimal at 25 % seawater. A medium, 1651 MA, similar to this was available from the American Type Culture Collection (ATCC). However, 1651 MA had to be supplemented with FBS to support growth of six ciliates isolated from parasitized turbot (Alvarez-Pellitero et al., 2004).

1.3.3 Media based on mammalian body fluids

For the ciliates that can survive inside teleosts, growth media that reflect the internal milieu of bony fish and support fish cell proliferation in vitro would seem to be appropriate starting points for culturing the ciliates. Unfortunately, a medium has yet to be developed specifically for growing fish cells. However, many media have been developed for mammalian cells. With very little modification, these media work well for the cells of bony fish (Bols et al., 2005). A brief review on using these media for teleost cells follows.

1.3.3.1 Basal media and sera for culturing teleost cells

The complete growth medium for the propagation of fish cells has two essential parts, a basal medium and a supplement (Bols et al., 2005). A basal medium constitutes an aqueous solution of buffering agents, bulk ions, and nutrients. Basal media have been constructed to mimic in vivo fluids, which would be primarily extracellular fluid and blood plasma (Ham, 1984). Generally, the inorganic blood constituents of mammals and fish are similar, although only a few fish species have been examined (Wolf and Ahne, 1982). For all basal media the nutrients include a hexose, amino acids, and vitamins, but many variations are possible and available commercially. Most of these, such as Minimum Essential Medium (MEM) or Eagle's MEM (EMEM), have a sodium bicarbonate (NaHCO_3) buffering system, which requires an atmosphere of 5 % CO_2 . Others are formulated specifically for use in free gas

exchange with air. One of these is Leibovitz's L-15, which maintains physiological pH through a combination of salts, high basic amino acid concentrations, and galactose as the hexose (Leibovitz, 1963). In order to support the proliferation of fish cell lines, the basal media needs to be supplemented. The most common supplement is a bovine serum, usually fetal bovine serum (FBS). For the routine maintenance of cell lines, antibiotics often are added to media. Usually these are penicillin at 100 I.U. with streptomycin at 100 µg/ml (Pen/Strep). Media like these have formed the basis of attempts to culture several ciliates of fish.

1.3.3.2 Basal media and sera for culturing ciliates

Miamiensis avidus (syn. *Philasterides dicentrarchi*) has been the most intensively studied ciliate for growth in basal media originally developed for mammalian tissue culture. Several additions to L-15 led to the first success (Iglesias et al., 2003). The additions were lipids (lecithin and Tween 80), nucleosides (adenosine, guanosine, cytidine and uridine), and glucose, and the undefined supplement of 10 % FBS. FBS was essential for growth and the lipids enhanced the growth with FBS. The medium was prepared at 10 and 27 ‰ salinity. Over 7 days the best growth was observed in 10 ‰ salinity at temperatures between 18 and 23 °C. Growth was negligible at 13 °C. A turbot cell homogenate together with FBS and lipids further enhanced growth but not if the homogenate had been autoclaved (Castro et al., 2007). The authors suggest that toxic substances released from broken cells impeded growth but were inactivated by autoclaving. In addition to L-15, MEM formed the basis of a culture medium for *M. avidus* (Harikrishnan et al., 2010). Growth occurred when the MEM was supplemented with yeast extract and FBS.

Other marine ciliates of fish have been cultured in media based on L-15. With supplements, L-15 allowed the axenic culture for up to 24 months of six *Philasterides* isolates from parasitized turbot (Alvarez-Pellitero et al., 2004). In L-15 alone, the ciliates survived for long periods but did not proliferate and instead became slender and acquired unusual shapes. Supplementing L-15 with turbot blood cells or fish brain extract supported growth. For convenient routine growth, the best medium was L-15 supplemented with 1.28

% artificial marine salts, 10 % heat-inactivated FBS, 1X Eagle's Basal Medium (BME) vitamin mixture, and 1 % ribonucleic acid from torula yeast. The final salinity was approximately 20 ‰. At 20 °C, growth was exponential between 72 and 96 h and stationary phase started at about 120 h. Another ciliate, *U. marinum*, was cultivated in media based on L-15 (Anderson et al., 2009). Growth was best in L-15 with FBS, lipids and an additional supplement of grouper tissue homogenate.

Two mammalian basal media, EMEM and L-15, have been tried with limited success to grow the obligate parasites, *I. multifiliis* and *C. irritans*. For *I. multifiliis*, which is from freshwater, EMEM supplemented in various ways was compared with simpler media, including water (Ekless and Matthews, 1993). For *C. irritans*, which is a marine ciliate, media were built on L-15 mixed with seawater (Yambot and Song, 2004).

Compared with water, EMEM extended the survival of all stages of *I. multifiliis*, but failed to support the in vitro growth and development of the parasite (Ekless and Matthews, 1993). In EMEM, theronts survived up to 5 days. In EMEM with 10 % FBS, trophonts survived for up to 16 days. The theronts failed to transform to trophonts.

L-15/seawater supplemented with sera supported *C. irritans* tomonts and trophonts in short-term cultures that either had an attachment substrate (solid medium) or not (liquid medium) (Yambot and Song, 2004). Fetal calf serum (FCS), tilapia serum or grouper serum were compared. The attachment substrate was a strip of trypticase soy agar (TSA) and was meant to mimic invasion of a fish host. Theronts transformed into trophonts, which enlarged in cultures with approximately 20 % FCS but not with other sera. Growth occurred in both liquid and solid media. After transformation, trophonts survived from 4 to 13 days. The trophonts that were raised in vitro were not observed to continue development into tomonts. To improve the culturing of *C. irritans* and *I. multifiliis*, investigators have explored the use of fish cell lines as support in co-cultures.

1.3.3.3 Teleost cell lines

Cell lines have been reported from about 75 of the estimated 29,000 teleost species. Several compilations of the species and tissue origins of fish cell lines have been published

(Wolf and Mann, 1980; Fryer and Lannan, 1994; Lee et al., 2009; Lakra et al., 2011). About 283 cell lines have been described and nearly all can be cryopreserved (Lakra et al., 2011). Many of these likely have been lost over time through inattention (Bols et al., 2005) but some are available from the ATCC. Formally, cell lines arise when primary cultures are passaged or subcultivated into new culture vessels (Schaeffer, 1990). For many fish species, serially subcultivating cell cultures leads to the spontaneous immortalization of the cells and a cell line (Bols et al., 2005). Some common fish cell lines are bluegill fin (BF-2), Chinook salmon embryo (CHSE-214), fathead minnow (FHM), and EPC, which is now known to be from fathead minnow but is still referred to as EPC (Winton et al., 2010).

Except for temperature, the environment for culturing piscine and mammalian cells is similar. Nearly all the fish cell lines are adherent, requiring a surface on which to attach, spread and grow. An exception is a monocyte/macrophage cell line, RTS11, from the rainbow trout spleen (Ganassin and Bols, 1998). Fish adherent cell lines are maintained on conventional tissue culture plastic manufactured for mammalian cells. Fish cell lines grow over wide temperature ranges, which vary with the species and have been referred to as the proliferation zones (Bols et al., 1992). Within the proliferation zones, growth is optimal over narrower ranges. For warm-water fish, this is 26-30 °C; for cold-water fish, this is 20-23 °C. The warm water fish cell lines, BF-2 and EPC, and the coldwater fish cell line, CHSE-214, have been explored as agents to support ciliate growth in vitro.

1.3.3.4 Co-culturing ciliates and fish cell lines

The maintenance of *I. multifilis* has been studied in co-culture with piscine cell lines and with fish tissue fragments, including skin, gill, and fin (Hurley, 1999; Pugovkin et al., 2001; Xu et al., 2000). One cell line partner was BF-2 in either EMEM or medium 119 with FBS (Xu et al., 2000; Pugovkin et al., 2001). BF-2 was compared with explant cultures of channel catfish tissues but the explants were superior in promoting ciliate growth (Xu et al., 2000). Theronts attached and penetrated the cells undergoing tissue explant outgrowth and trophonts began to grow. By contrast, theronts swam in the medium of BF-2 cultures, moved on the cell surface, and briefly adhered to the cells, but did not penetrate the monolayer. Although

initially supporting trophont development, the co-cultures of tissue fragments and *I. multifilis* deteriorated over time and the majority of the trophonts were dying by 48 h. The other cell line partner was EPC in either EMEM or L-15 that was supplemented or not with 8% rainbow trout serum and 8% mucus (Nielsen and Buchmann, 2000). With or without EPC and with or without the supplements, theronts transformed within 2 days into trophont-like stages. However, they grew and survived best when co-cultured with EPC in the presence of rainbow trout serum and mucus. Although surviving for up to 13 days, trophonts never developed into tomonts.

More success has been achieved in co-cultures for the marine counterpart of *I. multifiliis*. The entire life cycle of *C. irritans* was supported in a double-layered co-culture system with FHM cells (Yoshinaga et al., 2007). The system had as a medium, L-15 with FBS and penicillin/streptomycin, and was initiated by having the FHM cells attach and spread in culture dishes. After spreading, ultra-low-melting agarose was layered on top of the cells. A micropipette was used to inoculate theronts beneath the agarose and at the centre of the cell layer. Over 4 h, theronts transformed to trophonts. Trophonts appeared to detach and eat FHM cells and gradually grew. Some transformed to encysted tomonts. When transferred into seawater, some of the encysted tomonts released theronts. These theronts were able to infect fish. By using large culture dishes the theront production could be scaled up (Yoshinaga et al., 2007).

The in vitro growth of the scuticociliate *M. avidus* has been done conveniently in co-cultures with a salmon cell line. CHSE-214 in EMEM with 10 % FBS supported the growth of *M. avidus* in co-cultures at 20 °C (Lee and Kim, 2008ab, 2009; Jung et al., 2011).

1.4 Uses of ciliates cultures in fish biology

The culturing of free-living ciliates can allow the study and control of at least four aspects of fish biology. Firstly, they aid the study of ciliates as pathogens and disease-causing agents. Secondly, they could be exploited to study the contribution that ciliates make to nutrient cycling from fish carcasses. Thirdly, they may be used to study whether ciliates modulate the

transmission of viral and microbial pathogens of fish. Finally, cell cultures of ciliates can provide food for early life stages, the protozooplankton-ichthyoplankton link.

1.4.1 Ciliates as fish pathogens

Cell cultures can aid the study of the diseases caused by ciliates. As well as supplying the pathogen, they can be used to identify and study, host responses, pathogenic mechanisms, and cures.

1.4.1.1 Scaling up production and attenuation of pathogens

Cultures can potentially produce large quantities of ciliates for use in experiments to study the development of pathogenicity and immunity in fish. However, culturing has been noted to change the virulence of several pathogenic ciliates. For *U. marinum* from infected olive flounder, protease activity from short-term cultures was higher than activity from long-term cultures (Kwon et al., 2003). In the case of *Tetrahymena* sp. isolates from infected guppies, one isolate, Tet-NI 1, was attenuated after 15 months of culture, whereas another isolate, Tet-NI 6, was cultured for only a few months and remained virulent (Leibowitz et al., 2009). For *Philasterides*, the virulence of two isolates from turbot changed in different ways during long-term culture (Alvarez-Pellitero et al., 2004). After 35-42 passages, isolate A became attenuated, whereas after 20-42 passages, isolate B became more virulent. These phenomena deserve further study and can be exploited to develop vaccination strategies.

1.4.1.2 Host responses

The cell culture approach has been used to study responses of fish and identify possible host defense mechanisms. Cysteine proteinases were obtained from *M. avidus* cultures and added to primary cultures of turbot head kidney leucocytes (Paramá et al., 2007a). The leucocytes increased production of superoxide radicals and of mRNA for the proinflammatory cytokine interleukin-1 β , which mediates host inflammatory responses. When in contact with or phagocytized by *M. avidus*, turbot leucocytes were activated (Piazzon et al., 2011b). Activation included degranulation and a respiratory burst but these activities were insufficient to kill the ciliates. However complement that was activated

through the classical pathway was a potent killer of *M. avidus* (Leiro et al., 2008; Piazzon et al., 2011b). These results suggested that the humoral responses were more important than cellular immune responses in defense against *M. avidus* (Piazzon et al., 2011).

1.4.1.3 Pathogenic mechanisms

Ciliate cultures have been used to understand the mechanisms by which the ciliates evade or modulate the defense mechanisms of the host, with a focus on reactive oxygen species (ROS) and proteinases. Host leucocytes produce ROS to generally kill parasites but the ciliates appear to evade ROS by scavenging them. This has been observed in cultures of *U. marinum* and turbot head kidney leucocytes (Kwon et al., 2002) and of *M. avidus* and turbot peritoneal leucocytes (Leiro et al., 2004). Proteinase activities have been examined as virulence factors among *Tetrahymena* and scutitociliates. For a *Tetrahymena* sp. pathogenic to guppies, the virulence of different isolates correlated with their cysteine protease (Leibowitz et al., 2009). The avirulent Tet-NI 1 had no cysteine protease activity, whereas the virulent Tet-NI 6 had high levels of activity. Cultures of *M. avidus* have been used as source of proteinases and a way of evaluating the killing activity of sera from fish that had or had not been injected with the ciliate (Paramá et al., 2007a; Piazzon et al., 2011a). Through activation of the classical complement pathway, serum from vaccinated turbot killed *M. avidus* in culture (Leiro et al., 2008; Piazzon et al., 2011a). However, proteinases from *P. dicentrarchi* were able to degrade the antibodies and factors necessary for complement activation and to decrease killing of the ciliates. Proteases also inhibited leucocyte migration (Paramá et al., 2007b) and caused apoptosis of leucocytes (Parma et al., 2007a). These might be mechanisms by which ciliates evade the fish immune response.

Another mechanism by which histophagous ciliates might survive within their fish hosts is the possession of acid phosphatases. Modulation of phosphotyrosine signaling through protein tyrosine phosphatases (PTPs), such as PTP1b, is emerging as a general point of interaction between host and pathogen (Heneberg, 2012). Hints of this are seen with ciliates and fish as well. The pathogenicity of different *Tetrahymena* isolates correlated with their acid phosphatase activity, in addition to cysteine protease (Leibowitz et al., 2009). PTP1b

was detected in the plasma membrane of *M. avidus* (Salinas et al., 2011). The levels of PTP1b increased upon exposure to grouper skin mucus and leucocytes.

Destruction of fish cells by ciliates has been studied in co-cultures, including an investigation of a possible cytopathogenic mechanism. When a *Tetrahymena* sp. was co-incubated with a guppy fin cell line (Leibowitz et al. > 2010) and *M. avidus* with turbot fibroblast cell line (Paramá et al., 2004), the number of fish cells declined but the decrease was prevented by the addition of the cysteine protease inhibitor E64. These results suggest cysteine proteases have a role in the histolytic activity of the ciliates. In co-cultures with *P. dicentrarchi*, turbot leucocytes served as nourishment, supporting ciliate growth (Piazzon et al., 2011b), and grouper leucocytes were engulfed by the ciliates, up-regulating ciliate acid phosphatases (Salinas et al., 2011). The acid phosphatase activities might destroy leucocytes, but other mechanisms might be contributing as well. Work with free-living amoeba such as *Naegleria fowleri* and flagellated protozoa such as *Trichomonas vaginalis* suggest possible mechanisms to be explored in the future. Amoebae can injure and kill mammalian cells in culture by repeated “nibbling” and ingesting plasma membrane fragments (Sohn et al., 2010), a process which has been termed trophocytosis (Brown, 1978; 1979). In co-cultures with bovine epithelial cells, *T. vaginalis* clustered around the cells, causing mechanical stress, membrane damage and cell death (Midlej and Benchimol, 2010). Only necrotic cells were then phagocytized by *T. vaginalis*.

1.4.1.4 Chemotherapy

Cultures of *M. avidus*, *C. irritans* and *Tetrahymena* sp. have been used to study the efficacy of chemotherapeutic drugs and treatments. As no effective control exists for *M. avidus* in fish, many potential drugs have been screened for their effects on these ciliates in culture (Budiño et al., 2012; Harikrishnan et al., 2010; Iglesias et al., 2002; Jin et al., 2010; Leibowitz et al., 2010; Paramá et al., 2005; Paramá et al., 2007c). Formalin, hydrogen peroxide and resveratrol were among 17 drugs found to be toxic to the ciliates. However, unlike formalin and resveratrol susceptibility, the susceptibility to hydrogen peroxide varied considerably between different *P. dicentrarchi* isolates (Budino et al., 2012). Other toxic

treatments were chitosan microspheres cross linked with glutaraldehyde and containing beta-cyclodextrin (Paramá et al., 2005) and the anti-inflammatory drug indomethacin (Paramá et al., 2007c). Indomethacin reduced the growth of *P. dicentrachi* and eventually caused cell death, possibly by apoptosis (Paramá et al., 2007c). For *C. irritans*, the double-layered co-culture system with FHM cells was used to study the effects of antiprotozoal compounds on trophonts (Yoshinaga et al., 2011). This identified sodium salinomycin as a candidate drug for the control of *C. irritans* infections. In another study on *C. irritans* cultures, epigallocatechin gallate was found to kill theronts (Picón-Camacho et al., 2011). For *Tetrahymena* sp., the toxicity of eight chemicals to the ciliates was evaluated in cultures (Leibowitz et al., 2010). The most potent compound was niclosamide. These studies show the promise of the cell culture approach for identifying effective treatments and likely will be used more in the future.

1.4.2 Ciliates as fish passengers and carcass consumers

The process by which peritrich ciliates attach to fish is largely unknown but might be explored through organ cultures of fish skin or gill as well as cell cultures. Organ cultures would be maintained in very similar media as used for cell cultures and might allow attachment to be recorded more easily than on swimming fish.

Cell cultures also could be used in the future to study the role of ciliates independent of other microbes in the formation of fish floc. Ciliate cultures have been used for this purpose in the context of wastewater treatment. Capsule secretion by *T. thermophila* was shown to contribute to flocculation (Arregui et al., 2007). Ciliates have been grown in the laboratory on an undefined fish powder (Caillierte-Ethuin et al., 1998), fish homogenates (Anderson et al., 2009; Castro et al., 2007) and on fish cells (Piazzon et al., 2011b) so fish fragments might be expected to support ciliate cultures. These cultures could be used to investigate mechanisms by which ciliates form floc and the nature of the floc from fish tissues.

1.4.3 Ciliates interacting with fish microbial and viral pathogens

Culturing fish pathogens and ciliates together has been done on just a few occasions but should be a useful approach in the future for studying how ciliates can modulate the

interactions between pathogens and fish. Ciliate cultures have not been used to study fish viruses, although mammalian viruses have been studied on many occasions as pointed out earlier in section 1.2.3. However, they have been used on one occasion to study a fungal pathogen, the microsporidian *Glugea hertwigi* (Legault and Delisle, 1967), and in a preliminary study on a bacterial pathogen, *Yersinia ruckerii* (Tobback et al., 2007), which is presented here. Microsporidia need to infect animal cells, including fish cells (Monaghan et al., 2009), in order to complete their life cycle. Outside host cells, microsporidia exist as spores. *Tetrahymena* was shown to phagocytize *G. hertwigi* spores, but once inside ciliate lysosomes, *G. hertwigi* blocked lysosomal acidification (Weidner and Sibley, 1985), which is necessary for intracellular digestion and might be expected to inactivate spores. Whether internalization under these circumstances protects and moves spores in the environment would be interesting to explore. Internalization of *Y. ruckerii* does lead to digestion as shown in Figure 1.1. *Yersinia ruckerii* is the cause of enteric redmouth disease (ERM) (Tobback et al., 2007) and a strain expressing green fluorescent protein (gfp) has been constructed (Welch and Wiens, 2005). With other bacteria expressing gfp, engulfment and digestion by ciliates has been visualized by the appearance and disappearance of fluorescence over time (Power et al., 2006). This was also observed in cultures of gfp *Y. ruckerii* and *T. thermophila* (Pinheiro and Bols, unpublished results, Figure 1.1), which suggests that this ciliate engulfs and digests this fish bacterial pathogen.

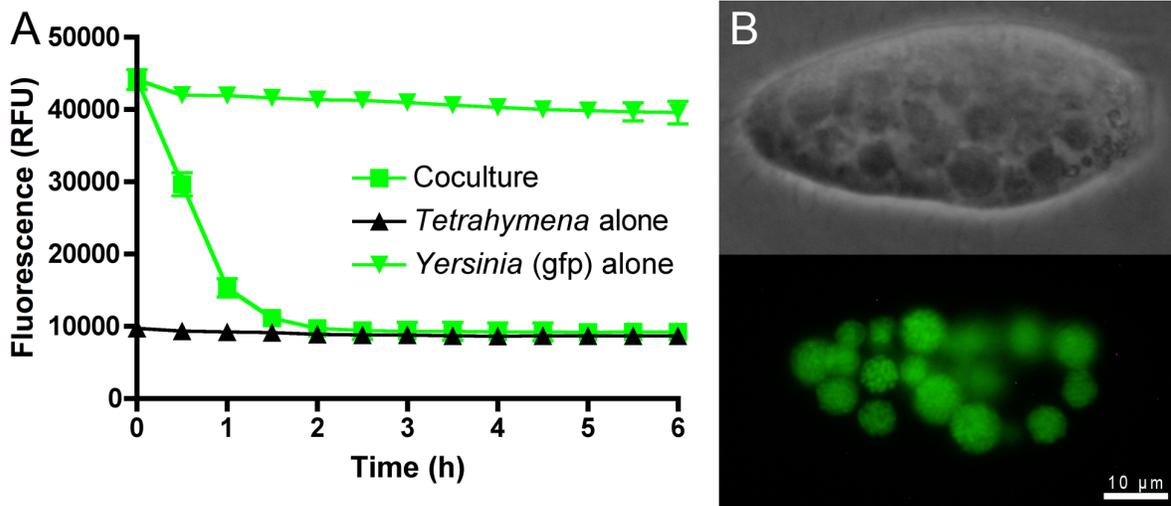


Figure 1.1 Co-culture of *Tetrahymena thermophila* and *Yersinia ruckeri*

A. Mixtures of *T. thermophila* and gfp-expressing *Y. ruckeri* were established and changes in fluorescence (480 nm excitation / 530 nm emission) monitored over a six hour period. Ciliate- and bacteria-free controls did not show decreases over this period, however the presence of ciliates together with bacteria quickly reduced fluorescence. These data are consistent with other examples of easily phagocytosed and digested bacterial prey (Power et al., 2006). Initial densities of organisms were 50,000 ciliates mL⁻¹, and 10⁶ cfu mL⁻¹ of bacteria. **B.** When viewed by epifluorescence microscopy, ciliates could be seen filled with fluorescent particles, localized to areas characteristic of food vacuoles. The images are taken soon after initiation of co-culture of bacteria and ciliate, and this fluorescence was no longer visible by 24 hours after mixture.

1.4.4 Ciliates as fish food: protozooplankton-ichthyoplankton link

The practical application of ciliates as fish larval feed has been considered for a long time and has become an established protocol for some species (Montagnes et al., 2010). Thinking on this subject has its origins in the 19th century (see review by May, 1970) and has mostly focused on larvae of marine fish that have the potential for aquaculture. For some larval marine fish, the small mouth opening makes ciliates an attractive alternative to other feed such as rotifers (Decamp et al., 2001). However, the most routine use of ciliates in fish husbandry has been with a freshwater species, zebrafish (*Danio rerio*). Zebrafish are used both by aquarists and by researchers, and zebrafish larvae are often raised on ciliates (Lawrence, 2007). In some cases, ciliates have been collected from the environment for feeding to fish larvae, but in other cases the ciliates have been grown in the laboratory for this purpose. Optimizing the production of ciliates in culture will make their use as larval feed in aquaculture, zebrafish husbandry and laboratory food chain studies more practical, convenient and controlled.

1.4.4.1 Scaling up cultures of freshwater ciliates

The mass cultivation or scale up of freshwater ciliates has been done primarily in order to produce commercially important enzymes (de Coninck et al., 2000; Munro, 1985; Kiy, 1998), but the methods might also be used to produce ciliates for use as fish larval feed. Large-scale production has been explored with *T. thermophila*, *T. pyriformis* and *T. rostrata* (Dive et al., 1989). Usually a variety of undefined supplements have been tested in order to reduce or replace peptones so that growth can be maximized but costs for running large reactors can be kept low. Good growth has been achieved with skimmed milk (Saliba et al., 1983), hemoglobin hydrolysate (Dive et al., 1989), and yeast extract (Ethuin and de Coninck, 1996). Other undefined supplements have been less successful (Caillieret-Ethuin et al., 1998). In medium with a fish powder supplement, the ciliates only grew slightly. This was attributed to the loss of amino acids during fish powder preparation (Caillieret-Ethuin et al., 1998). On the other hand, yeast extract together with 0.5 % glucose supported ciliate

densities as high as 1.7×10^6 cells/mL in 100 L fermentors (bio-reactors) at 28 °C (de Coninck et al., 2000). Other milestones in *Tetrahymena* scale up have been the use of a defined medium and of an airlift reactor with a working volume of 1200 L (Hellenbroich et al., 1999).

However, the culturing of ciliates for use as feed might require more than just scaling up production. For the rearing of zebrafish larvae, both *Paramecium multimicronucleatum* Powers and Mitchell, 1910, and *T. pyriformis* were helpful feed supplements (Lawrence, 2007; Speksnijder and Bijmolt, 1995). However, *Tetrahymena* were easier to grow, and *Paramecium* appeared to have some nutritional limitations. *Paramecium* alone did not satisfy the nutritional requirements for the transition of zebrafish from larvae to juveniles (Lawrence, 2007). The suggestion was made that because the ciliates had been grown up on bacteria they lacked essential minerals such as iodine for the larval/juvenile transition (Lawrence, 2007). Clearly the culturing of ciliates to support fish larval growth has several fascinating scientific issues, but the engineering of ciliate production on a large scale is being developed.

1.4.4.2 Scaling up cultures of marine ciliates

For saltwater, one of the most intensively studied ciliates is the hypersaline ciliate *F. salina* (Guermazi et al., 2008; Pandey et al., 2004; Rhodes and Phelps, 2008). Different feeds for the ciliate have been tried, including egg custard, yeast, and nanoplanktonic green algae. Egg custard (10 mg/mL) yielded the highest densities, 90 ciliates/mL (Pandey and Yeragi, 2004). Mass cultures of 300 L were achieved but the maximal population growth was found in 200 L cultures (Pandey and Yeragi, 2004).

1.5 Summary and Future

The culturing of some free-living ciliates, such as *T. thermophila*, has a long history and is easy, whereas culturing some pathogenic ciliates, such as *Ichthyophthirius multifiliis*, is difficult, but for both of these extremes there are many interesting issues to explore in the future. The culturing of fish ciliates can be viewed as being done in media that is based on either freshwater, seawater, or vertebrate bodily fluids together either with bacteria, fish cells,

or organic matter that is either undefined, such as proteose peptone, or defined. For species like *T. thermophila*, the end goal of straightforward culturing has been achieved but questions could still be asked about monoxenic and axenic cultures of this species. In monoxenic cultures are *E. coli* the best food or do other bacterial species, or even other microbes such as yeast, support better growth? In axenic cultures, the components of defined media might still be optimized to support more vigorous growth. For obligate parasites with complex life cycles, such as *I. multifiliis*, more sophisticated culture systems need to be developed to obtain all stages. Possibly this requires improvements in fish cell culturing because a particular ciliate life cycle stage might need a specific fish cell type. In final analysis, some parasites might prove impossible to culture but efforts to do so can nonetheless provide insights into their life cycle.

The culturing of fish ciliates aids studies of ciliate/fish interactions and holds out the possibility of revealing new ones (some described in Table 1.2). Cultures allow many aspects of ciliate pathogenicity to be examined. These include how histophagous ciliates kill fish cells and what drugs might protect the fish cells from the histophages. Cultures could be used to examine processes after fish death because ciliates can eat dead matter and might participate in flocculation of fish carcasses, which is important in nutrient cycling. Cultures can be used to study the relationships of free-living ciliates, and even pathogenic ones, with other fish pathogens, viral, bacterial, or fungal. This approach is only beginning but promises to reveal the contrasting actions of ciliates in protecting and inactivating pathogens. This knowledge could be used to understand possible roles of ciliates in disease transmission and how to control the spread of diseases. Cultures could be a source of feed for fish larvae because large-scale cultures of both freshwater and marine ciliates have been achieved. Finally, cultures might be used to delineate new relationships, with xenohormesis being an example. Xenohormesis is the sensing of chemical cues from one species by another (Howitz and Sinclair, 2008). This could reveal new compounds that might be used to promote fish growth and health.

1.6 Goals

This thesis is meant to be a beginning and hopefully a foundation for the in vitro approach to exploring ciliate/fish interactions. The five thesis goals and the chapters in which they are covered are as follows:

I. Assess the survival & growth of *Tetrahymena thermophila* in media used for animal cell culture (Chapter 2). This goal might be considered foundational because once accomplish the technique could be tried on other ciliates and perhaps other protozoa.

II. Assess how *T. thermophila* & fish cells survive & grow when cultured together in animal media (Chapter 3). This goal also can be considered foundational because once achieved other ciliates and other animal cells could be examined.

III. Assess the survival of animal cells from different groups of vertebrates in co-culture with three *Tetrahymena* species (Chapter 4). This goal uses the results of Chapters 1 and 2 to begin looking at specific ciliates and at animal cells from different animal groups and tissue types.

IV. Determine whether *T. thermophila* inactivates, activates, or has no effect on a fish rhabdovirus, VHSV (Chapter 5). In this goal the results from Chapters 2, 3 and 4 are used to set up an experimental approach for beginning to study the possible interactions between fish viruses and ciliates.

V. Determine whether *T. thermophila* inactivates, activates, or has no effect on a fish aquareovirus, CSV (Chapter 6). The final goal uses the results from the other four Chapters to set up an experiment approach for studying for the first time how ciliates might activate and transfer a specific fish virus in water.

Table 1.1 Fish ciliates that are mentioned in this chapter and their relationship to fish

| Species | Context in this article | Conventional relationship to fish |
|---|--|--|
| Family Balanionidae Small and Lynn, 1985 | | |
| Genus <i>Balanion</i> sp. Wulff, 1919 | Section 1.4, feed for red snapper larvae | Prey Item |
| Family Balantiididae | | |
| Genus <i>Balantidium</i> Claparéde and Lachman, 1858 | Section 1.2, endocommensals within the gut of fish | Endocommensal |
| <i>Balantidium ctenopharyngodonis</i> Chen, 1955 | Section 1.2, typically harmless but identified to cause intestinal lesions in older fish | Endocommensal |
| Family Chilodonellidae Deroux, 1970 | | |
| Genus <i>Chilodonella</i> sp. Strand, 1928 | Section 1.2, obligate ectoparasite in freshwater and brackish water | Ectoparasite |
| <i>Chilodonella hexasticha</i> (Kiernik, 1909) Kahl, 1931 | Section 1.2, facultative ectoparasite | Ectoparasite |
| <i>Chilodonella piscicola</i> Zacharias, 1894 | Section 1.2, facultative ectoparasite | Ectoparasite |
| Family Climacostomidae Repak, 1972 | | |
| <i>Fabrea salina</i> Henneguy, 1890 | Section 1.4, feed red snapper larvae | Prey Item |
| Family Scyphidiidae Kahl, 1933 | | |
| | | Prey Item |

Family Epistylididae Kahl, 1933

Epistylis lwoffii Fauré-Section 1.2, ectocommensal, opportunistic parasite in poor water conditions Ectocommensal
Fremiet, 1943

Family Euplotidae Ehrenberg, 1838

Genus *Euplotes* sp. Müller, Section 1.4, seen inside gut of larvae Prey Item
1786

Family Ichthyophthiriidae Kent, 1881

Ichthyophthirius multifiliis Section 1.2, obligate freshwater endoparasite; III, cultured; Section IV Endoparasite
Fouquet, 1876

Family Hartmannulidae Poche, 1913

Brooklynella hostilis Lom Section 1.2, obligate ectoparasite of marine fish Ectoparasite
and Nigrelli, 1970
Brooklynella sinensis Gong Section 1.2, recent isolate with no parasitism yet identified Free-living
and Song, 2006

Family Holophryidae Perty, 1852

Cryptocaryon irritans Section 1.2, obligate marine ectoparasite; III, cultured; Section IV, Ectoparasite
Brown, 1951 chemotherapy

Family Leegaardiellidae Lynn and Montagnes, 1988

Genus *Leegaardiella* sp. Section 1.4, feed for fish larvae Prey Item
Lynn and Montagnes, 1988

Family Mesodiniidae Jankowski, 1980

Myrionecta rubra Section 1.4, feed for fish larvae Prey Item

Jankowski, 1976

(syn. *Mesodinium rubrum*

Lohmann, 1908)

Family Scyphidiidae Kahl, 1933

Prey Item

Family Orchitophryidae Cépède, 1910

Genus *Paranophrys* sp. Section 1.3, cultured

Free-living

Thompson and Berger,

1965

Family Parauronematidae Small and Lynn, 1985

Miamiensis avidus Section 1.2, facultative marine parasite; Section 1.3, cultured; Section 1.4, connection with infection of seahorse with IPNV, immune response in the host

Ectoparasite

Philasterides dicentrarchi

Dragesco et al., 1995)

Parauronema virginianum Section 1.3, cultured

Ectoparasite

Thompson, 1967

Family Parameciidae Dujardin, 1840

Genus *Paramecium* sp. O.F. Section 1.3, cultured; Section 1.4, potential fish feed supplement

Prey Item

Müller, 1773

Paramecium Section 1.4, feed for zebrafish larvae

Prey Item

multimicronucleatum

Powers and Mitchell, 1910

Family Philasteridae Kahl, 1931

| | | |
|---|---|--------------|
| Genus <i>Philasterides</i> sp. Kahl, 1931 | Section 1.4, change in pathogenicity with in vitro culture age | Ectoparasite |
| <i>Philasterides dicentrarchi</i> Dragesco et al., 1995 (syn. <i>Miamiensis avidus</i> Thompson and Moewus, 1964) | Section 1.2, facultative marine parasite; 1.3, cultured; Section 1.4, immune response in the host, chemotherapy | Ectoparasite |

Family Pseudocohnilembidae Evans and Thompson, 1964

| | | |
|--|---|--------------|
| <i>Pseudocohnilembus persalinus</i> Evans and Thompson, 1964 | Section 1.2, marine ciliate isolated from ovarian fluid of freshwater rainbow trout | Ectoparasite |
|--|---|--------------|

Family Scyphidiidae Kahl, 1933

Prey Item

Family Strombidiidae Fauré-Fremiet, 1970

| | | |
|---|--|-----------|
| Genus <i>Strombidium</i> sp. Claparède and Lachmann, 1859 | Section 1.3, cultured; Section 1.4, feed for fish larvae | Prey Item |
| <i>S. capitatum</i> (Leegaard, 1915) Kahl, 1932 | Section 1.4, feed for fish larvae | Prey Item |

Family Tetrahymenidae Corliss, 1952

| | | |
|--|--|-------------|
| Genus <i>Tetrahymena</i> sp. Furgason, 1940 | Section 1.2, facultative parasite of various fish larvae; Section 1.4, phagocytize <i>Glugea hertwigi</i> spores, attenuation of pathogenicity with in | Free-living |
|--|--|-------------|

| | | |
|---|--|---------------|
| | vitro culture age, immune response in the host, chemotherapy | |
| <i>T. corlissi</i> Thompson, 1955 | Section 1.2, facultative parasite of various fish; | Ectoparasite |
| <i>T. geleii</i> Furgason, 1940 (syn. <i>T. pyriformis</i> Ehrenberg, 1830) | Section 1.4, inactivated poliovirus | Free-living |
| <i>T. pyriformis</i> Ehrenberg, 1830 | Section 1.1; Section 1.2, facultative parasite of various fish; Section 1.3, cultured; Section 1.4, potential fish feed supplement, contribution to floc formation, inactivating a variety of animal viruses | Free-living |
| <i>T. thermophila</i> Nanney and McCoy, 1976 | Section 1.1, 1.3, cultured | Free-living |
| <i>T. rostrata</i> Kahl, 1926 | Section 1.3, cultured; 1.4, feeding on fish powder | Ectoparasite |
| Family Tintinnidae Claparède and Lachmann, 1858 | Section 1.4, feed for fish larvae | Prey Item |
| Family Tontoniidae Agatha, 2004 | | |
| <i>Laboea strobila</i> Lohman, 1908 | Section 1.4, feed for fish larvae | Prey Item |
| Genus <i>Tontonia</i> sp. Fauré- Fremiet, 1914 | Section 1.4, feed for fish larvae | Prey Item |
| Family Trichodinidae Claus, 1951 | | |
| Genus <i>Trichodina</i> sp. Ehrenberg, 1830 | Section 1.2, freshwater and marine ectocommensals, opportunistic parasite in poor water conditions | Ectocommensal |

Family Uronematidae Thompson, 1964

Uronema marinum Section 1.2, facultative marine parasite; Section 1.3, cultured; Section 1.4, Ectoparasite
Dujardin, 1841 culture and study of tissue destruction, immune response in the host

Table 1.2 Ciliates whose interactions with fish might be understood better through cell cultures

| Species | Known relationship to fish | Representative References |
|---|--|---|
| <i>Balantidium</i> spp. (i.e. <i>B. jocularum</i>) | Endocommensal facultative parasites; ten species are found to infect freshwater and marine fish | Basson and Van As, 2006; Grim, 2006 |
| <i>Brooklynella hostilis</i> | Ectoparasite; causes “brooklynellosis: marine counterpart to “chilodonellosis” | Noga, 2010 |
| <i>Capriniana piscium</i> | Suctorian ectocommensal found heavily infesting gills and body muculature of environmentally stressed marine fish | Cheung et al., 1980 |
| <i>Chilodonella piscicola</i> , <i>C. hexastichia</i> | Ectoparasites or freshwater and brackish water fish; causes “chilodonellosis” | Basson and Van As, 2006; Noga, 2010 |
| <i>Cryptocaryon irritans</i> | Obligate ectoparasite of marine fish with polymorphic life cycle; no current method for propagating without the use of live fish | Tookwinas, 1990; Colorni and Burgess, 1997 |
| Family Epistylididae | | |
| • <i>Epistylis lowffi</i> | Stalked ectocommensal that may damage fish in poor water conditions; linked to “red sore disease” resulting from a secondary infection by the bacterium, <i>Aeromonas hydrophila</i> | Hazen et al., 1978 |
| • <i>Apiosoma micropteri</i> | Stalked ectocommensal; alleged to cause fingerling mortalities due to overgrowth on gill surface | Basson and Van As, 2006 |
| <i>Ichthyophthirius multifiliis</i> | Obligate ectoparasite of freshwater fish with polymorphic life cycle; no current method for propagating without the use of living fish | Dickerson and Dawe, 2006; Matthews, 2005 |
| <i>Paramecium</i> spp. (i.e. <i>P. multimicronucleatum</i>) | No known parasitic relationship but occasionally found in low numbers on skin or gills; model organism; along <i>T. pyriformis</i> shows potential as a potential fish feed | Speksnijder and Bijmolt, 1995; Lawrence, 2007 |

supplement

| | | |
|--|--|---|
| <i>Pseudocohnilembus persalinus</i> | Endosymbiont in oliver flounder and capable of free-living in marine water; recently found as an endosymbiont in freshwater in rainbow trout | Jones et al., 2010 |
| <i>Tetrahymena thermophila</i> | No known relationship; model organism; fish bath immunization noted to impart protection against <i>I. multifiliis</i> | Wolf and Markiw, 1982 |
| Family Trichodinidae <ul style="list-style-type: none">• <i>Trichodina</i> spp.• <i>Trichodinella</i> spp.• <i>Tripartiella</i> spp.• <i>Dipartiella</i> spp.• <i>Paratrichodina</i> spp.• <i>Hemitrichodina</i> spp.• <i>Vauchomia</i> spp. | Ectocommensals, that may overpopulate and damage fish skin and gills under poor water conditions; limited ability to survive free of the host; include marine and freshwater species | Basson and Van As, 2006 |
| Ciliates found to be ingested by larval fish <ul style="list-style-type: none">• <i>Myrionecta</i> spp.• <i>Tontonia</i> spp.• <i>Leegaardiella</i> spp.• <i>Laboea</i> spp.• <i>Strombidium</i> spp.• <i>Euplotes</i> spp.• <i>Balanion</i> spp.• <i>Fabrea salina</i>• Members of Family Tintinnida | Found present within the gut of feeding fish, members of these genera hold the potential to be additives to commercial fish feeds | Ohman et al., 1991; Nagano et al., 2000; Hunt von Herbring and Gallager, 2000; Figueiredo et al., 2007; Rhodes and Phelps, 2008 |

Chapter 2

Survival and growth of *Tetrahymena thermophila* in media that are conventionally used for piscine and mammalian cells

2.1 Introduction

Tetrahymena have perhaps the longest history of successful laboratory culture of any eukaryotic cell. In nature these ciliates are largely free-living, feeding on bacteria in freshwater streams and ponds, which have osmolalities in the 10 to 50 mOsm/kg H₂O range (Elliott, 1973). In the laboratory, water with simple supplements, such as proteose peptone and either yeast or liver extract, supports their growth, making them the first single cell eukaryote to be grown without any other organisms (axenically) (Lwoff, 1923). Replacing the general supplements with specific chemical components also supports proliferation, making *Tetrahymena* the first eukaryotic cell to be grown in a chemically defined medium (Kidder and Dewey, 1951). However, their vigorous proliferation in simple buffered media with the inexpensive undefined supplements was probably critical in making these ciliates a popular laboratory organism, especially *Tetrahymena thermophila* (Asai and Forney, 1999).

Concurrent with the history of culturing free-living, single-cell eukaryotes, the science of culturing cells from multicellular animals, primarily vertebrates, emerged. One important advance was the development of basal media. These contain nutrients, buffering agents, and ions, to yield aqueous solutions with an osmolality of approximately 300 mOsm/kg H₂O (Ham, 1984). Although many types of basal media are commercially available, Leibovitz's L-15 stands out because its buffering capacity is maintained under normal atmospheric conditions, and thus without the need for a CO₂ incubator (Leibovitz, 1963). To support the growth of vertebrate cells, basal media commonly have to be supplemented with animal sera, usually fetal bovine serum (FBS) (Griffiths, 1987). Serum arises from the coagulation of blood and is formed in vivo during wounding and pathogen invasion to provide factors that co-ordinate restoration of barrier functions (Oikonomopoulou et al., 2012). In vitro, serum

supports the growth of animal cells in multiple ways, including the provision of polypeptide growth factors, hormones and vitamins (Ham, 1984).

Although a few tetrahymenids have been known for some time to parasitize gastropod molluscs (Brooks, 1968), insects (Lynn et al, 1981) and fish (Hoffman et al., 1975), reports of *Tetrahymena* species infecting vertebrates have increased considerably over the last 15 years, with infections becoming an economic problem in the ornamental fish trade (Leibovitz and Zilberg, 2009). When guppies swimming abnormally were examined, ciliates were found inside muscles, internal organs, eye socket, and spinal cord (Imai et al., 2000). Treating healthy guppies with acetic acid to wound skin, followed by exposure to the ciliates, led to infections (Ponpornpisit et al., 2000). Infections in other tropical fish have been reported (Thilakarante et al., 2003; Leibowitz et al., 2005), including the important research species, zebrafish (Astrofsky et al., 2002). Less is known about infections in other vertebrate groups, but experimental infections of frog tadpoles have been reported (Thompson, 1958) and surprisingly a species has been found in the bladder of a dog (Lynn et al., 2000). These results suggest that at least some tetrahymenids have the potential to move from being free-living in freshwater to living inside vertebrates as opportunistic pathogens.

To develop experimental culture systems for investigating how tetrahymenids make the transition to life inside vertebrates and how the host cells respond, I have begun by determining the ability of *T. thermophila* to survive hyperosmotic challenges and to grow in a basal medium, L-15, for vertebrate cells. *T. thermophila* was chosen because more is known about the cell biology of this species, including the existence of mutants, than for other tetrahymenids (Asai and Forney, 1999). FBS enhanced the survival of this species during hyperosmotic shocks and was essential for it to grow in L-15.

2.2 Materials and Methods

2.2.1 *Tetrahymena thermophila*

Axenic cultures of *T. thermophila* B1975 were obtained from Dr. D. Lynn (Department of Integrative Biology, University of Guelph, Guelph, ON). A temperature-conditional strain of *T. thermophila*, NP1 was acquired from the Tetrahymena Stock Center (Cornell University, Ithaca, NY). This mutant is incapable of phagocytosis when grown above 30 °C, but carries out phagocytosis similarly to B1975 below 30 °C (Orias and Pollock, 1975). Ciliates were routinely maintained at 22 °C in the ciliate growth medium, proteose peptone yeast extract (PPYE), and enumerated using a Coulter Z2 electronic particle counter (Beckman Coulter, Brea, CA) as described previously (Pinheiro et al., 2007). The PPYE medium consisted of 0.125% (w/v) dextrose (Sigma-Aldrich, St. Louis, MO), 0.5% (w/v) proteose peptone (BD Biosciences, Durham, NC) and 0.5% (w/v) yeast extract (Becton-Dickinson) dissolved in MilliQ water. The measured osmolality of PPYE was 82 ± 2 mOsm/kg H₂O (n = 3).

2.2.2 Direct transfer of *Tetrahymena* to new media

For direct transfer of ciliates to hyperosmotic medium (> 290 mOsm/kg H₂O) from PPYE, which is considered normosmotic for *Tetrahymena* (~20 to 80 mOsm/kg H₂O), the ciliates were prepared as follows. Cultures of *T. thermophila* B1975 that were grown to mid- to late-log phase were collected by centrifugation for 10 min at 1,000 x g. Pelleted ciliates were washed three times, each time with one volume of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Sigma-Aldrich; 24 ± 4 mOsm/kg H₂O; n = 3), and enumerated. Washed ciliates were resuspended in test media, generally at a final volume of 15 mL, in a non-tissue culture treated 75 cm² flask (BD Biosciences). Ciliate-containing flasks were maintained at 22 °C, unless otherwise noted, on an orbital shaker (50 rpm). Special care was taken to ensure flasks were placed vertically, rather than horizontally. This was done to limit the potential for interface-mediated death, which is a non-programmed cell death due to lysis at the point of contact between the medium and air above and is described by Hagemester et al., (1999). The density of ciliates at the beginning each experiment

varied, and is specifically noted for each section, but was generally adjusted to either low (1,500 or 5,000 cells/mL), medium (25,000 cells/mL), or high density (150,000 cells/mL).

2.2.3 Effect of hyperosmotic media on ciliate viability

Hyperosmotic media may impact ciliate viability due to stress caused by the change in osmolality and/or differences in nutrient content between media designed for culture of ciliates and animal cells. To investigate this, ciliates were first added to media used to culture *Tetrahymena* that were made hyperosmotic by adding NaCl. *Tetrahymena thermophila* B1975, prepared as described above, were added to PPYE and 10 mM HEPES, which are normosmotic, or PPYE with 0.7 % NaCl (318 ± 1 mOsm/kg H₂O; n = 3) and HEPES with 0.8 % NaCl (309 ± 6 mOsm/kg H₂O; n = 3), which are hyperosmotic. Addition of NaCl to these media was done as described previously (St Denis et al., 2010). The initial ciliate density was 25,000 cells/mL. Cell counts were taken at 0, 3, 8, and 24 hours.

Similarly prepared *T. thermophila* B1975 were also added to a hyperosmotic medium conventionally used for piscine and mammalian cell culture, L-15 basal medium and changes to cell number occurring over a short period of time (3 h) were monitored. Basal L-15 was Leibovitz's L-15 with 2 mM L-glutamine from Hyclone (Sigma-Aldrich), with added penicillin (100 IU/mL) / streptomycin (100 µg/mL) solution (Sigma-Aldrich). The measured osmolality of L-15 basal medium was 324 ± 2 mOsm/kg H₂O (n = 3). Change in the density of ciliates was assessed after 3 h in hyperosmotic medium, and compared to ciliates in PPYE and HEPES with NaCl. The influence of starting density was investigated by running parallel mixtures of two initial ciliate densities for each medium; 25,000 and 150,000 cells/mL.

2.2.4 Direct transfer of *Tetrahymena* into serum-supplemented hyperosmotic media.

Routine use of L-15 in the maintenance of animal cell culture conventionally involves supplementing the medium with fetal bovine serum (FBS). Thus, *T. thermophila* B1975 were prepared for direct transfer as described above, and were added to FBS-supplemented L-15. Changes to cell number were assessed over 24 h and compared to ciliates moved to hyperosmotic PPYE and HEPES. Supplementation with FBS involved the addition of 10 %

(v/v) FBS (Sigma-Aldrich). The measured osmolality of L-15 with FBS was 332 ± 8 mOsm/kg H₂O

(n = 3), PPYE/NaCl with FBS was 337 ± 6 mOsm/kg H₂O (n = 3), and HEPES/NaCl with FBS was 343 ± 6 mOsm/kg H₂O (n = 3). Cell density was assessed at 0, 8, and 24 h after mixture. Ciliates were added at 25,000 cells/mL.

The initial density appeared to influence the immediate effects of direct transfer to hyperosmotic medium, especially at low ciliate numbers. To assess the immediate effects at low density, *T. thermophila* B1975 were added at 1,500 cells/mL directly from PPYE to L-15 basal or L-15 with FBS and changes to morphology and swimming behaviour were observed microscopically with an inverted microscope, within five minutes of transfer to media. Observations were compared to ciliates remaining in PPYE. Proliferation of ciliates initiated in L-15 basal, and L-15 with FBS at a low density (5,000 cells/mL) was also assessed over a period of 24 h, and compared to ciliates maintained in normosmotic PPYE and HEPES. Culture density was measured after 0, 4, 8, and 24 h after mixing.

2.2.5 Ability of sera to support ciliate growth in normosmotic PPYE and HEPES

To assess FBS as an additive to promote growth of *T. thermophila* B1975, two incomplete growth media were made from PPYE components, 0.5 % (w/v) proteose peptone (PP) alone, and 0.5 % (w/v) yeast extract (YE) alone. Ciliates were transferred directly from PPYE, as described above, and resuspended in PP or YE media, with and without added 10 % FBS. Cultures were initiated at 25,000 cells/mL. Density was measured after 24 h in these media. The addition of FBS to the nutrient-free, starvation buffer HEPES was similarly assessed for the ability of FBS to support proliferation, along with commercially dialyzed FBS, dFBS (10,000 MW cut-off; 10 %; Sigma-Aldrich). Alternative sera to FBS were also explored for their ability to support ciliate growth in HEPES. Sera from horse, rabbit, pig (porcine), and chicken were used to supplement normosmotic HEPES, at a serum concentration of 10 % (v/v). *Tetrahymena thermophila* B1975 were added at a density of 25,000 cells/mL to these media, and density was assessed at 0 and 24 h.

2.2.6 Pre-transfer acclimation of *Tetrahymena* to high osmolality in an intermediate medium.

An acclimation procedure was developed to facilitate the adaptation of ciliates to hyperosmolality, similar to that of St Denis et al (2010). This involved an acclimation period in an intermediate medium, prior to adding *T. thermophila* to hyperosmotic medium. This intermediate medium was PPYE with added L-15 components, hereafter referred to as PPYE/L-15. Aseptically, powdered L-15 (Sigma-Aldrich) was added to autoclave-sterilized PPYE medium, following manufacturer's directions. The osmolality of this medium was between 300 and 380 mOsm/kg H₂O. Special care was taken to avoid filter sterilization of this medium, as this has been demonstrated to impact growth of *Tetrahymena* (Rasmussen and Kludt, 1970). Pre-treated cells were prepared as follows; mid- to late-log cultures of *T. thermophila* B1975 or NP1 in PPYE were centrifuged at 1,000 x g for 10 minutes, and the pellet was resuspended in PPYE/L-15. The density of these overnight cultures was generally greater than 50,000 cells/mL. These cells were maintained for 18 - 24 hours on an orbital shaker (50 rpm). After this period, cells were washed with an equal volume of phosphate buffered saline, three times, by centrifugation. Washed cells were enumerated and added to test medium, generally at a final volume of 15 mL, in non-tissue culture treated 75 cm² flasks.

To assess the acclimation procedure on subsequent ciliate growth in hyperosmotic medium, acclimated cells were resuspended in either L-15 basal or L-15 with 10 % FBS. Initial density was 5,000 cells/mL. Cell number was determined immediately after inoculation, and again after 24 hours. Both strains of *T. thermophila*, B1975 and NP1, were maintained at either 22 or 30 °C. The initial acclimation period was maintained at the same temperature as the following growth trial. For example, ciliates to be assayed for proliferation at 30 °C, were acclimated for 18 - 24 hours at 30 °C. This was done to ensure the population of the temperature-conditional mutant NP1 would be phagocytosis-deficient.

2.2.7 Effects of acclimation on bacterivory

Phagocytosis and digestion of bacterial prey, bacterivory, by *T. thermophila* was assessed using a fluorometric method (Power et al., 2006). *Tetrahymena thermophila* B1975 were added to a population of gfp-expressing *Escherichia coli* XL-1 (*pET-gfp*). Bacteria were maintained in Luria-Bertani broth supplemented with 100 µg/mL ampicillin (Sigma-Aldrich) at 37 °C. Cultures were harvested by centrifugation (3,000 x g for 10 min), washed and resuspended in normosmotic HEPES. Bacterial fluorescence at 480 nm excitation/ 530 nm emission was assessed, and the culture was diluted to 100,000 relative fluorescence units (RFU). To the bacteria, 100,000 ciliates/mL were added in either PPYE with added 1 % penicillin - streptomycin solution (Sigma-Aldrich), basal L-15 with penicillin-streptomycin, or L-15 with 10 % FBS and penicillin-streptomycin. Antibiotics were added to these media to prevent the proliferation of *E. coli* XL-1 during the test period. Ciliates were transferred directly from PPYE, as described above. Ciliate / bacteria mixtures were added to a 96-well microplate (final volume: 200 µL; 6 replicate wells per trial). Ciliate-free bacterial controls, and ciliate-only controls were concurrently run and each control contained added penicillin-streptomycin solution. Microplate gfp fluorescence was read every 15 minutes, for 2 hours using a Victor ³V fluorescent plate reader (Perkin-Elmer, Waltham, MA). Data from three independent trials were first normalized to the percentage of *Tetrahymena*-free fluorescence for the respective trial, then pooled to find the mean at each time point.

2.2.8 Statistics

For each assessment of ciliate proliferation, data were collected from multiple independent trials, with the number of replicates indicated as *n* number in the text. Data were, where noted, expressed as the percentage of the measured mean starting ciliate density. Specific statistical tests are described for each experiment in the results and figure legends, and all tests were conducted using GraphPad Prism v.4.0c for Macintosh. Linear and non-linear regressions were similarly conducted using this software, and R^2 values are reported in the text.

2.3 Results

2.3.1 Effect of hyperosmotic media on ciliate viability

Tetrahymena thermophila B1975, wildtype, was examined for viability after being switched from a normosmotic medium to either a starvation buffer or a growth medium that was made hyperosmotic. The starvation buffer was 10 mM HEPES (24 ± 4 mOsm/kg H₂O; n = 3); the growth medium, PPYE (82 ± 2 mOsm/kg H₂O; n = 3). Normosmotic solutions for this ciliate were defined as those with osmolalities ranging from 20 to 90 mOsm/kg H₂O, which would encompass values for the usual habitat of these ciliates (i.e. freshwater). Any media with osmolalities similar to those of piscine and mammalian plasma and commercial animal cell culture media (~300 mOsm/kg water) were defined as hyperosmotic. HEPES and PPYE were made hyperosmotic by adding NaCl. When switched from PPYE to 10 mM HEPES with 0.8 % NaCl (309 ± 6 mOsm/kg H₂O), *T. thermophila* almost immediately started to lose their normal shape and by 3 h nearly all ciliates were round and nonmotile and cell debris was evident. A significant drop in cell number was seen by 3 h. By 8 h the number had declined further and almost no cells were present by 24 h (Figure 2.1). When transferred to PPYE with 0.7 % NaCl (318 ± 1 mOsm/kg H₂O), some ciliates initially had abnormal swimming and shapes, and by 3 h most ciliates were round and swam noticeably slower. These cultures had some cellular debris and fewer cells at 3 and 8 h. After 24 h in this medium, debris from dead cells was still apparent but cells looked and swam normally. Therefore, nutrients and/or osmolytes in PPYE appeared necessary for some ciliates to survive transfer to a hyperosmotic medium.

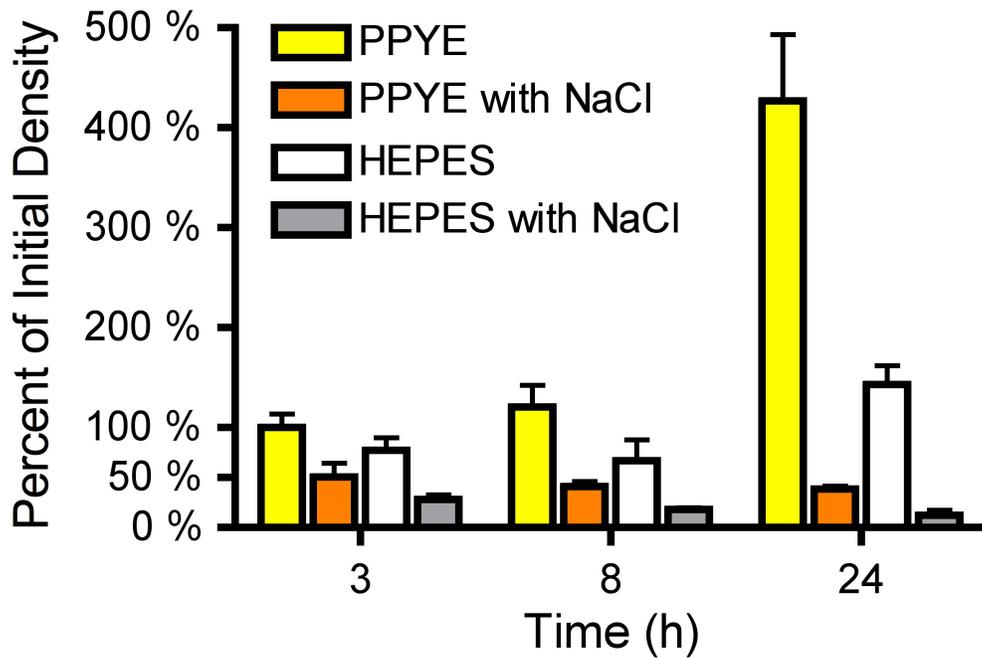


Figure 2.1 Proliferation of *Tetrahymena* in hyperosmotic media

Proliferation of ciliates was investigated in ciliate growth medium, PPYE, or an organic buffer, 10 mM HEPES, that had been artificially made hypertonic with NaCl. Cultures were initiated at 25,000 cells/mL, and maintained at 22 °C. Cell density, expressed as the percent of the respective starting density for each sample, is plotted on the graph as the mean with standard deviation (n = 3 or greater).

2.3.2 Effect of transfer to L-15 without or with fetal bovine serum on ciliate viability

When transferred from PPYE to L-15 (324 ± 2 mOsm/kg H₂O, $n = 3$) some *T. thermophila* B1975 died but others persisted and survival was better if the L-15 was supplemented with fetal bovine serum (FBS). Upon being placed in L-15, some ciliates almost immediately began to swim erratically, such as swimming in circles, and to adopt unusual shapes (Figure 2.2). These abnormalities were more apparent when the cultures had been initiated in L-15 at a medium- and lower initial density (25,000 cells/mL or less) than at high (150,000 cells/mL or more) ciliate density. Monitoring cell number with a Coulter counter confirmed a decrease in ciliate number after 3 h, and microscopy of cultures showed some cells had died in medium-density (25,000 cells/mL) cultures but not in high-density cultures (Figure 2.3). Within an hour or two of cultures being initiated at a low ciliate density (5,000 ciliates/mL) in L-15, most cells were round and moving slowly. The cultures had cell debris and motionless ciliates that were no longer phase bright, suggesting some cell death.

After 24 hours, death was observed in low-density cultures in L-15 basal, with the cultures containing $32.0 \% \pm 1.1 \% (\pm \text{SD}, n = 3)$ of the starting cell number (Figure 2.4). Even after 24 h in L-15 at a low density, some ciliates with a normal morphology and motility were seen. Overall survival was better in L-15 than previously shown in 10 mM HEPES with 0.8 % NaCl, despite the solutions having similar osmolalities. Therefore, some L-15 nutrients must promote survival in hyperosmotic media. In L-15 with 10 % FBS, most ciliates swam normally and had the usual slipper shape, cell debris was largely absent, and cell number was maintained or increased over the 24 h. When the cultures were started at 25,000 cells/mL and maintained at room temperature, cell number had increased $2.1 \pm 0.6 (\pm \text{SD}, n = 3)$ fold by 24 h (Figure 2.5A). When cultures were started at 5,000 cells/mL, most ciliates had the usual slipper shape and swam normally after 24 h. However, ciliate number was only maintained and did not increase over 24 h (Figure 2.4). Therefore, FBS must have additional components that support survival of *Tetrahymena*, and when the ciliate density was high FBS supported *T. thermophila* B1975 proliferation in the hyperosmotic medium, L-15.

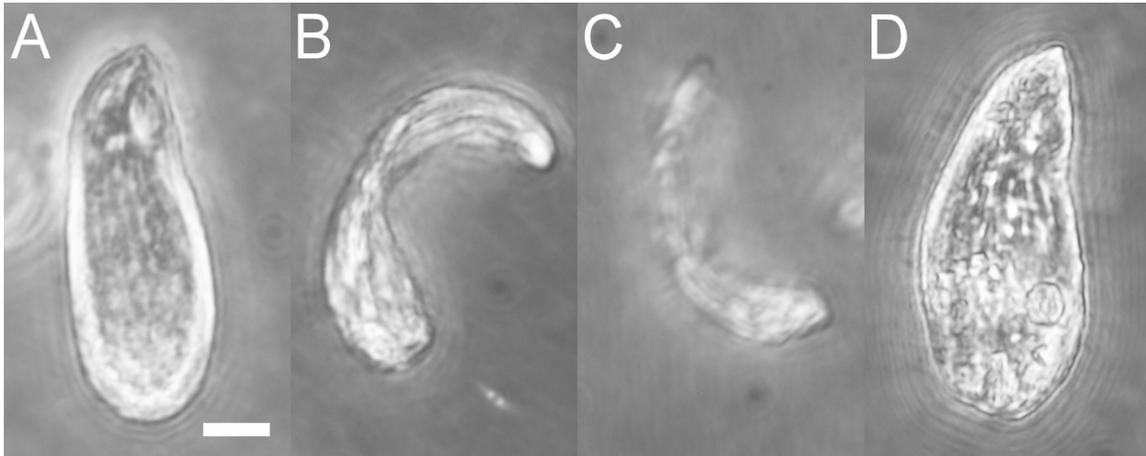


Figure 2.2 Immediate morphological changes to *Tetrahymena* when transferred to hyperosmotic media

Tetrahymena thermophila B1975 were moved from an overnight culture in PPYE to either sterile PPYE (A), L-15 basal medium (B), L-15 + 10 % FBS (C), or PPYE / L-15 intermediate medium (D); in all cases starting density was 1,500 cells/mL. Cultures were maintained at 22 °C. Micrographs were taken 5 minutes after transfer; bar = 10 µm. By this time only ciliates moved to PPYE and PPYE / L-15 remained motile. Those moved to L-15 + FBS regained a very sluggish motility at approximately 4 hour after transfer.

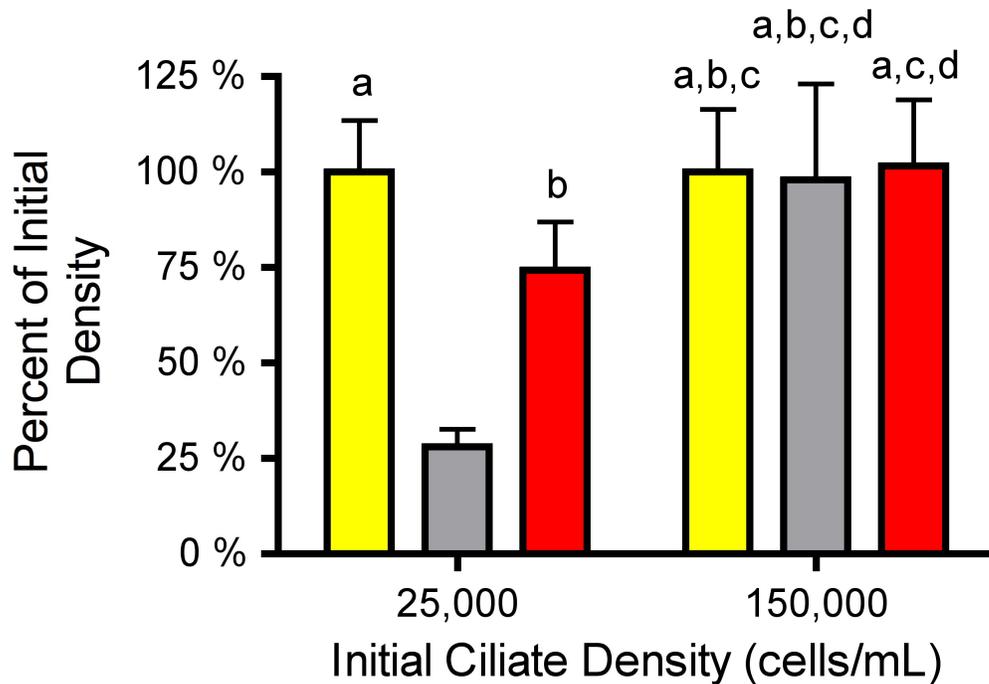


Figure 2.3 Reduction of cell number shortly after movement to hyperosmotic media

Changes to density of *Tetrahymena thermophila* B1975 were investigated from two initial ciliate densities (25,000 and 150,000 cells/mL), 3 h after movement from ciliate growth medium, PPYE, to hyperosmotic media. These media were 10 mM HEPES with 0.8 % NaCl (309 ± 6 mOsm/kg H₂O) shown as grey bars, or L-15 basal medium (324 ± 2 mOsm/kg H₂O) shown as red bars. Ciliates were also maintained for 3 h in normosmotic growth medium, PPYE (82 ± 2 mOsm/kg H₂O), shown as yellow bars. Data are presented as the percent of initial density, and plotted as the mean, with standard deviation ($n = 9$ for 25,000 cells/mL and $n = 6$ for 150,000 cells/mL). Bars having the same letters are statistically similar ($p > 0.05$; One-way ANOVA with Bonferroni Multiple Comparisons test).

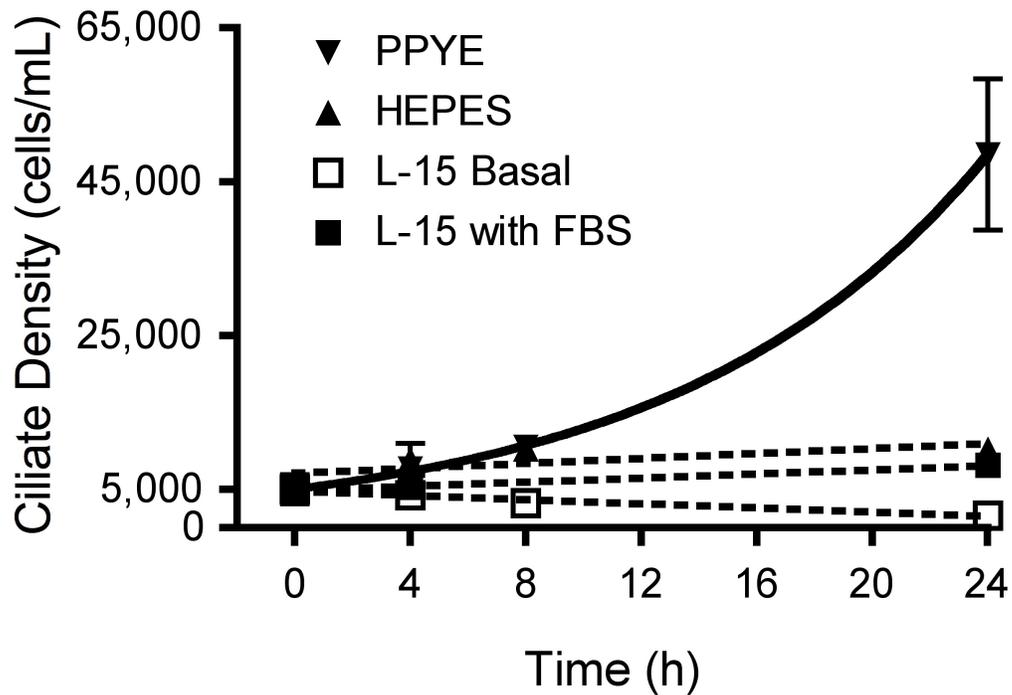


Figure 2.4 Proliferation of *Tetrahymena* in animal medium

Tetrahymena thermophila B1975 were maintained overnight in PPYE then moved directly to either 10 mM HEPES (▲), L-15 basal (□), or L-15 with FBS (■). Cultures were initiated at 5,000 cells/mL, and maintained at 22 °C. A control sample was also transferred to PPYE (▼). Proliferation was assessed using an electronic particle counter, over 24 hours at 22 °C. Data points represent mean cell number per mL, with standard deviation (n = 3). Nonlinear regression, with exponential growth model shown for PPYE ($R^2=0.9497$). Linear regression shown for HEPES, L-15 basal, and L-15 with FBS ($R^2=0.4688$; $R^2=0.9600$; $R^2=0.9953$, respectively).

2.3.3 Effect of FBS on the killing of ciliates by hyperosmotic PPYE and HEPES

The effect of FBS on *Tetrahymena* was evaluated over 24 h in two other media (PPYE and 10 mM HEPES) made hyperosmotic by the addition of NaCl. When ciliates were put in PPYE/0.7 % NaCl either with (337 ± 6 mOsm/kg H₂O, n = 3) or without FBS (318 ± 1 mOsm/kg H₂O, n = 3), the cultures with FBS did better over the next 24 h. With FBS, most ciliates at 3 h had some motility, although they had become round in both cultures. With FBS, cell number, relative to the starting number, did not decline significantly at 8 h and at 24 h had increased (Figure 2.5B). When ciliates were put in 10 mM HEPES/0.8 % NaCl with (343 ± 6 mOsm/kg H₂O) and without 10 % FBS (308 ± 5 mOsm/kg H₂O), the cultures with FBS had a more normal appearance and motility over the next 24 h. Over the first few hours, the ciliates moved slowly but retained their normal shape. Over 8 h the ciliate numbers did not decline from the starting number as they did without FBS (Figure 2.5B). At 24 h with FBS, the ciliates had normal shapes and motility and had increased in number, whereas without FBS most ciliates were dead. Therefore, FBS appeared to contain nutrients and/or osmolytes necessary for *T. thermophila* B1975 to survive transfer to hyperosmotic media.

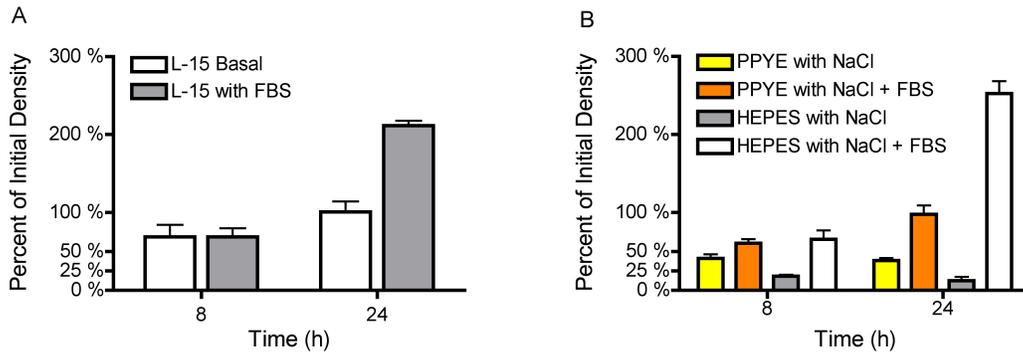


Figure 2.5 Protection of *Tetrahymena* from hyperosmotic shock by FBS

Serum was added to ciliates that were transferred to either animal cell culture medium (**A**) or NaCl-supplemented media (**B**), to assess the effect on cell proliferation. Cultures were initiated at 25,000 cells/mL, and maintained at 22 °C. Cell density, expressed as the percent of the respective starting density for each sample, is plotted on the graph as the mean with standard deviation (n = 3). For ciliates in L-15 with FBS, there was no significant difference in density compared to L-15 basal found after 8 h ($p > 0.05$; unpaired t-test). FBS had a protective effect for ciliates in supplemented PPYE or HEPES, as FBS-supplemented cell densities were greater than FBS-free data for each respective base medium ($p < 0.05$; unpaired t-test).

2.3.4 Ability of sera to support ciliate growth in normosmotic PPYE and HEPES

As FBS could promote survival in hyperosmotic medium by supporting growth, the ability of sera to stimulate ciliate growth was investigated in normal growth medium and in starvation buffer. A supplement of 10 % FBS increased the number of cells accumulating over 24 h in cultures with PPYE, PP and YE (Figure 2.6). This suggests FBS can aid growth by providing supplemental factors at least. As a result, the ability of FBS to support ciliate growth was examined further in a starvation buffer, 10 mM HEPES. When washed twice and placed in HEPES alone, ciliate number remained largely unchanged over 24 h. A brief survey was done to see whether commercial sera from mammals and birds would support *Tetrahymena* growth in HEPES. In 10 mM HEPES with sera at 10 % (v/v) from rabbit, pig, horse, and chicken, the ciliates increased over 24 h in number from 25,000 cells/mL to over 50,000 cells/mL (Figure 2.7). With 10 % FBS, HEPES supported over 24 h a 2.7 ± 0.3 (\pm SD; n = 6) fold increase in the number of ciliates. With a serum supplement of 10 % dFBS (10,000 MW cutoff), HEPES supported only a 2.1 ± 0.2 (\pm SD; n = 9) fold increase in ciliate number, significantly less than the undialyzed FBS ($p < 0.01$; one-way ANOVA with Bonferroni Multiple Comparisons test). These results suggest that both low and high molecular-weight components in FBS support growth of *T. thermophila* B1975 in normosmotic media.

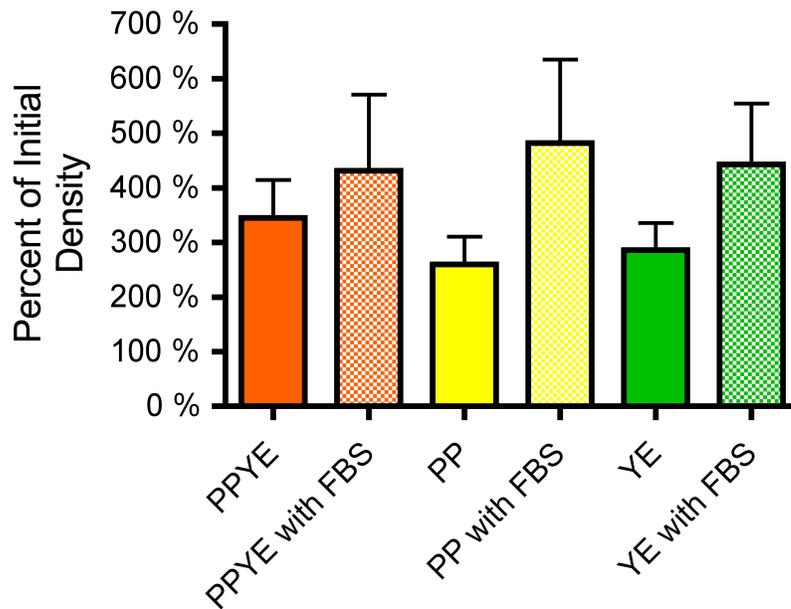


Figure 2.6 Supporting *Tetrahymena* proliferation in normosmotic nutrient-rich media with FBS

Cell proliferation was measured for ciliates in complete growth medium, PPYE, with the presence of FBS after 24 h at 22 °C. Reduced-nutrient media, made from either proteose peptone (PP) alone or yeast extract (YE) alone, were also assessed for increased proliferation with FBS-supplementation. Addition of 10 % FBS significantly increased proliferation compared to FBS-free media in PP and YE media ($p < 0.05$; unpaired t test), but not complete PPYE ($p > 0.05$; unpaired t test). Initial ciliate density was 25,000 cells/mL, and data are presented as the percent of initial density and plotted as the mean, with standard deviation ($n = 6$).

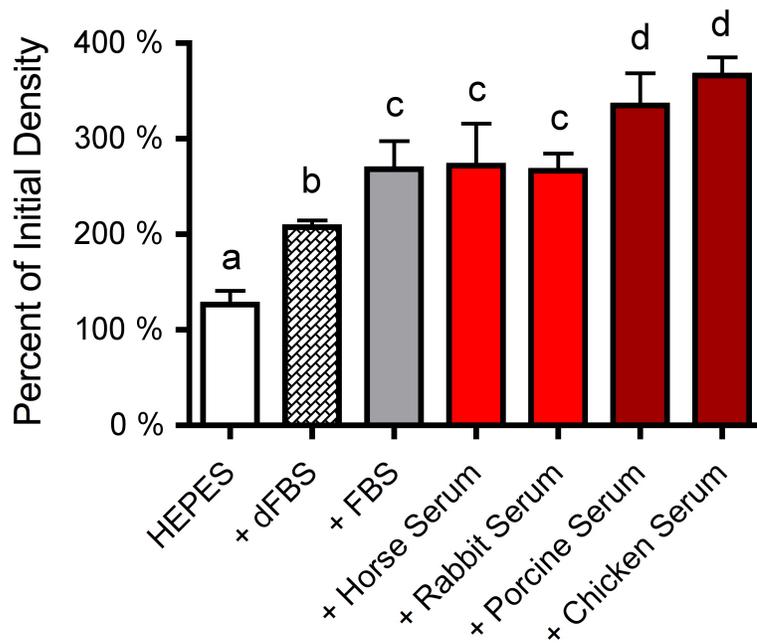


Figure 2.7 Alternative serum supplements for promoting *Tetrahymena* proliferation

Sera from a variety of animal sources were added to 10 mM HEPES to assess their capabilities to promote ciliate proliferation. Dialyzed FBS (dFBS; 10,000 MW cutoff) was also assessed for promotion of proliferation. All sera were added to HEPES at a final concentration of 10 %. Cultures were initiated at 25,000 cells/mL, and cell density was measured after 24 h at 22 °C. Data are presented as the percent of initial density, and plotted as the mean, with standard deviation (n = 6; n = 10 for dFBS). Bars having the same letters are statistically similar ($p > 0.05$; One-way ANOVA with Bonferroni Multiple Comparisons test).

2.3.5 Acclimating *T. thermophila* B1975 to grow in L-15 with FBS from a low cell density

At even a low starting cell density (5,000 cells/mL), ciliates could be adapted to grow in L-15 with FBS. This was done by prior growth of B1975 (wild type) to mid or late logarithmic phase for at least 24 h either in PPYE with 10 % FBS (PPYE/FBS) or in a mixture of PPYE and L-15 (PPYE/L-15), which is referred to as acclimation, and initiating cultures in L-15 or L-15/FBS at 5,000 ciliates/mL. Without an acclimation regimen, ciliate number did not increase over time. This was observed whether the ciliates were in L-15 or L-15/FBS at either 22 °C or 30 °C as illustrated in Figures 2.8A and 2.8B (white bars). With an acclimation regimen, ciliate number did increase as illustrated in figures 2.8A and 2.8B (dark bars). The ciliates grew in L-15/FBS at either 22 °C (Figure 2.8A) or 30 °C (Figure 2.8B). Therefore, even at a low density, wild type *T. thermophila* B1975 were capable of acclimating and growing in L-15/FBS.

2.3.6 Acclimating *T. thermophila* NP1 to grow in L-15 with FBS from a low cell density

A similar acclimation regimen also was used with the mutant *T. thermophila* NP1, which phagocytizes and forms food vacuoles at temperatures below 30 °C, but not at 30 °C to 37 °C. After being grown to mid or late logarithmic phase in PPYE/L-15 at either 22 °C or 30 °C, NP1 was incubated in L-15 or L-15/FBS at 22 °C or 30 °C. Without acclimation, ciliate number increased only in L-15/FBS at 30 °C (Figure 2.8D). With acclimation, NP1 proliferated in L-15/FBS at both temperatures (Figures 2.8C and 2.8D), but at 30 °C the increase was the same as without acclimation (Figure 2.8D). This suggests that the lack of phagocytosis appeared to help ciliates survive and proliferate upon being switched immediately to L-15/FBS. By contrast after a period of acclimation, ciliates without phagocytosis gained no advantage for growth in L-15/FBS. Overall the results suggest that the ciliates can grow in L-15/FBS without phagocytosis.

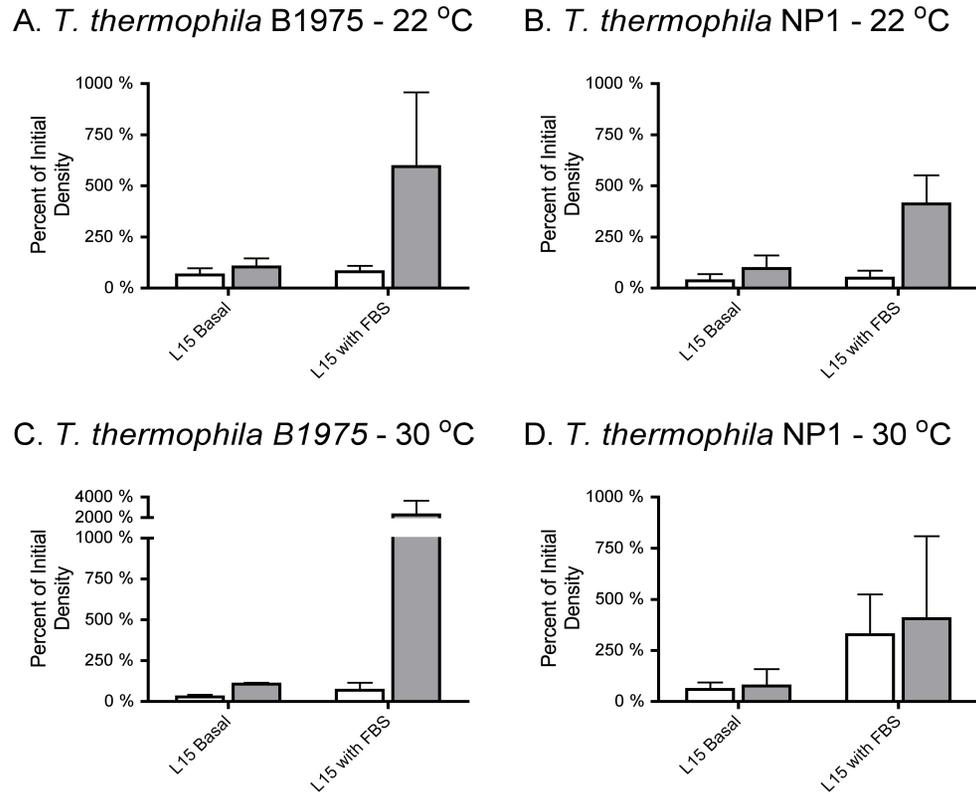


Figure 2.8 Comparing acclimation to direct transfer of ciliates to hyperosmotic media

Ciliates were transferred from PPYE overnight culture (**white bars**), or acclimated for 24 hours in PPYE/L-15 (**grey bars**) prior to movement into hyperosmotic media. Cultures were initiated at 5,000 cells/mL. After 24 hours in either L-15 or L-15 with FBS, cell number was determined. Panels **A** and **C** represent proliferation of *T. thermophila* B1975 at either 22 or 30 °C, respectively. Panels **B** and **D** represent proliferation of *T. thermophila* NP1 at either 22 or 30 °C, respectively. Data are presented as percent of initial density, with standard deviation (n = 3 or greater).

2.3.7 Phagocytosis and bacterivory in L-15 with FBS

Tetrahymena thermophila B1975 maintained at 22 °C in L-15 or grown in L-15 with FBS were still capable of phagocytosis and bacterivory. Within minutes of the ciliates being transferred to L-15 with or without FBS or after 24 h in L-15 with or without FBS, fluorescent beads (1 µm) were added to the cultures. Ten minutes later, greater than 90% of the ciliates had accumulated beads in phagosomes. Likewise, in these media the ciliates engulfed *E. coli* XL-1 (*pET-gfp*), which expressed green fluorescent protein (GFP). Culturing the ciliates and the bacteria together brought about declines in the fluorescence of the cultures over 2 h (Figure 2.9), indicating that the bacteria were being eaten and digested, which is bacterivory. When moved directly to hyperosmotic media, the ciliates showed only short-term decreases in their bacterivory compared to ciliates in PPYE, and by the end of the 2 h period had reduced bacterial fluorescence similarly to those in PPYE. Therefore, *T. thermophila* was capable of performing a characteristic physiological activity of ciliates in L-15/FBS.

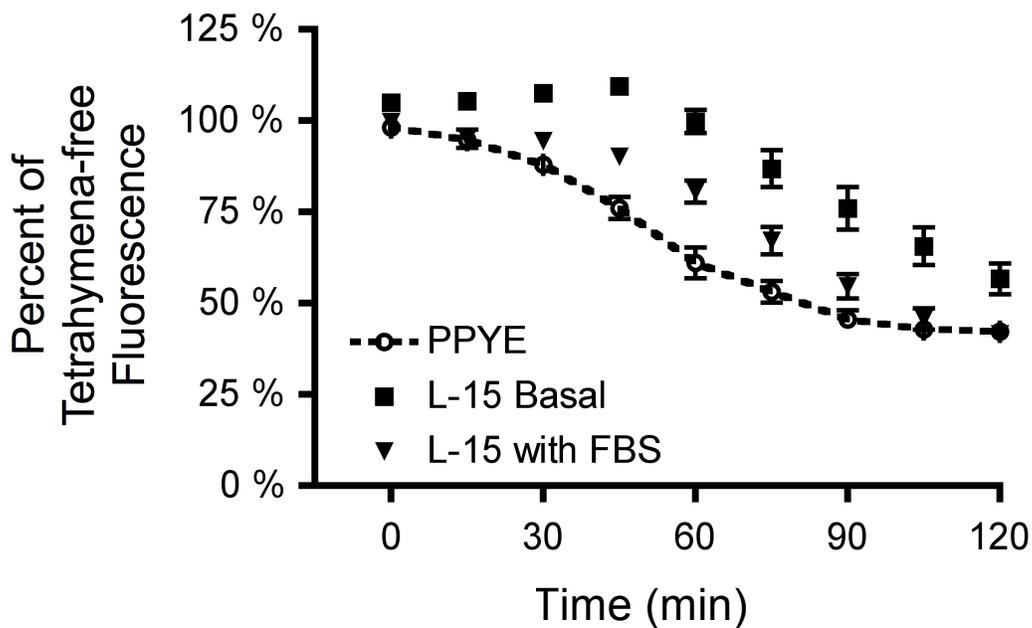


Figure 2.9 Varying rates of bacterivory in hyperosmotic media

Tetrahymena thermophila B1975 were combined with *gfp*-expressing *E. coli*, in PPYE (○), L-15 basal (■), or L-15 + FBS (▼) and change in fluorescence was assessed over 2 hours. Prior to adding bacteria, ciliates were transferred directly from PPYE. Data are normalized to the *Tetrahymena*-free value for medium, and presented as the percent of the initial value. Data points represent the mean of three independent trials, each including 6 duplicate wells. Error bars indicate standard error.

2.4 Discussion

This is the first report of ciliate viability being impaired by a shift to a hyperosmotic medium that is similar in composition to the interstitial or tissue fluids of vertebrates. Previously, high concentrations of NaCl in water or simple salt solutions were shown to inhibit swimming, phagocytosis, and proliferation and to distort morphology and eventually kill ciliates (Dunham, 1964; St Denis et al., 2010). In this study, a basal medium developed for mammalian cells was used. This was L-15, which like most basal media for mammalian cells, was developed to reflect interstitial fluids and has an osmolality of approximately 300 mOsm/kg H₂O and is composed of salts, amino acids, hexose, and vitamins (Ham, 1984). L-15 caused abnormalities in the shape and swimming of *Tetrahymena thermophila* and a loss of viability. However, these effects could be ameliorated by adding fetal bovine serum (FBS) to the L-15 and by transferring at high ciliate density.

How FBS and high ciliate density made the immediate transition of ciliates to hyperosmotic L-15 more successful is unclear at this time, but both could be contributing restorative osmolytes. The responses to hyperosmotic medium are much better studied for the cells of vertebrates (Burg et al., 2007). The cells shrink, followed by either cell death or a regulatory volume increase (RVI) and cell survival. For RVI, transporters are activated for the rapid uptake of inorganic osmolytes such as K⁺ and Cl⁻ and of organic osmolytes such as inositol and α -glycerophosphorylcholine and an influx of water to restore volume (Burg et al., 2007). FBS contains osmolytes (Griffiths, 1987; Honn et al., 1975), as do ciliates (Stock et al., 2002). Osmolytes might be released by the ciliates and their levels would be higher in medium in which the density of the ciliates was higher, so the transition to L-15 was better at high ciliate densities.

Phagocytosis appeared unnecessary for *T. thermophila* to make the switch to L-15/FBS. At 30 °C the phagocytic deficient mutant NP1 made the direct transition to L-15/FBS. In fact, NP1 did so better than wild type B1975. Possibly NP1 and B1975 differ in their uptake of solutes under conditions where NP1 does not undergo phagocytosis, at temperatures above 30 °C. This might cause NP1 to better take up osmolytes that allow the cells to more rapidly recover upon being switched to L-15/FBS. However, once established in L-15/FBS at 30°C,

B1975 proliferated much better than NP1, so phagocytosis appeared to help the ciliates acquire nutrients from L-15/FBS necessary for proliferation.

Bacterivory appeared to be less sensitive to disruption by transfer to L-15 than by transfer to high NaCl, as shown by St Denis et al., (2010). Previously, bacterivory was impaired immediately upon being switched to 0.10 % NaCl (St Denis et al., 2010) but in the current study bacterivory continued in ciliates after being switched to L-15 and to L-15/FBS. For mammalian cells, the responses to NaCl hypertonicity can differ from the responses to the hypertonicity due to organic solutes (Burg et al., 2007). Thus the physiological functions impaired in the ciliates by hyperosmotic solutions might depend on the composition of the hyperosmotic media.

This is the first report of serum from vertebrates supporting the growth of *T. thermophila* in hyperosmotic media, including media with an osmolality level (300 mOsm/kg H₂O) that is normally maintained in the blood and body fluid of mammals and of many fish. Studies on the development of defined media have identified several biochemical components essential for *Tetrahymena* growth (Kidder and Dewey, 1951; Orias et al., 2000). These include nucleosides, trace metals, glucose, vitamins, salts and amino acids. The basal medium, L-15, for piscine and mammalian cells, has these components except nucleosides and trace metals, and the lack of these might account for the inability of this basal medium to support ciliate growth without a supplement of serum. Sera of vertebrates would have all these components (Griffiths, 1987; Honn et al., 1975) and so one of the ways that FBS likely supported ciliate growth in L-15 was by providing these essential low molecular weight components.

However, serum might also be providing polypeptide growth factors. Several polypeptides that are found in mammalian sera have been reported to stimulate the proliferation of ciliates in media with conventional osmolalities for freshwater ciliates (< 100 mOsm/kg H₂O). These include insulin (Christensen et al., 1996; Csaba, 2000) and α 2-macroglobulin (Hosoya et al., 1995). Thus FBS could act in several ways to support *T. thermophila* proliferation and might be essential for growth in hyperosmotic media.

Prior growth in PPYE/L-15 allowed *T. thermophila* to grow better upon being switched to L-15/FBS than ciliates that had been switched directly from PPYE to L-15/FBS. In a preliminary way, other hyperosmotic media were tested as acclimation regimens, but PPYE/L-15 appeared superior (data not shown). The osmolytes, nutrients, and/or growth factors in PPYE might better support the changes needed to acclimate the ciliates for growth in hyperosmotic media in general and/or in L-15 specifically. What these changes are is a matter of speculation. The maintenance of low intracellular concentrations of Na⁺ and Cl⁻ are considered to be the key to salt adaptation by ciliates (Dunham, 1964). Possibly PPYE/L-15 better supports the increased expression of transporters that maintain low intracellular Na⁺ and Cl⁻ concentrations.

This is the first report of *T. thermophila* being able to make the transition to a basal medium (L-15) that has been developed for mammalian cells and to proliferate in this medium when supplemented with mammalian serum (FBS). The results raise questions about the biology of *T. thermophila* and provides new experimental avenues. As serum would form at wound sites of injured fish, which would be sites where ciliates might be able to enter the vertebrate body, and serum helped ciliates to survive the transition to medium that has the composition of vertebrate body fluids, this suggests that *T. thermophila* has the potential to be an opportunistic pathogen of vertebrates. Cell lines originating from frog, fish, and reptiles are routinely grown in L-15 with FBS, and with the ability to maintain *T. thermophila* in L-15, this makes possible the interactions of ciliates and animal cells to be studied in vitro.

Chapter 3

Co-culturing *Tetrahymena thermophila* and fish cell lines to study interactions between ciliates and fish

3.1 Introduction

Ciliates can have symbiotic relationships with fish that vary greatly in complexity (Lom and Dyková, 1992). An example would be movement of ciliates over the surface of fish gills, feeding on desquamated epithelial cells. Other ciliates are described as facultative parasites or opportunistic histophagous pathogens, with the capacity to enter and eat fish tissues in circumstances of host stress or wounding (Paramá et al., 2003). An example of this is scuticocilatoriasis caused by *Philasterides dicentrarchi* (syn. *Miamiensis avidus*). This disease has only become apparent with the development of aquaculture for marine fish and has caused significant losses around the world (Jin et al., 2009). The most complex relationships involve *Cryptocaryon irritans* or *Ichthyophthirius multifiliis*, which are responsible for the economically important “white spot disease” and “ich” in respectively marine and freshwater teleosts (Matthews, 2005). These are obligate parasites, with free-living stages in water and parasitic stages within the fish epidermis.

Although the interaction between ciliates and the cells of multicellular animals is poorly understood, animal cell cultures could provide a convenient *in vitro* platform for studying the interactions and could be a way of maintaining ciliates that are difficult to grow. Indeed this approach has begun to be taken with the obligatory parasites *C. irritans* and *I. multifiliis* because they are difficult to culture by themselves. All the life cycle stages of *C. irritans* developed in an *in vitro* system that consisted of a monolayer of the fathead minnow cell line (FHM) with an overlay of agarose (Yoshinaga et al., 2007). Co-culture of *I. multifiliis* and fish cells has shown progress (Nielsen and Buchmann, 2000) but culture of the complete life cycle *in vitro* has not yet been accomplished. How histophagous ciliates interact with animal cells *in vitro* is unknown. Therefore in this thesis, I report *in vitro* procedures that would

allow ciliates and animal cells to be cultured together and to use the co-cultures to study ciliate/animal cell interactions.

Tetrahymena thermophila was chosen as the species to develop and study ciliate/animal cell co-cultures for several reasons, even though this species has yet to be described as parasitic or histophagous. Firstly, more is probably known about the general cell biology of this ciliate than any other (Asai and Forney, 1999). Secondly *T. thermophila* can be cultured axenically. Thirdly, growth of these ciliates under different conditions has been explored intensively (Christensen et al., 2001; Hellung-Larsen et al., 2000), and recently they have been shown to grow in media that were originally created for mammalian cells (Chapter 2). Fourthly, mutants of *T. thermophila* exist for cellular functions that would be expected to be important in histophagy, such as phagocytosis and mucocyst development (Orias and Pollack, 1975; Orias et al., 1983). Finally, several members of the genus *Tetrahymena* have been found to be parasites of a gastropod mollusk (Brooks, 1968), insects (Lynn et al., 1981) and fish (Ferguson et al., 1987; Imai et al., 2000; Astrofsky et al., 2002; Leibowitz et al., 2005), and one was discovered in the bladder of a dog (Lynn et al., 2000).

This chapter reports findings where *T. thermophila* was placed in co-culture with monolayers of epithelial cell lines from fish and rabbit, and were shown to completely destroy all cell monolayers. The ciliates had to be able to swim into contact with the monolayer to initiate destruction and phagocytosis was required to completely clear the monolayer from cultures.

3.2 Materials and Methods

3.2.1 Ciliates and cell lines

Three strains of *Tetrahymena thermophila*, B1975, NP1, and SB255, were used. *Tetrahymena thermophila* B1975 or wild type were obtained from Dr. D. Lynn (Department of Integrative Biology, University of Guelph, Guelph, ON). The *T. thermophila* strains NP1 and SB255 were acquired from the Tetrahymena Stock Center (Cornell University, Ithaca, NY). Mutant NP1 is a temperature-conditional mutant for oral apparatus development (Orias and Pollock, 1975) and SB255 is a mucocyst-deficient strain (Orias et al., 1983). The ciliates were maintained in PPYE as described previously (Pinheiro et al., 2007). PPYE (protease peptone, yeast extract) consisted of 0.125 % (w/v) dextrose (Sigma-Aldrich, St. Louis, MO), 0.5 % (w/v) proteose peptone (BD Biosciences, Durham, NC) and 0.5 % (w/v) yeast extract (BD Biosciences) dissolved in MilliQ water. Growth was done routinely at 22 °C. However, NP1 is incapable of phagocytosis when grown at ≥ 30 °C, and this is a reversible mutation (Orias and Pollock, 1975). Therefore for experiments to exploit this deficiency, the ciliates were grown for at least 18 h at 37 °C prior to use.

Three piscine cell lines, ZEB2J, RTgill-W1, and CHSE-214, and one mammalian cell line, RK-13, were used (detailed in Table 3.1). ZEB2J and RTgill-W1 were developed in this laboratory and RTgill-W1 has been deposited in the American Type Culture Collection (ATCC, Manassas, VA). ZEB2J originated from zebrafish blastula-stage embryos expressing enhanced green fluorescent protein (GFP) under the constitutive control of the zebrafish β -actin promoter (Xing et al., 2008). RTgill-W1 was derived from the rainbow trout gill (Bols et al., 1994). CHSE-214 is from Chinook salmon embryos (Lannan et al., 1984) and RK-13 is from the rabbit kidney and both were obtained from the ATCC. All cell lines have an epithelial-like morphology. The cell culture supplies were from Sigma-Aldrich, except fetal bovine serum (FBS), which was prepared by Hyclone (Invitrogen, Carlsbad, CA). The basal medium was Leibovitz's L-15, and the complete medium had 10 % FBS, 2 mM L-glutamine, and penicillin (100 IU/mL) / streptomycin (100 μ g/mL). Routine growth was done in 75 cm²

tissue culture flasks (BD Biosciences) in an atmosphere of air. ZEB2J and CHSE-214 were maintained at 18 °C; RK-13 cells, at 37 °C.

Table 3.1 Animal cell lines used to generate monolayer cultures

| Cell Line: | Species of origin | Tissue of Origin: | Cell Morphology: | Incubation Temperature: | Reference: |
|-------------------|---|--------------------------|-------------------------|--------------------------------|--------------------------|
| RTgill-W1 | Rainbow trout (<i>Oncorhynchus mykiss</i>) | Gill | Epithelial | 22 °C | Bols et al, 1994 |
| CHSE-214 | Chinook salmon (<i>Oncorhynchus tshawytscha</i>) | Embryo | Epithelial | 22 °C | Fryer et al., 1965 |
| ZEB2J | Zebrafish (<i>Danio rerio</i>) | Embryo | Epithelial | 26 °C | Xing et al., 2008 |
| RK-13 | Rabbit (<i>Oryctolagus cuniculus</i>) | Kidney | Epithelial | 37 °C | McCarthy et al., 1963 |

3.2.2 General initiation of ciliate/animal cell cultures

The most common vessels for setting up co-cultures were 12-well tissue culture plates, but in some cases 25 cm² flasks and plastic Leighton tubes with coverslips were used (BD Biosciences). In most cases, the cell lines were plated into culture vessels first and allowed to proliferate to completely cover the growth surfaces. For 12-well plates, approximately 5.0×10^5 animal cells were added per well in L-15 with FBS. Usually 1 to 2 days later the growth surface was covered, or had become confluent, at which point the ciliates were added. The ciliates were collected from mid or late logarithmic phase cultures in either PPYE, PPYE with 0.7 % NaCl, PPYE with 10 % FBS or PPYE/L-15 as described in Chapter 2. For 12-well plates, approximately 1,500 ciliates were added per well, although as few as 150 ciliates was tested. The culture temperature depended on the cell line and is mentioned in the Results section.

3.2.3 General monitoring of ciliate/animal cell cultures

Ciliate/animal cell cultures were monitored in several ways. Observations of cultures were made periodically with a phase contrast microscope and continuously through video micrography. For video micrography, a Nikon Coolpix E8400 camera was used, mounted to a Nikon Eclipse TS100 inverted phase contrast microscope using a Nikon Coolpix MDC mount. Videos were recorded at 30 frames per second, in a MOV file format. Video files were combined and labeled, and converted to AVI file formats using Apple iMovie software, but otherwise unmodified. Magnification is indicated for each video. When ZEB2J cells were in the culture, cultures were observed additionally with an inverted Nikon TE300 fluorescence microscope. For counting ciliates in cultures, a Z2 Coulter Particle Counter /Size Analyzer (Coulter, Luton, UK) was used.

3.2.4 Ciliate swimming and monolayer destruction

The importance of motility in the destruction of monolayers by ciliates was tested by co-culturing ciliates and animal cells at low temperature. As *T. thermophila* swam poorly at 14 °C and hardly at all at 4 °C, cultures of *T. thermophila* B1975 on CHSE-214 monolayers

were set up as described in section 3.2.2 and immediately placed at 14 °C and 4 °C and monitored for up to a week.

3.2.5 Necessity for physical contact between ciliates and monolayer

The necessity for physical contact between ciliates and cells of the monolayer for monolayer destruction was studied in 12-well culture plates with culture inserts (BD Biosciences). The inserts were placed into wells to divide each well into an upper chamber and a bottom chamber separated by a barrier with either 0.4 µm or 8.0 µm pores. Monolayers of CHSE-214 were allowed to develop on the growth surface of each well, which was the bottom chamber. Approximately 1,500 ciliates were then added to the top chamber and the monolayer in the bottom chamber was viewed daily with an inverted phase contrast microscope.

3.2.6 Effect of monolayer breaks on development of monolayer destruction

Monolayers were deliberately damaged in order to determine the effect of monolayer integrity on the actions of ciliates in co-cultures. Co-cultures were set up with CHSE-214 as described in section 3.2.5, but just before the addition of ciliates, either a sterile glass Pasteur pipette or a metal inoculation loop was used to damage monolayers. The pipette was drawn or scored across monolayers in approximately straight lines. The metal inoculation loop was heated using a Bunsen burner and touched to the monolayer to create a circular burn. Cultures were observed for up to a week by phase contrast microscopy.

3.2.7 Role of ciliate phagocytosis on animal cell monolayer destruction

Expression of GFP by the fish cell line ZEB2J (Xing et al., 2008), and the *T. thermophila* mutant strain NP1 were used to investigate the role of ciliate phagocytosis on monolayer destruction. ZEB2J cells can be visualized by fluorescence microscopy and were used to determine whether ciliates could engulf fish cells at 22 °C and 4 °C. NP1 is a temperature conditional mutant for oral apparatus development and is incapable of phagocytosis when grown and assayed for phagocytosis at 37 °C (Orias and Pollock, 1975). At 37 °C the rabbit

kidney cell line, RK-13, was used because most fish cell lines die at this temperature (Bols et al., 1992). For these studies, co-cultures of ciliate and animal cells were initiated and monitored as described previously.

3.2.8 Role of soluble cytolytic factor release on animal cell monolayer integrity

The ciliates and dying fish cells were both tested for their ability to release factors that could be damaging or cytolytic to fish cells. Media-conditioned by the ciliates or by ciliates and fish cells were prepared.

L-15 with FBS medium in which 100,000 ciliates/mL had been maintained for 24 h was collected, syringe filtered (0.2 µm pore size; Pall Corporation, Ann Arbor, MI), and added to monolayer cultures of either CHSE-214 or RTgill-W1 in 12-well or 96-well plates. The monolayers were examined with an inverted phase contrast microscope for changes in appearance and signs of cell death. For the 96-well plates, the cell viability was monitored 24 h later with two indicator dyes, alamar blue (Medicorp. Science Inc., Montreal, PQ) and 5'-carboxyfluorescein diacetate acetoxyethyl ester (CFDA-AM; Molecular Probes, Eugene, OR). These were used as described in detail by Dayeh et al, (2003). Relative fluorescence values were measured with a CytoFluor Series 4000 microplate reader (Applied Biosystems, Foster City, CA) at excitation and emission wavelengths of 530 nm / 590 nm for alamar blue, and 485 nm / 530 nm for CFDA-AM. The data from three trials were collected and are plotted as mean with standard deviation (n = 18), and an unpaired t test was used to compare the mean fluorescence from monolayers in conditioned versus control media.

In order to generate medium that possibly had accumulated cytolytic factors from dying fish cells, *T. thermophila* B1975 was cultured on monolayers of CHSE-214 until approximately 25 % of each monolayer was destroyed. The media from these co-cultures was collected, syringe filtered (0.2 µm pore size), and added to confluent monolayers of CHSE-214. The monolayers were examined with an inverted phase contrast microscope for changes in appearance and signs of cell death.

A final approach for investigating whether soluble cytolytic factors had a role in monolayer destruction was to determine whether the mutant *T. thermophila* SB255, which

has a reduced number of secretory mucocysts (Orias et al., 1983) and is expected to secrete less material into the medium, had a reduced capacity to destroy fish cell monolayers.

3.2.9 Monolayer recovery from ciliate actions

Recovery of a monolayer from ciliate-mediated damage was assessed by growing a confluent monolayer of CHSE-214 cells on a plastic cover slip within a Leighton tube (Sigma-Aldrich) and adding *T. thermophila* B1975 (1,500 ciliates/mL). After approximately 25 % of the monolayer was destroyed (referred to as phase 2 damage), the cover slip was removed from the Leighton tube and rinsed by immersing in sterile L-15 with 10 % FBS and 1 % pen/strep, three times, to remove ciliates. Cover slips were placed inside a sterile Leighton tube and fresh growth media was added. Areas of monolayer damage were observed for changes to morphology and signs of reattachment using an inverted microscope.

3.2.10 Proliferation of ciliates on CHSE-214 monolayers

Live and dried CHSE-214 monolayers in 25 cm² culture flasks were tested for their ability to support the proliferation of *T. thermophila* B1975. For dried monolayers, confluent cultures were rinsed with phosphate buffered saline and then allowed to dry at room temperature; these monolayers are referred to as “dead”. A control flask for the dead monolayer was a flask in which no cells had been grown but which had been incubated with L-15 with FBS. Each flask was inoculated with approximately 15,000 ciliates in 10 mL of L-15 with FBS for the living monolayer, to ensure survival of the animal cells, and in a balanced simple salt solution without nutrients, Osterhout’s solution (Osterhout, 1906) for the dead monolayer and incubated at 22 °C. After 72 h, ciliate number was counted again and the percentage of initial density after this time was calculated. This change in cell number, expressed as the percentage of initial density [$100 \times (\text{density at 72 hours} / \text{initial density})$], was graphed, and represents the mean of three trials with standard error. The increase in cell number from initial density after 72 hours ($\text{density at 72 hours} - \text{initial density}$) was also calculated for each treatment, and these data were compared between *T. thermophila* maintained in media only and those maintained alongside a confluent monolayer of CHSE-214. Data were analyzed for significant variation between ciliate proliferation in media alone

and alongside CHSE-214 cells using unpaired t tests, with GraphPad Prism software (v4, GraphPad Software).

3.3 Results

3.3.1 General observations of ciliates behaviour on fish cell cultures

When *T. thermophila* was cultured on monolayers of fish epithelial cell lines, the ciliates continued to swim and proliferate, whereas the monolayers died, being completely consumed over several days. Different numbers of ciliates were added to monolayers in L-15 with or without FBS after the *T. thermophila* had been grown in either PPYE, PPYE with NaCl, or in PPYE/L-15. Adding as few as 150 ciliates to a monolayer in L-15 with FBS within a culture well of a 12-well plate destroyed the monolayer and adding more ciliates generally sped up destruction. Ciliates that had been grown in PPYE with either NaCl or L-15, destroyed monolayers more quickly than ciliates that had been grown in PPYE alone, with similar numbers of ciliates added initially. When the monolayer was in L-15 alone, destruction was delayed for up to a week. Monolayer destruction was explored in more detail under standard conditions: 1,500 *T. thermophila* on a monolayer of approximately 5×10^5 fish cells with 1 ml of L-15 with FBS per well of a 12-well plate (Table 3.2). Monolayer destruction was divided into disruptive, destructive, and clearing phases.

3.3.2 Disruptive phase

During the first 24 to 36 h at 22 °C, the swimming of many ciliates changed from movement throughout the entire volume of the medium to gliding over the surface of the monolayer and the monolayer began to show small breaks (Figure 3.1A). The damage appeared to be caused by groups of ciliates repeatedly bumping or pushing their anterior end into the monolayer (video available as Appendix A). Although each collision between a ciliate and the monolayer was short lived, these kinds of physical contacts appeared necessary for the small breaks to develop and necessary for the development of the next stage or phase in the destruction of fish cell cultures.

3.3.3 Destructive phase

The next step was the expansion of the small disruptions to about 90 % demolition of the monolayer, and is referred to as the destructive phase. Large groups of ciliates aggregated and hovered around spots of damaged monolayer (Figure 3.1B,C). Many ciliates became bloated and filled with phase-dark vacuoles. They had a tendency to swim slowly along the surface of the monolayer and are referred to as grazers. At the end of the destructive phase, which took approximately 3 days at 22 °C, a monolayer no longer existed but single cells and patches of cells remained attached to the plastic growth surface, and intact and broken cells floated in the medium.

3.3.4 Clearing phase

The final step in the destruction of the animal cell cultures was the clearance of all visible signs of animal cells, either as a monolayer on the culture surface or in suspension as dislodged cells (Figure 3.1D). In this phase, ciliates appeared more numerous by microscopy and contained many phase-bright vacuoles (Figure 3.1E). At 22 °C clearance was achieved between 3 and 4 days. When the ciliates either in L-15 with FBS or in a simple salt solution were added to a dried monolayer of CHSE-214 cells, the ciliates were able to clear the cultures of all cells and cellular debris. Therefore ciliates could clear both living and dead animal cell monolayers.

Table 3.2 Summary of monolayer destruction by multiple strains of *T. thermophila*.

| | | <i>Tetrahymena thermophila</i> strain | | | | | |
|------------|-------------------------|---------------------------------------|---------------------------------|------------------------|---------------------------------|------------------------|--|
| | | B1975 | | SB255 | | NP-1 | |
| Cell Line: | Incubation Temperature: | Monolayer Cleared (h): | Ciliate Morphology / Behaviour: | Monolayer Cleared (h): | Ciliate Morphology / Behaviour: | Monolayer Cleared (h): | Ciliate Morphology / Behaviour: |
| CHSE-214 | 22 °C | 72 | Grazers | 72 | Grazers | 72 | Grazers |
| RK-13 | 37 °C | 144 | Grazers | n.d. ^a | n.d. | Not cleared | Mix of swimmers and grazers ^b |

^a No data.

^b Grazers typified by increased cell size compared to ciliates in growth medium (~3-4 times increase), a morphology bloated by phase-dark vacuoles, slow motility, and the tendency to swim along the surface of the monolayer. Swimmers swim through the entire volume of media without a noticeable attraction to the monolayer surface.

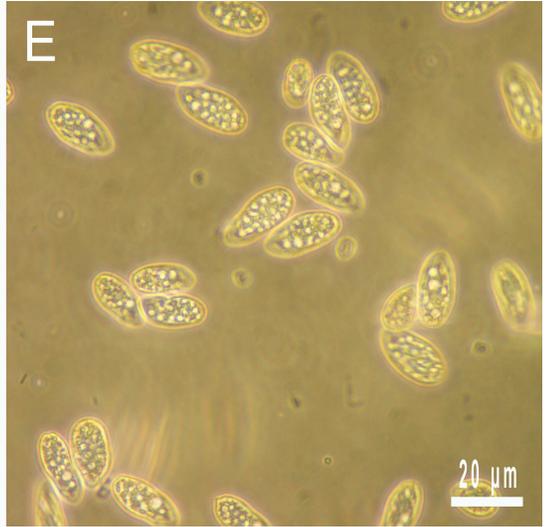
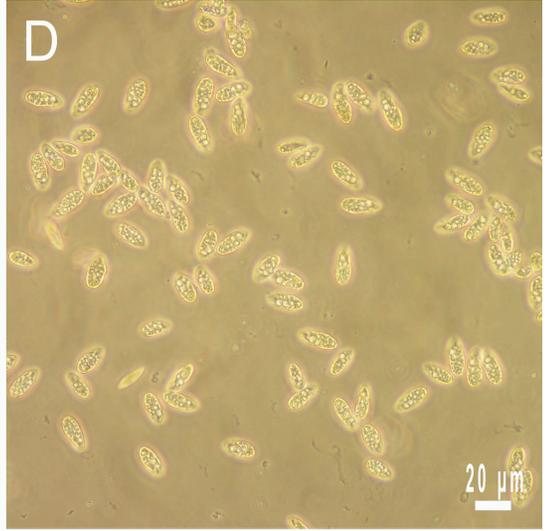
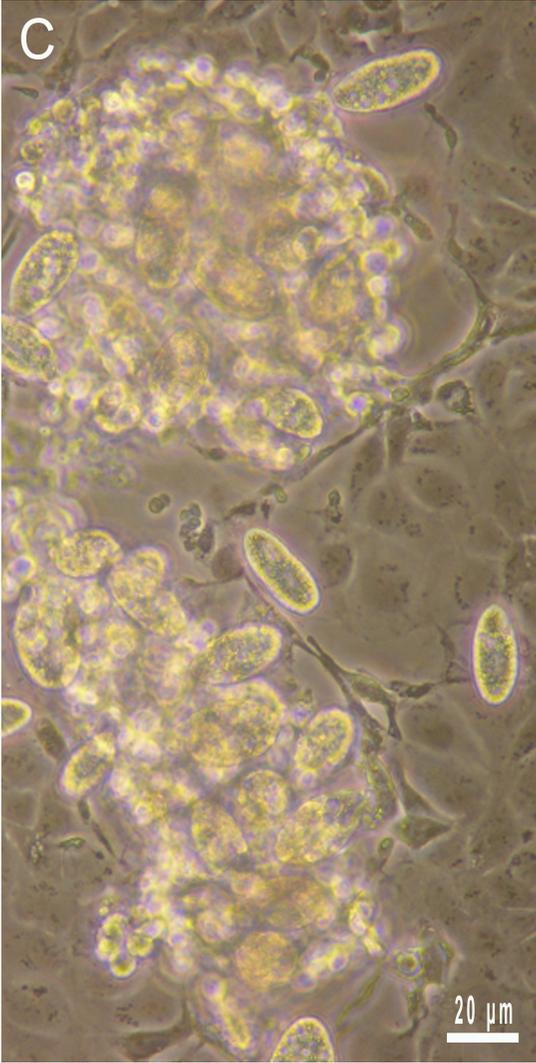
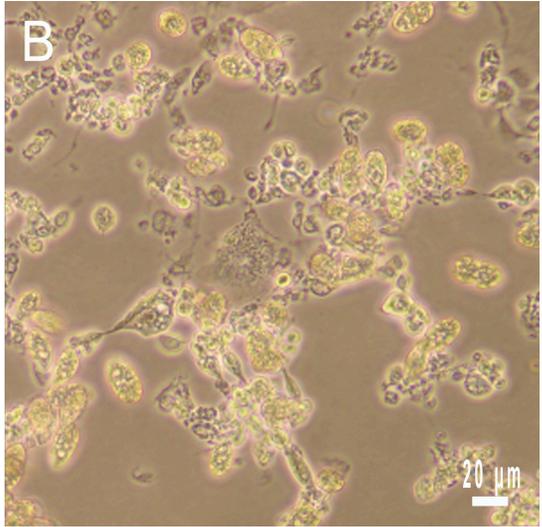
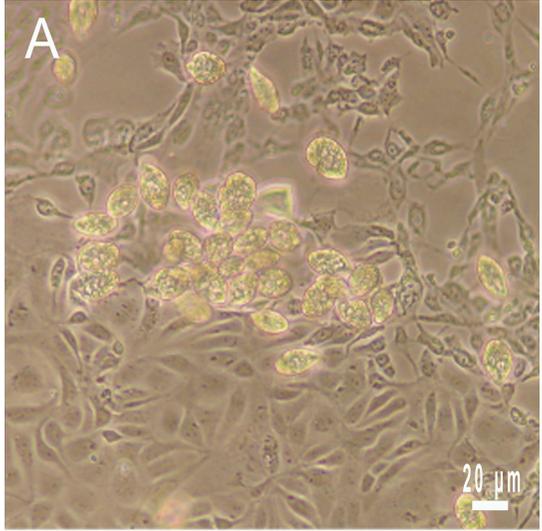


Figure 3.1 Phases of ciliate-borne damage to CHSE-214 cell monolayer

Phase 1 damage (**A**), where only small breaks could be seen in the monolayer, exposed the smooth growth surface below. This occurred for *T. thermophila* in CHSE-214 prior to 24 hours of exposure to ciliates. Phase 2 damage (**B**) was considered when the majority of the monolayer had been destroyed. During this phase, small areas of intact fish cells were surrounded by string-like remnants of destroyed cells. This occurred for CHSE-214 before 72 hours. Aggregates of ciliates could be seen surrounding areas of damaged fish cells during phase 1 and 2 damage (**C**). Ciliates seemed quite active, but appeared to spin within the aggregation of ciliates and swim forward and backward slowly, without ever leaving this area; contrary to long, straight swim paths when damage was absent. At 72 hours, following monolayer destruction, no cellular remains of animal cells are visible (**D**). Remaining ciliates swim actively and contain many phase-bright vacuoles (**E**).

3.3.5 Ciliate motility and monolayer destruction

Incubating cultures at 14 °C, slowed ciliate swimming and the development of monolayer destruction. When *T. thermophila* B1975 was added to CHSE-214 at 14 °C, the time for the initial small breaks in the monolayer to appear was greatly increased (first breaks in the monolayer occurring after 72 hours), and the monolayer was only partially destroyed (~ 50% clearance) by 144 h. This was compared to complete clearance by 72 h at 22 °C. When co-cultures were placed at 4 °C, the ciliates were very sluggish, and hardly swam. These ciliates did not probe the monolayer and minor breaks in the monolayer did not develop. Therefore, the minor monolayer breaks appeared to be initiated by direct physical contact with the ciliates, brought about by ciliate's vigorous swimming.

3.3.6 Necessity for physical contact between ciliates and monolayer

When CHSE-214 monolayers were co-cultured with the ciliates but physically separated by porous culture inserts, the CHSE-214 monolayers remained intact if the pore size was 0.4 µm but ultimately deteriorated if the pore size was 8.0 µm. With 0.4 µm pore-size inserts, the ciliates remained in the top chamber of the co-culture and the monolayer remained intact and normal in appearance in the bottom chamber. With 8.0 µm pore-size inserts, the ciliates remained in the top chamber of the co-culture and the monolayer remained intact for several days but by 4 days some ciliates had moved to the bottom chamber and the monolayer began to develop small breaks that over several more days expanded until the monolayer was destroyed. These results suggest that *T. thermophila* B1975 initiated monolayer destruction by making physical contact with the cells.

3.3.7 Effect of monolayer breaks on development of monolayer destruction

Artificially making breaks in a CHSE-214 monolayer prior to adding the ciliates resulted in the ciliates concentrating along the edge of lesions (Figure 3.2A) and in monolayer destruction being sped up. The monolayer breaks were made with either a sterile Pasteur pipette tip or a flamed inoculating loop, and if ciliates were not added, the monolayers reformed. With either damaging treatments, many more ciliates were seen “grazing” in the

lesion areas relative to areas of intact monolayer (Figure 3.2B). The creation of lesions caused the ciliates to completely clear monolayer cultures approximately 24 h earlier.

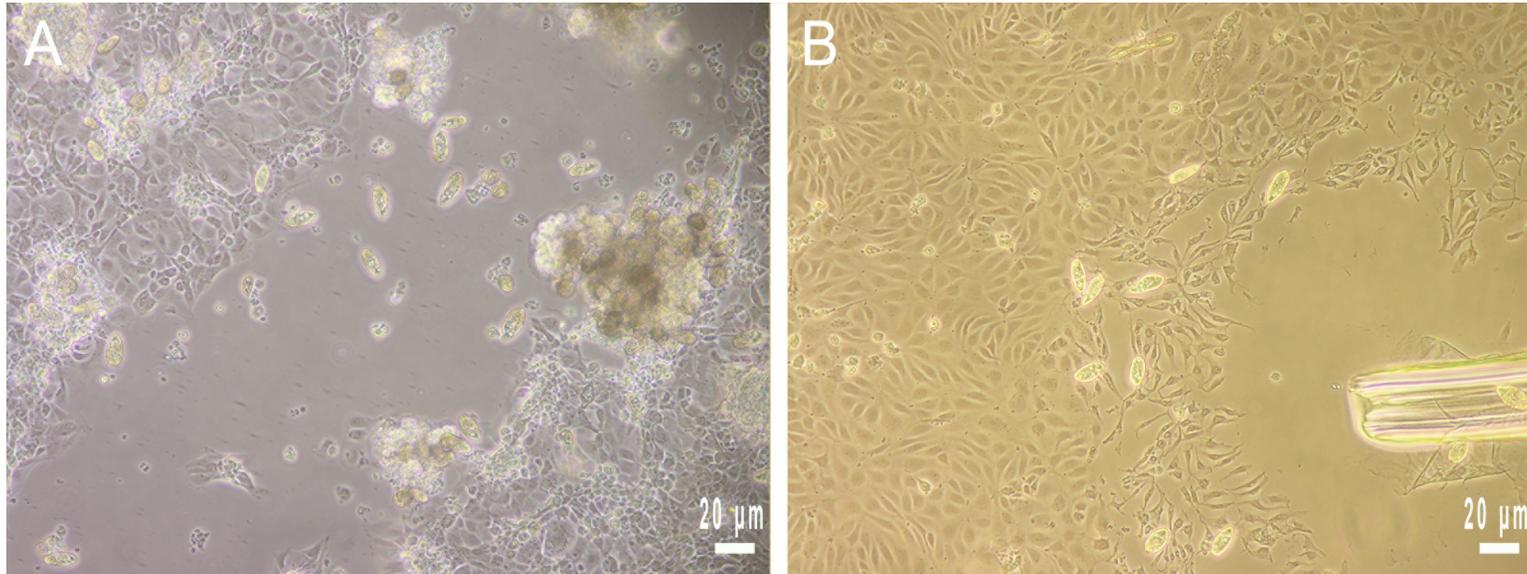


Figure 3.2 Reaction to cellular disruption by *T. thermophila* B1975

Ciliates in the presence of fish cell monolayers that were disrupted, either by physical scoring with a glass pipette (A) or burning (B), were more commonly found near areas of damage, than in undamaged areas. Within one hour following ciliate inoculation, many more *T. thermophila* could be seen in the immediate vicinity of damaged areas, with slower swimming speeds and many turns, as compared to non-damaged areas of a CHSE-214 monolayer. Observations were conducted using an inverted phase microscope.

3.3.8 Ciliate phagocytosis and the disruptive phase

Phagocytosis by ciliates was essential for the ciliates to destroy cell monolayers. This was illustrated with *T. thermophila* mutant strain NP1, which phagocytoses at temperatures below 30 °C but not at 37 °C. At 18 °C, NP1 destroyed monolayer cultures of CHSE-214 in the same manner and timeframe as the wild type B1975. When NP1 and B1975 were grown for 24 h at 37 °C and then added to monolayer cultures of the rabbit kidney cell line, RK-13, at 37 °C, the monolayers were destroyed over 6 days by B1975 but not by NP1. NP1 swam in RK-13 cultures at 37 °C and after 24 h about 50 % of the ciliates grazed over the monolayer. For wildtype B1975, this grazing behaviour had appeared to dislodge or loosen cells in a monolayer, but for NP1 at 37 °C this behaviour was insufficient for monolayer destruction. These results suggest that concurrent cell dislodgement and phagocytosis by the ciliates were needed to initiate monolayer obliteration and continue onto the destructive and clearance phases.

3.3.9 Ciliate phagocytosis and the destructive and clearance phases

Tetrahymena thermophila was able to phagocytose fish cells at 18 and 22 °C but not at 4 °C. The fish cells were ZEB2J, which fluoresce because they express GFP. Cellular debris from disrupted ZEB2J also continued for a time to fluoresce, although less intensely. When added to a monolayer culture of ZEB2J or to a suspension of ZEB2J, ciliates at 18-22 °C were seen 24 h later to have engulfed fluorescent material, which had the localization and the approximate size of food vacuoles (Figure 3.3). When incubated with disrupted ZEB2J cells, the ciliates acquired less intensely fluorescent vesicles. Thus *T. thermophila* appear to have the capacity to phagocytose cultured cells and their debris.

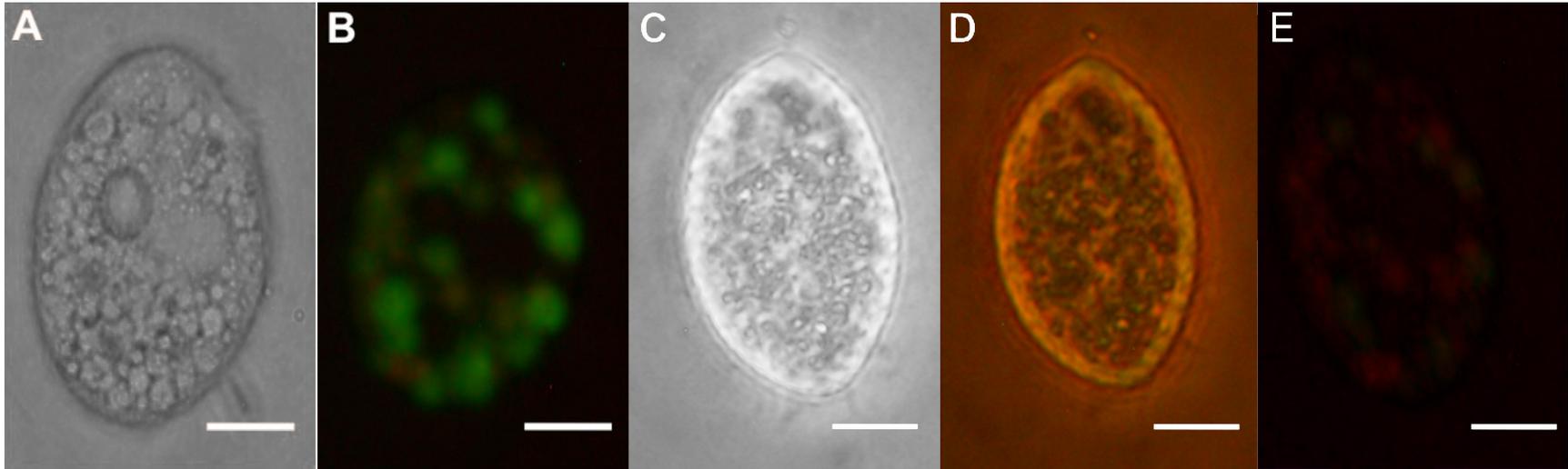


Figure 3.3 *Tetrahymena thermophila* added to ZEB2J monolayers contained engulfed fluorescent fish cell remains

When maintained alongside the GFP-expressing ZEB2J cell line at 22 °C, *T. thermophila* B1975 caused significant damage to the monolayer and by 24 h could be seen to contain fluorescent material in cellular compartments (A, B). These had the size and shape of food vacuoles. When maintained at 4 °C for 24 h, no fluorescent material could be seen inside the ciliates (C, D, E). Pictures were taken of fixed cells using an inverted, fluorescence microscope with panels B, D and E using a B-2A filter (excitation, 450 nm; emission, 520 nm); E is (very faintly) illuminated. Scale bar is 10 μ m.

3.3.10 Lack of a role for soluble cytolytic factor release in monolayer destruction

The addition of medium conditioned by ciliates alone or by ciliates and dying fish cells to monolayers of fish epithelial cells had no effect on the viability of the fish cells. Monolayers of CHSE-214 and RTgill-W1 in ciliate-conditioned L-15 with FBS remained adherent with their characteristic epithelial-like shape for at least 7 days. When RTgill-W1 cultures were evaluated for cell viability with the indicator dyes, alamar blue for metabolic activity, and CFDA-AM (Figure 3.4), for plasma membrane integrity, no differences were seen between cultures in ciliate-conditioned medium for 24 h and cultures in control medium, as judged by unpaired t test ($p > 0.05$). Medium from ciliate/CHSE-214 co-cultures in which approximately 25 % of the CHSE-214 had been destroyed by the ciliates also had no effect when applied to intact monolayer cultures of CHSE-214 and observed for up to a week.

The mutant *T. thermophila* SB255, which has few mucocytes (Orias et al., 1983) and is expected to secrete little material into the medium, still destroyed CHSE-215 monolayers over the same time frame as the wildtype *T. thermophila* B1975. This suggests secreted material has a minor or no role in monolayer destruction.

3.3.11 Recovery from the disruptive and destructive phases

When co-cultures of *T. thermophila* B1975 and CHSE-214 were incubated until minor or major breaks in the CHSE-214 monolayer had developed and then the ciliates were removed, the monolayers recovered. Recovery was possible as long as approximately 10 % of the monolayer still remained when incubation with the ciliates was terminated.

3.3.12 Proliferation of ciliates on CHSE-214 monolayers

Monolayers of CHSE-214 supported the growth of *T. thermophila* B1975. Ciliates added to living monolayers in L-15 with FBS proliferated much more than in just L-15 with FBS (Figure 3.5). After 72 h ciliate number was nearly ten times greater in cultures with CHSE-214 than in L-15/FBS alone. When applied to a dried monolayer of CHSE-214 to which a simple salt solution had been added, the ciliates cleared the culture of all signs of cellular

debris and increased in number (Figure 3.6). Therefore fish cells could supply all the nutrients necessary to support the proliferation of *T. thermophila* B1975.

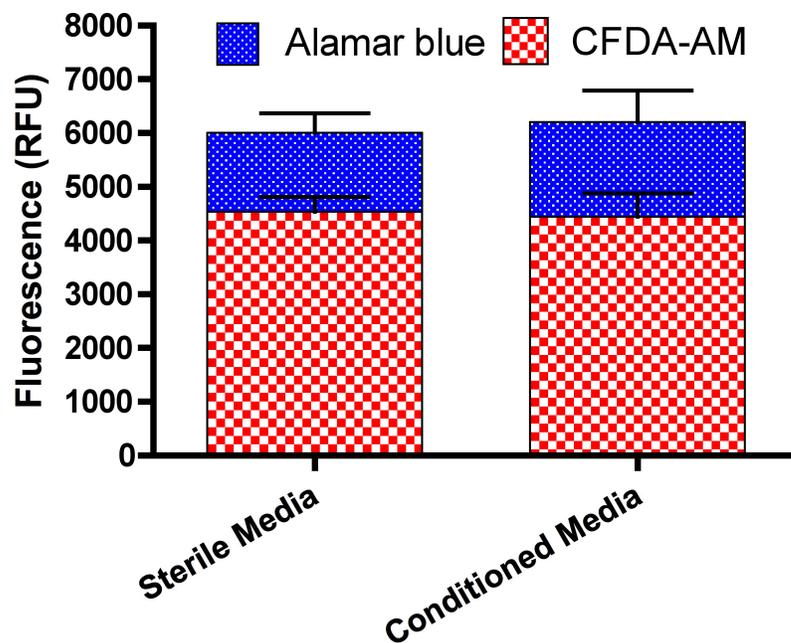


Figure 3.4 Effect of medium conditioned by ciliates on the viability of RTgill-W1 cells

RTgill-W1 cultures in 96 well plates were exposed to conditioned medium for 24 h before being evaluated for viability with alamar Blue and CFDA-AM, which measure metabolic activity and membrane integrity, respectively. There was no significant difference between unconditioned medium and conditioned medium fluorescence value, for either dye determined by t test ($p > 0.05$). Fluorescence was measured at 530 nm excitation and 590 nm emission for alamar Blue, and 485 nm excitation and 530 nm emission for CFDA-AM. Bars represent the plotted mean of these data (\pm SD; $n = 18$).

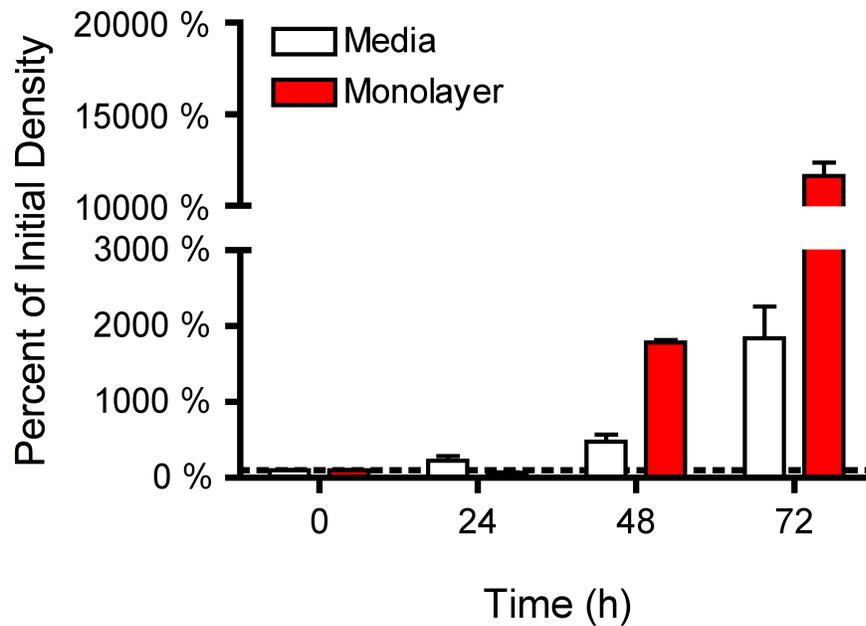


Figure 3.5 Ciliate proliferation in animal cell co-culture

Tetrahymena thermophila were added to either cell culture medium (L-15 + 10 % FBS with antibiotics) alone, or to confluent monolayers of CHSE-214 in medium. Initial density of ciliates was approximately 1,500 cells/mL, and cell counts were conducted every 24 hours for 72 hours. Unfilled bars represent ciliates grown in medium alone, and solid bars indicate animal cell co-maintenance. Data are presented as the mean (\pm SE) of the percent of initial density after 72 hours. The dotted line is presented and indicates 100 % of initial ciliate density.

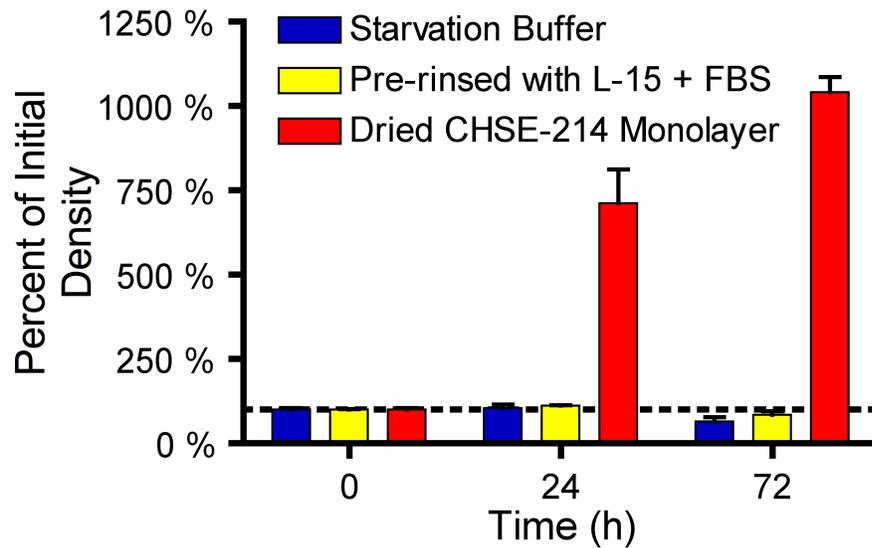


Figure 3.6 Dried fish monolayer as a nutrient source

Tetrahymena thermophila suspended in a nutrient-free starvation buffer were added to either sterile flasks (blue bars), L-15 with FBS-rinsed flasks (yellow bars), or to flasks containing dried CHSE-214 monolayers (red bars). Ciliate density was determined and is expressed as the percent of the corresponding sample initial value. Data presented as mean \pm SE ($n = 3$). Ciliate proliferation in contact with the dried monolayer was significantly higher than either of the other two culture conditions ($p < 0.001$; one-way ANOVA with Bonferroni post-test), while there was no statistical significance between proliferation in starvation buffer alone versus L-15 with FBS-rinsed flasks ($p > 0.05$). The dotted line indicates 100 % of initial ciliate density.

3.4 Discussion

In co-cultures of *Tetrahymena thermophila* and monolayers of epithelial cell lines either from Chinook salmon, rainbow trout, zebrafish or rabbit, the ciliates flourished, swimming and proliferating, and the monolayers died, being completely consumed. As discussed below, the mechanism(s) behind the monolayer destruction appears not to involve the release of soluble cytolytic factors but requires that the ciliates make physical contact with the animal cells through swimming and that ciliates carry out phagocytosis.

3.4.1 Role of physical contact through swimming in monolayer destruction

Culture conditions that reduced ciliate swimming increased protection of animal cell monolayers, suggesting that the physical contact necessary to initiate monolayer destruction occurred through swimming. At low temperatures, 14 and 4 °C, swimming of the ciliates slowed or stopped respectively and monolayer destruction slowed or was absent, respectively. The nature of the contact brought out by swimming was the repetitive bumping of the anterior end of the ciliates into the monolayer or at the sides of the monolayer, if the monolayer had discontinuities. What occurs during the transitory physical contacts or “kisses of death” between the attached animal cells and the ciliates that start monolayer obliteration could be “mechanical stress” or “enzymatic lysis” or both.

Mechanical stress appears to contribute to the killing of animal cells by some protozoa and this could be the case for *T. thermophila* as well, but ciliates likely generate mechanical stress by different mechanisms. *Naegleria fowleri*, *Acanthamoeba castellanii*, and *Trichomonas vaginalis* generate mechanical stress that contributes to cell killing by attaching to the animal cells and pulling on microvilli through the formation of food cups during the process of phagocytosis (Sohn et al., 2010). This process of killing animal cells through nibbling and ingesting plasma membrane fragments has been termed trophocytosis (Brown, 1979; Midlej and Benchimol, 2010; Sohn et al., 2010). By contrast the ciliates do not attach to the animal cells but repeatedly bump into them, especially the anterior end of the ciliates, which could generate mechanical stress.

The repetitive and continuous poking of the monolayer by the front end of ciliates might generate mechanical stress and animal cell plasma membrane disruption by repeatedly impaling the membranes with cilia and/or by vortices of medium causing sheer over localized membrane regions. The vortices could be created by medium being swept into the cytopharynx by cilia of the oral apparatus in an attempt to begin phagocytosis. This speculation is supported by the results with the *T. thermophila* mutant NP1, which at 37 °C does not have a normal oral apparatus and is incapable of phagocytosis (Orias and Pollock, 1975). At 37 °C both NP1 and the wild type strain B1975 swam similarly over a monolayer of rabbit kidney cells, including the continuous probing of the monolayer. With B1975 the monolayer was obliterated; yet, with NP1 the monolayer remained intact. At 37 °C the oral apparatus of NP1 has several abnormalities. The arrangement of cilia is disorganized and the buccal cavity is described as being very shallow or non-existent and no undulating membrane is observed (Orias and Pollock, 1975). Perhaps these abnormalities prevent the generation of vortices with sufficient force to disrupt animal cell membranes and begin monolayer destruction.

At the same time, contact with animal cells might cause the activation of surface lytic enzymes or discharge of lytic mucocyst enzymes, such as proteases in the ciliates. Activation of membrane-associated enzymes by contact with animal cells has been postulated to explain the cytopathogenicity of *Entamoeba histolytica* (Eaton et al., 1969). The discharged enzymes might act only locally and fail to remain active in the medium at large so that medium conditioned by the ciliates did not have the capacity to destroy cell monolayers. Such a mechanism could explain why a cysteine protease inhibitor slowed the destruction of fish cells co-cultured with a *Tetrahymena* spp. (Leibowitz et al., 2010) or with *P. dicentrarchi* (Paramá et al., 2004a).

3.4.2 Phagocytosis and monolayer destruction

Concurrent with the dislodgement or loosening of cells in monolayers, phagocytosis by ciliates was needed to initiate, continue, and complete monolayer destruction. This was supported by experiments with the *T. thermophila* mutant NP1, which phagocytoses at

temperatures below 30 °C but not at 37 °C (Orias and Pollock, 1975). Like the wild type strain B1975, NP1 destroyed monolayers and cleared the medium of debris in cultures with animal cells at room temperature. At 37 °C monolayers of rabbit kidney cells were destroyed by B1975 but with NP1 remained intact. When the zebrafish cell line, ZEB2J that expresses green fluorescent protein (GFP), was co-cultured at room temperature with *T. thermophila*, fluorescent ciliate food vacuoles were seen, indicating internalization of the monolayer. However determining whether *T. thermophila* distinguished between live cells, dying cells, dead cells, or cellular fragments for internalization was difficult because ZEB2J fragments, as well as ZEB2J cells, were fluorescent. However the ciliates could internalize dead cells as illustrated by their ability to clear a dried monolayer of fish cells. With the flagellate *Trichomonas vaginalis*, necrotic but not living animal or apoptotic cells were phagocytosed (Midlej and Bechimol, 2010), whereas amoeba internalized both living and dying epithelial cells (Martinez-Palomo et al., 1985).

3.4.3 Role of soluble factors on cell monolayer destruction

The release of soluble cytolytic factors has been shown to contribute to the destruction of animal cell monolayers in co-cultures with some protozoa, and although a number of enzymes could be potential cytolytic factors. The release of cytolytic enzymes has been thought to be at least partially responsible for the destruction of mammalian cells in culture by the amoebae, *Naegleria fowleri* (Visvesara and Callaway, 1974) and *Acanthamoeba castellanii* (González-Robles et al., 2006), and the flagellate, *Trichomonas vaginalis* (Singh et al., 2004). *Tetrahymena thermophila* releases enzymes into the medium and some have the potential to kill cells. These include phospholipases, which might be responsible for the hemolytic activity of the ciliates (Florin-Christensen et al., 1985), and, for *T. pyriformis* strain HSM, ribonucleases (Rothstein and Blum, 1974) which from other microorganisms can be cytotoxic to mammalian cells (Arnold, 2008). However, several observations rule out the accumulation of cytolytic enzymes from the ciliates into the medium as the mechanism of monolayer destruction. When fish cells and ciliates were co-cultured in the same medium but separated physically by a porous insert that blocked ciliates from moving between the top ciliate chamber to the bottom CHSE-214 chamber, no monolayer destruction ensued.

Likewise monolayers remained intact when fish cell cultures received conditioned-media in which the ciliates had been maintained. As well, the mutant strain *T. thermophila* SB255 that was expected to secrete less material into the medium destroyed CHSE-214 monolayers as rapidly as wildtype *T. thermophila* B1975. Therefore the release of cytolytic factors for animal cells into the medium by ciliates does not appear to contribute to monolayer destruction.

Whether the behaviour of ciliates in destroying monolayers is modulated by factors released by fish cells remains to be investigated. For example, once the monolayer began to come apart, more ciliates appeared to attack the monolayer, resulting in a frenzy of feeding and increasing monolayer deterioration. Possibly damaged or stressed fish cells released factors that attracted ciliates. The movement of a cell in response to the recent death of another cell has been termed necrotaxis (Bessis and Burte, 1964). Previously *T. pyriformis* has been shown to move away from lysed cells of *T. pyriformis* and *Euglena* sp., which is negative necrotaxis (Ragot, 1993). Possibly positive necrotaxis is the response of *T. thermophila* B1975 to dead or dying fish cells. The culture systems developed here should allow the nature of such phenomena to be investigated in the future. Interestingly, whole turbot blood has chemoattractants for *Philasterides dicentrarchi* (Paramá et al., 2004b), and *T. thermophila* has been shown to be attracted to some mammalian cytokines (Köhidaï and Csaba, 1998).

3.4.4 Proliferation of ciliates on CHSE-214 monolayers

Fish cell monolayers provided nutrients to support the proliferation of *T. thermophila*. The monolayers were a source of growth-promoting substances and of particulate matter, which would have stimulated phagocytosis and food vacuole formation (Rasmussen and Kludt, 1970; Rasmussen and Moedweg-Hansen, 1973). The presence of growth-promoting substances was demonstrated in two ways. Firstly, living fish cell monolayers enhanced *T. thermophila* growth in L-15 with FBS, and therefore, would have been providing additional growth factors and nutrients to the ciliates. This is similar to reports of other ciliate species growing in basal media that had fish cells, such as leucocytes (Piazzon et al., 2011).

However, in the current study, the ciliates had to remove the fish cells from a plastic surface to obtain the growth factors and nutrients. Secondly, dead fish cell monolayers in a simple salt solution supported ciliate growth. In this case, the monolayer of fish cells was the sole source of growth factors and nutrients for *T. thermophila* growth, albeit ciliates did not proliferate as much as when co-cultured with a living monolayer. This is a clear demonstration that fish cells have all the nutrients and growth factors necessary for *T. thermophila* to proliferate.

3.4.5 Histophagy and potential pathogenicity of *T. thermophila*

This study shows that *T. thermophila* is histophagous in vitro and raises the possibility of *T. thermophila* being a pathogen of freshwater vertebrates. In the past, ciliates have been described as being histophagous based on observing them eat tissues in vivo and on their relatedness to known histophagous species (Strüder-Kypke et al., 2001). On the basis of genetic criteria, *T. thermophila* has not been grouped with the histophagous tetrahymenas (Strüder-Kypke et al., 2001). However, the current study demonstrates that if given access to the cells of a vertebrate in vitro, *T. thermophila* can consume them completely as illustrated with monolayers of epithelial cells and fibroblasts. Likely the repertoire of *T. thermophila* enzymes is important for this destruction, but swimming and phagocytosis also were identified as critical activities. In experimental demonstrations of tetrahymenosis in fish and in spontaneous outbreaks, a key seems to be a break in the integrity of the integumentary system, such as in wounding (Thompson, 1958; Ponporpisit et al., 2000; Leibovitz et al., 2005). The behaviour of *T. thermophila* in vitro suggests that this species can take advantage of a chance to enter a vertebrate and to grow and cause damage to organ systems. Yet to date, *T. thermophila* has not been associated with tetrahymenosis in fish. However, in some tetrahymenosis infections, the ciliate responsible has not been identified to the species level (Leibovitz et al., 2005) so possibly the involvement of *T. thermophila* in disease outbreaks has gone unrecognized.

Tetrahymena thermophila has been co-cultured for the first time with the cells of fish and mammals and shown to destroy them completely over several days at either room temperature or at 37 °C. Given the model organism status of *T. thermophila*, such cultures might be used in the future to study how ciliates in general interact with aquatic vertebrates at the cellular level, including the discovery of possible treatments. For example, the current study suggests a simple treatment would be to keep fish at low temperatures. The cultures also might be used to study the response of vertebrate cells to the ciliates prior to their consumption and whether some vertebrate cells or some organization, such as an extensive extracellular matrix, might be less susceptible to destruction. In turn they can be used to investigate how ciliates invade and destroy fish tissues. For example, the ciliate parasite of fish *Ichthyophthirius multifiliis* has been described as invading the host epithelium through repeated bumping and a swirling boring motion (Ewing et al., 1985), which bears some resemblance to what has been described here in vitro with *T. thermophila*. Finally, co-cultures of animal cells and ciliates can be used to determine whether all *Tetrahymena* species have the capacity for histophagy or whether this property is restricted to just some species.

Chapter 4

Some but not all *Tetrahymena* species destroy monolayer cultures of cells from a wide range of tissues and species

4.1 Introduction

The tetrahymenas are ciliates, belonging to the kingdom Protista (Lynn, 2008), and several species cause a disease in fish that has been called tetrahymenosis (Pimenta Leibovitz et al., 2005). The disease has been reported most frequently in tropical fish during the course of aquarium trade, resulting in sporadic but serious economic losses. Infections have been seen in the guppy (*Poecilia reticulata*), cichlids, black mollies, and tetras (Hoffman et al., 1975; Imai et al., 2000; Johnson, 1978). However the ciliates could impact other types of fish aquaculture, including the farming of Atlantic salmon (Ferguson et al., 1987). Most often the disease-causing ciliate has been identified only to the level of genus, simply as *Tetrahymena* sp. and the pathogenic mechanism described broadly as tissue destruction. Thus, which ciliates interact with the fish cells, and how, is poorly documented.

One way to study ciliate/fish interaction at the cellular level is to use co-cultures of ciliates and fish cells (Chapter 1). This was recently done with *Tetrahymena thermophila* (Chapter 3), which is a species that is widely used as a model organism in cell biology but usually not considered a fish pathogen. Surprisingly, *T. thermophila* destroyed cell lines from a wide range of fish species and tissues by a mechanism that appeared to involve phagocytosis. These results raise the question of whether the destruction of fish cells in vitro is something all tetrahymenas can do or is an ability restricted to just some species, perhaps those that have been previously associated with health problems in fish.

Therefore the behavior of two additional tetrahymenas was examined in co-cultures with fish cell lines. One is *T. corlissi*, which has been associated with disease and appears to be the most widespread cause of tetrahymenosis among cases that have characterized the

parasite (Imai et al., 2000; Pimenta Leibowitz et al., 2005). The other, *T. canadensis*, which has also been called *T. pyriformis* syngen 7, has never been associated with fish.

Tetrahymena canadensis is a species originally isolated from streams and has not been studied extensively, but is related to another histophagous, or tissue-feeding, *Tetrahymena*, *T. rostrata* (Lynn and Strüder-Kypke, 2006). Close evolutionary relatedness between histophagous, or tissue-feeding, *Tetrahymena* and non-histophages does not necessarily suggest an ability to feed on tissue however, as this form of feeding seems to have arisen multiple times across the genus. Nonetheless, the close relatedness to *T. rostrata*, along with the lack of research into the cell biology of *T. canadensis*, makes it an interesting candidate to investigate ciliate-fish interactions.

Here two species of *Tetrahymena* showed differences in histophagy. *Tetrahymena corlissi* could destroy most fish cells, whereas *T. canadensis* could not. Additionally, there were clear differences in the behaviour of each of these species when in co-culture with an animal cell monolayer; *T. corlissi* showing a grazing behaviour also seen with other monolayer-destroying species (Chapter 3), *T. canadensis* showing no apparent attraction to the monolayer surface. These results indicate that only some Tetrahymenids have the capacity to destroy fish cells in vitro. In turn, this research suggests that animal cell cultures could be convenient for screening ciliate species for their potential to be histophagous and for studying the mechanisms by which ciliates destroy animal cells.

4.2 Materials and Methods

4.2.1 *Tetrahymena* spp.

Axenic cultures of *Tetrahymena corlissi*, *T. canadensis*, and *T. thermophila* B1975 (kindly provided by Dr. D.H. Lynn, Department of Integrative Biology, University of Guelph, Guelph, Ontario, Canada) were maintained at 22 °C in the ciliate growth medium, proteose peptone yeast extract (PPYE) prior to use, as described in Chapter 3 (Section 3.2.1).

4.2.2 Animal Cells

In general, animal cell lines were grown to confluency in 75 cm² tissue culture flasks (BD Biosciences, Durham, NC) in Liebovitz's L15 culture medium (Sigma-Aldrich, St. Louis, MO) supplemented with fetal bovine serum (FBS, Sigma-Aldrich) and 1% penicillin-streptomycin solution, [henceforth referred to as *pen/strep*] (10,000 units/ml penicillin, 10 mg/mL streptomycin, Sigma-Aldrich). Insect cells (Sf9) were grown in Grace's Insect medium (Invitrogen, Carlsbad, CA) with added FBS and 1% pen/strep. Incubation temperatures and FBS concentrations differed by cell type and are presented, along with a list of all cell types used, in Table 4.1.

Table 4.1 Animal cell lines used to generate monolayer cultures

| Cell Line: | Species: | Tissue of Origin: | Cell Morphology: | Incubation Temperature: | Reference: |
|---------------------|---|--------------------------|-------------------------|--------------------------------|-------------------------|
| Sf9 ^a | Fall Armyworm (<i>Spodoptera frugiperda</i>) | Ovary | epithelial ^b | 26 °C | Vaughn et al., 1977 |
| RTG-2 | Rainbow Trout (<i>Oncorhynchus mykiss</i>) | Mixed Gonads | Fibroblastic | 22 °C | Wolf and Quimby, 1962 |
| RTgutGC | Rainbow Trout | Intestine | Epithelial | 22 °C | Kawano et al., 2011 |
| RTH-149 | Rainbow Trout | Hepatoma | Epithelial | 22 °C | Fryer et al., 1981 |
| RTgill-W1 | Rainbow Trout | Gills | Epithelial | 22 °C | Bols et al., 1994 |
| RTS-11 ^b | Rainbow Trout | Spleen | Monocyte/ Macrophage | 20 °C | Ganassin and Bols, 1998 |
| CHSE-214 | Chinook Salmon (<i>Oncorhynchus tshawytscha</i>) | Embryo | Epithelial | 22 °C | Fryer et al., 1965 |
| ZEB2J | Zebrafish (<i>Danio rerio</i>) | Embryo | Epithelial | 26 °C | Xing et al., 2008 |
| EPC | Fathead Minnow (<i>Pimephales promelas</i>) ^c | Epithelioma Papulosum | Epithelial | 22 °C | Fijan et al., 1983 |
| A6 | African Clawed | Kidney | Epithelial | 22 °C | Rafferty and |

| | | | | | |
|-------|-----------------------------------|----------------------|--------------|-------|-------------------------|
| | Frog (<i>Xenopus laevis</i>) | | | | Sherwin, 1969 |
| L-929 | Mouse (<i>Mus musculus</i>) | Connective Tissue | Fibroblastic | 37 °C | Sanford et al., 1948 |
| HeLa | Human (<i>Homo sapiens</i>) | Cervix | Epithelial | 37 °C | Scherer et al., 1953 |

^a Growth medium was Grace's Insect Medium

^b Grows loosely adherent and in suspension

^c Note ATCC correction for species of origin (Winton et al., 2010).

4.2.3 Exposing animal cells to *Tetrahymena* spp.

Following a prior method for preparing *Tetrahymena* for animal cell co-culture (section 3.2.2), either *T. corlissi*, *T. canadensis*, or *T. thermophila* were added to animal cells to assess monolayer destruction. Briefly, exposure was carried out in 12-well tissue culture plates (Becton-Dickinson, Franklin Lakes, NJ) and wells were seeded at a cell density of 5.0×10^5 animal cells/mL in animal cell growth medium (see Table 4.1). Ciliates were added at a final density of 1,500 ciliates/mL. In the case of semi-adherent cell lines (RTS-11 and Sf9), 18-24 h was allowed prior to adding ciliates to ensure cell proliferation, and ciliate inoculum was suspended in animal cell culture medium and added directly to pre-existing media in each well, to obtain a final density of 1,500 ciliates/mL. Co-cultures were maintained at the animal cell line optimal temperature, and observations of monolayer integrity, ciliate morphology, and behaviour were conducted daily, using an inverted microscope. For video micrography of *Tetrahymena* and animal cell co-incubations, a Nikon Coolpix E8400 camera was used, as described in section 3.2.3. Video files are presented as Appendix B.

4.2.4 Artificially-damaged monolayers as a means to stimulate histophagy

Pre-existing damage to the animal cell monolayer was tested for the ability to attract ciliates, and promote further monolayer destruction using two methods. Co-incubations were set up using CHSE-214 as described in section 4.2.3, but at the time of ciliate addition a sterile glass Pasteur pipette was used to score the monolayer. Alternatively, prior to the addition of ciliate-containing media, a metal inoculation loop was heated using a Bunsen burner and touched to the monolayer to create a circular burn. In both cases, observations of the ciliates were made using an inverted microscope, with special attention paid to the attraction of ciliates to areas of monolayer damage compared to undamaged areas in the same well.

Monolayer recovery from approximately 25 % ciliate-mediated damage was assessed by growing a confluent monolayer of CHSE-214 cells on a plastic cover slip within a Leighton tube (Sigma-Aldrich) and adding *T. corlissi* or *T. canadensis* (1,500 ciliates/mL). After damage had occurred to the monolayer, the cover slip was removed from the Leighton tube and rinsed by immersing in sterile L-15 with 10 % FBS and 1 % pen/strep, three times, to

remove ciliates. Washed cover slips were placed into a sterile Leighton tube and fresh growth media was added. Monolayer damage was observed for changes to morphology and signs of reattachment using an inverted microscope.

4.2.5 Histophagy at low temperature

The effect of low-temperature on *T. corlissi*, or *T. canadensis* was tested by adding ciliates to a confluent CHSE-214 monolayer, as described in section 4.2.3, and immediately moving to either 4 °C or 14 °C. Observations were conducted daily using an inverted microscope, over 144 h.

4.2.6 Animal monolayers as a source of nutrition

Two species of *Tetrahymena*, *T. corlissi* and *T. canadensis* were transferred to animal cell growth medium L-15 with 10% FBS and antibiotics and 1 % pen/strep and were added either to confluent monolayers of CHSE-214, or to sterile cell culture flasks (25 cm² growth surface in both cases), and maintained at 22 °C. The initial density was 1,500 cells/mL and was determined using a Coulter Z2 Electronic Particle Counter (Coulter, Luton, UK), with an upper and lower size limit of 35 µm and 15 µm, respectively. After 72 h, ciliate number was counted again. The change in cell number, expressed as the percentage of initial density [$100 \times (\text{density at 72 hours} / \text{initial density})$], was graphed, and represents the mean of three trials with standard error. The increase in cell number from initial density after 72 hours [$(\text{density at 72 hours} - \text{initial density})$] was also calculated for each treatment, and these data were compared between *Tetrahymena* spp. maintained in medium only and those maintained alongside a confluent monolayer of CHSE-214. Data were analyzed for significant variation between ciliate proliferation in medium alone and alongside CHSE-214 cells using unpaired t tests, using GraphPad Prism soft (v4, GraphPad Software).

4.3 Results

4.3.1 Destruction of cell lines from a range of fish tissues

In total, eight fish cell lines were tested with each of the three species of *Tetrahymena*. Each of these ciliate species had a different capability for destroying monolayers when

considering their action over all cell lines tested (Table 4.2). An expanded investigation of *T. thermophila* for its ability to destroy monolayers increased the range of cell lines destroyed from those tested previously (Chapter 3), with no undisturbed cell line yet to be found. These included epithelial and fibroblast cell lines from different organs and from four species. Destruction was observed at 20 °C, 22 °C, 26 °C, and 37 °C. Out of the eight fish cell lines, five were destroyed by *T. corlissi*, and these were from a range of species, cell morphologies, and organs. Destruction was observed at 20 °C, 22 °C, and 26 °C, but not 37 °C. The stages of destruction and the swimming behaviour of *T. corlissi* when in contact with the monolayer - referred to as “grazing” - was typical of what occurred with *T. thermophila*, and has been described previously (Chapter 3).

In contrast to the other two species, *T. canadensis* did not cause damage to any cell line, under any tested circumstance, though the ciliates remained active and motile throughout the duration of these trials. The swimming behaviour of this species was also markedly different, as ciliates remained swimming actively throughout the entire volume of culture medium (video available as Appendix B). There was no apparent attraction of the ciliate to the surface of the monolayer, as was apparent with the other *Tetrahymena* species. When *T. canadensis* was maintained alongside the gfp-expressing cell line ZEB2J (Figure 4.1), ciliates were actively motile and no accumulation of dark vacuoles was noted, as was the case with *T. corlissi* (data not shown). However the presence of faint green fluorescence was present. Throughout the *T. canadensis* co-cultures, no visible negative effects could be seen via microscope analysis of the monolayer, and no damage occurred to any of the tested monolayers.

Table 4.2 Summary of Monolayer Destruction by Three Species of *Tetrahymena*

| Cell Line: | <i>Tetrahymena</i> species | | | | | |
|---------------------|-----------------------------|---------------------------------|------------------------|---------------------------------|------------------------|--|
| | <i>T. thermophila</i> B1975 | | <i>T. canadensis</i> | | <i>T. corlissi</i> | |
| | Monolayer Cleared (h): | Ciliate Morphology / Behaviour: | Monolayer Cleared (h): | Ciliate Morphology / Behaviour: | Monolayer Cleared (h): | Ciliate Morphology / Behaviour: |
| RTG-2 | 120 ^a | Grazers ^b | Not cleared | Swimmers ^c | Not cleared | Grazers, but no monolayer damage |
| RTgutGC | 120 | Grazers | Not cleared | Swimmers | Not cleared | Grazers, but no monolayer damage |
| RTH-149 | 120 | Grazers | Not cleared | Swimmers | 144 | Grazers |
| RTgill-W1 | 72 | Grazers | Not cleared | Swimmers | 120 | Grazers |
| RTS-11 ^d | 120 | Grazers | Not cleared | Ciliates decreased in number | 144 | Grazers |
| CHSE-214 | 72 ^a | Grazers | Not cleared | Swimmers | 168 | Grazers |
| ZEB2J | 72 ^a | Grazers | Not cleared | Swimmers | 216 | Initial cell swelling; grazers developing by 48h |
| EPC | 120 | Grazers | Not cleared | Swimmers | Not cleared | Swimmers |

| | | | | | | |
|------------------|----|--|-------------|-------------------------|-------------|-------------------------|
| A6 | 48 | Grazers | Not cleared | Swimmers | Not cleared | Swimmers |
| L929 | 96 | Grazers | Not cleared | Swimmers | Not cleared | Swimmers |
| Sf9 ^d | 72 | Grazers | Not cleared | Swimmers | 48 | Grazers |
| HeLa | 72 | Initial swelling, rapid development of grazers | Not cleared | Cells swelled, immotile | Not cleared | Cells swelled, immotile |

^a Originally presented in Chapter 3

^b Grazers typified by increased cell size compared to ciliates in growth medium (~3-4 times increase), a morphology bloated by phase-dark vacuoles, slow motility, and the tendency to swim along the surface of the monolayer.

^c Swimmers typified by elongated morphology, active motility, and tendency to swim through suspension, rather than at monolayer surface.

^d RTS-11 and Sf9 grow in suspension and does not form a monolayer, thus clearance refers to the removal of animal cells from suspension.

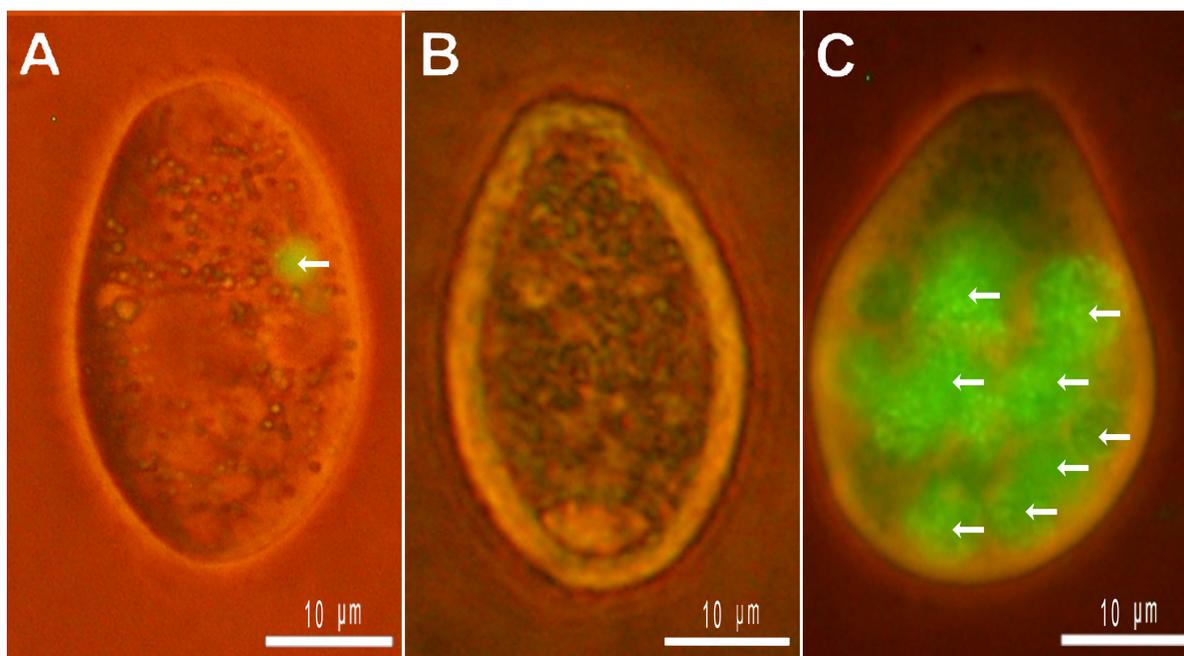


Figure 4.1 *Tetrahymena canadensis* added to ZEB2J monolayers contained engulfed fluorescent fish cell remains

When maintained alongside the GFP-expressing ZEB2J cell line for 48 h, *T. canadensis* did not cause damage to the monolayer but did accumulate some fluorescence inside the cell when the temperature was 22 °C (A), but not at 4 °C (B). For comparison, *T. thermophila* maintained with ZEB2J at 22 °C for 48 h showed large clusters of fluorescent material (C). Pictures were taken of fixed cells using an inverted, fluorescence microscope with a B-2A filter (excitation, 450 nm; emission, 520 nm).

4.3.2 Destruction of cell lines from other vertebrates

Of the three *Tetrahymena* species, only *T. thermophila* was able to destroy monolayer cultures of cell lines from other vertebrates (Table 4.2). These cell lines were the frog epithelial cell line, A6, the murine fibroblast cell line, L-929, and the human cell line, HeLa. The co-cultures with the murine and human cell lines were maintained at 37 °C in L-15 with 10 % FBS, which is not normally a medium used to grow them. Ciliates completely destroyed the A6, L-929, and HeLa cell monolayers by 48, 72, and 96 hours respectively. Neither *T. corlissi* nor *T. canadensis* were able to destroy these cell lines. Both species of ciliate swam throughout the media in the A6 and L-929 cell lines, and adopted a rounded morphology and stopped swimming when co-incubated with HeLa.

4.3.3 Destruction of an insect cell line, Sf9

Both *T. thermophila* and *T. corlissi* destroyed cells belonging to cultures of the lepidopteran cell line Sf9. These cells were a mixture of adherent and suspended cells, and ciliates did not appear to preferentially destroy either cell morphology. Maintained at 26 °C, *T. thermophila* and *T. corlissi* destroyed all adherent and suspended cells by 72, and 48 hours respectively. The presence of *T. canadensis* did not cause damage to Sf9 cells, and ciliates remained actively motile.

4.3.4 Artificially-damaged monolayers do not stimulate histophagy by *T. canadensis*

When *T. canadensis* was added to a CHSE-214 monolayer that was damaged either physically using a sterile Pasteur pipette, or using a flamed metal inoculating loop, the ciliates continued to swim throughout the monolayer with no obvious attraction to the areas of damage. In the case of *T. corlissi*, the ciliates displayed grazing behaviour soon after being added to the damaged monolayer. *Tetrahymena corlissi* destroyed the monolayer soon after addition and total destruction of the monolayer occurred much earlier compared to intact monolayers; 24 hours earlier in the case of the burn, and ~24 hours earlier in the case of

pipette lesions. Damaged monolayers to which *T. canadensis* had been added did not experience further damage, rather monolayers were seen to recover from damage and begin to re-grow in the damaged areas. The pre-damaged monolayers grew in these conditions and returned to confluency, despite the presence of *T. canadensis* in the medium.

4.3.5 Histophagy at low temperature

When added to CHSE-214 at 14 °C, for *T. corlissi* the decrease in temperature improved their ability to destroy the monolayer, with complete destruction by 120 h compared to 168 h at 22 °C. *Tetrahymena corlissi* remained actively motile throughout the trial. Reducing the temperature to 14 °C did not affect the inability of *T. canadensis* to destroy the monolayer. Lower temperatures (4 °C) caused *T. corlissi* to become nonmotile quickly (<1 h) and no recovery was seen over 72 h and there was no damage to the monolayer. At 4 °C, *T. canadensis* retained active motility and could be seen to phagocytose fluorescent beads (1 µm) when mixed with the ciliates, but did not accumulate fluorescent material when maintained on a monolayer of ZEB2J (Figure 4.1).

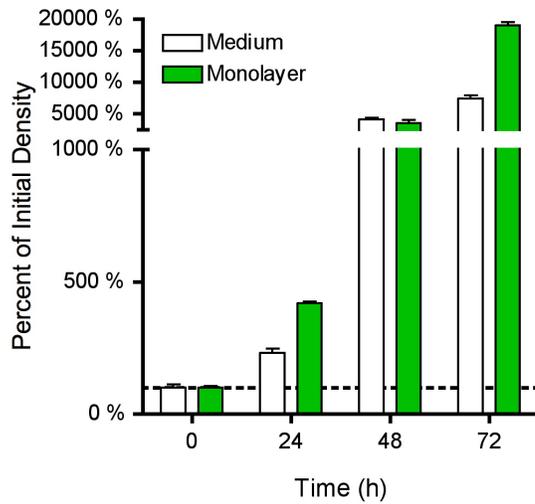
4.3.6 Animal monolayers as a source of nutrition

To contrast the survival and proliferation of *T. corlissi* and *T. canadensis* when in contact with a cell monolayer, both species were added to confluent monolayers of the fish cell line CHSE-214 and ciliate density measured over time. As *T. corlissi* destroyed the monolayer, ciliates became notably more numerous compared to a parallel culture maintained in fish cell culture medium (L-15 with 10 % FBS with antibiotics) alone, and this is reflected in the cell proliferation data (Figure 4.2). This species increased in cell number over the 72 h trial period, and by the end of the trial cell density remained significantly higher when in the presence of CHSE-214 than those in medium alone ($p < 0.0001$; unpaired t test). Proliferation of *T. canadensis* in cell culture medium alone was much reduced compared to the other tetrahymena, and did not significantly increase from the initial density ($p > 0.05$; unpaired t test). Proliferation was stimulated when the ciliates were in contact with CHSE-214, and proliferation in this treatment over 72 h was significant when compared to the initial

density ($p < 0.0001$; unpaired t test). Even with the stimulatory effect of the animal cells, *T. canadensis* proliferation over 72 h was much less than observed with *T. corlissi*.

A lysate of CHSE-214 cells was also produced and added to nutrient-free ciliate starvation buffer, to investigate if the animal cells could solely support ciliate proliferation (Figure 4.3). The lysate protein concentration at time 0 was 184 $\mu\text{g}/\text{mL}$; approximately that of a confluent 25 cm^2 flask of the fish cell monolayer. When the linear portion of these growth curves, the period from 0 to 24 hours, was fit with a linear regression and compared to starvation buffer alone using an F test, both *T. corlissi* and *T. canadensis* showed significant differences between the regression of the two growth conditions (*T. corlissi* $F = 59.83$, $P = 0.016$; *T. canadensis* $F = 91.77$, $P = 0.011$). Comparing cell density after 48 h showed each species having significantly greater number in the presence of CHSE-214 lysate than in starvation buffer alone ($p < 0.05$ for both species; unpaired t tests).

A. *Tetrahymena corlissi*



B. *Tetrahymena canadensis*

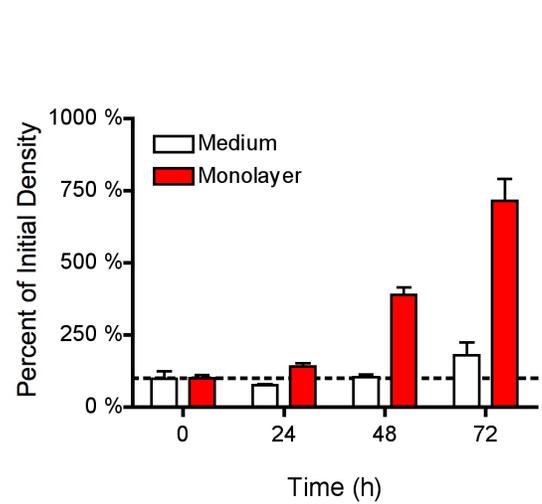


Figure 4.2 Ciliate proliferation in animal cell co-culture

Either *T. corlissi* (A) or *T. canadensis* (B) were added to cell culture medium (L-15 with 10 % FBS) alone (white bars), or to confluent monolayers of CHSE-214 in medium (coloured bars). Initial density of ciliates was approximately 1,500 cells/mL, and cell counts were conducted every 24 hours for 72 hours. White bars represent ciliates grown in medium alone, and coloured bars indicate animal cell co-maintenance. Data are presented as the mean (\pm SE) of the percent of initial density. The dotted line indicates 100 % of initial ciliate density.

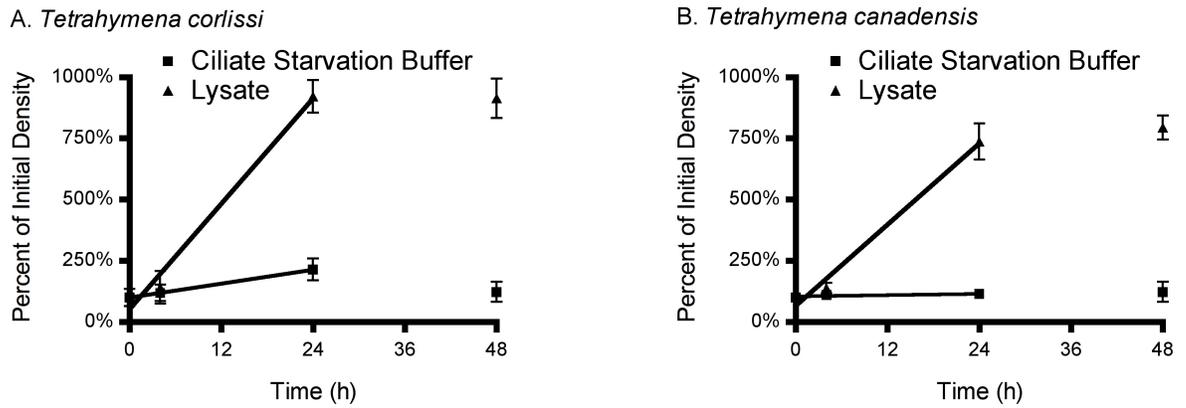


Figure 4.3 Growth of *Tetrahymena* in fish cell lysate

Two species of *Tetrahymena*, *T. corlissi* (A), *T. canadensis* (B), were maintained in CHSE-214 lysate-supplemented medium at 22 °C. Linear regressions were created for the first 24 hours of starvation buffer and lysate-supplemented data, with R^2 values of 1.000 and 0.9876 for *T. corlissi*, 0.7057 and 0.9898 for *T. canadensis*; starvation buffer and lysate respectively. Data points are the mean (\pm SD) of three independent trials, expressed as a percentage of their respective initial ciliate density.

4.4 Discussion

4.4.1 Stark contrasts in the potential for histophagy across species of *Tetrahymena*

Clear differences in the behaviour of *Tetrahymena* when in contact with fish cells have been observed. Unfortunately, a lack of detailed studies of the differences between species of *Tetrahymena* makes conclusions as to the underlying cell biology causing these differences difficult. Nonetheless, the expanded view of histophagy among the *Tetrahymena* is discussed below.

4.4.2 Destruction of cell lines from a range of fish, and other vertebrate tissues

Three *Tetrahymena* species were tested for their ability to destroy cell monolayers, from a range of animals and organs, and in a variety of conditions. The facultative parasite of ornamental guppy, *T. corlissi* was able to destroy five of eight tested fish cell lines, the sole insect cell line tested, but no mammalian cell lines. The most susceptible cell lines were that of gill (RTgill-W1) and embryonic (CHSE-214, ZEB2J) origins; the gill cell lines stands out as it originates from a common target for ciliate parasites, the fish gill surface (Mathews, 2005; Paramá et al, 2003). Further, the non-adherent monocyte/macrophage cell line RTS-11 was destroyed by 120 hours, suggesting that the destructive mechanism does not require a monolayer and these cells may feed on cells in suspension. The invertebrate cell line Sf9 was also destroyed and has a similar suspended morphology to RTS-11. As initially shown, section 3.3.1, *T. thermophila* is capable of consistently destroying animal cell monolayers in culture. It was notable that, although both *T. thermophila* and *T. corlissi* showed similar behaviours when in contact with monolayers, the former of the two *Tetrahymena* destroyed the cell lines in a notably shorter period than *T. corlissi*, with only the insect cell line, Sf9, as the exception to this.

4.4.3 Destruction of cell lines from other vertebrates

Human, mouse, rabbit, and frog cell lines were also assessed for their ability to be destroyed by *Tetrahymena*. Again, in all cases the cell monolayers were destroyed in a manner that matched the destruction of teleost monolayers. Additionally, the mammalian cell lines were kept at 37 °C, which is closer to the growth optimum for *T. thermophila* and these more active ciliates may have hastened the destruction of the monolayers, compared to the fish cell lines maintained at lower temperatures. Additionally, culturing of mammalian cell lines is not generally performed using L15 medium, and this may have caused stress on these cells that facilitated their destruction by the ciliate, though there were no visible signs of stress in ciliate-free cultures. This work provides a proof of concept for the co-maintenance of mammalian cells with free-living, potentially parasitic, ciliates. This system would also be a potential model for studying the general pathology of the known parasitic ciliate causing disease in humans, *Balantidium coli*.

4.4.4 *Tetrahymena canadensis*, a free-living isolate incapable of tissue destruction

The inability of *T. canadensis* to destroy animal cell monolayers may be a result of several factors. Phagocytosis of fluorescent beads and active swimming by *T. canadensis* in the co-culture medium, even at 4 °C, suggests that the ciliates are able to successfully carry out phagocytosis in animal cell culture medium. Physical separation of monolayer and ciliates prevented monolayer destruction by either *T. corlissi* or *T. thermophila*, suggesting that although enzyme exudates may play a role in destruction, physical contact between ciliates and monolayers is required. *Tetrahymena canadensis* was consistently found swimming throughout the entire volume of medium and, contrary to *T. thermophila* and *T. corlissi*, was rarely found swimming along the surface of the monolayer. This swimming behaviour may be a result of factors released by the animal monolayer that acts as chemoattractants to histophagous cells, but to which *T. canadensis* remains less sensitive. A variety of factors released by animal cells stimulate *T. thermophila*, even at the femtomolar scale, such as insulin (Csaba et al., 2007). Unfortunately, the lack of detailed physiological study

specifically on *T. canadensis* rules out conclusions as to species-specific physiology. Another explanation may be geotaxis, whereby movement of the cell is directed by gravity, specifically *T. canadensis* may exhibit negative geotaxis and swim upwards in the culture vessel. Negative geotaxis has previously been described in *T. pyriformis* (Noever et al., 1994), and because of the low volume of medium, other explanations for directed movement of the ciliates, namely dissolved oxygen, chemical, or light gradients, are not expected to have been a major factor. Detailed study of the physiology of *T. canadensis* has yet to be carried out. Here I show differences in the behaviour of this species compared to *T. thermophila* and *T. corlissi*, when maintained in culture with animal cells. These differences were, swimming of *T. canadensis* was random throughout the medium above the monolayer surface, proliferation was reduced in high-osmolality animal cell culture medium compared to *T. thermophila*, and *T. canadensis* was unable to destroy cell culture monolayers.

The co-existence of *T. canadensis* with all tested cell monolayers when the two were combined was notable, as there was no apparent adverse effects to either organism over an extended period (nine days representing the longest co-culture in this study). This is the first instance, to our knowledge, of such an interaction - or rather, lack thereof. Studies of *T. canadensis* have traditionally focused on mating studies, and more recently on genetic and barcoding studies (Strüder-Kypke et al., 2001; Lynn and Strüder-Kypke, 2006; Chantangsi et al., 2007; Chantangsi and Lynn, 2008). This lack of cell biology literature specific to *T. canadensis* hinders the attempt to describe why these ciliates cannot destroy animal monolayers. What has emerged here is an *in vitro* system of maintaining *T. canadensis* alongside animal cell monolayers that can provide a platform to investigate the destruction of tissues by these ciliates or allow an extended co-maintenance of the two organisms side-by-side.

Three species of *Tetrahymena* showed differing capabilities for causing monolayer destruction under different incubation conditions, such as temperature and culture medium. These include physiological temperatures relevant to the host of interest, including both fish and humans. One *Tetrahymena* species showed a complete inability to negatively affect

animal cell lines, while the ciliates thrived and proliferated. Across these *Tetrahymena* spp., easily observable behaviors correlated with destruction of the monolayers.

The use of free-living ciliates, like *Tetrahymena* spp., in a system such as this offers various advantages which would not exist with other currently available culturable histophages. Certain species of *Tetrahymena*, such as *T. corlissi* have a range of life styles that may include free-living and bacterivorous life stages, becoming facultative parasites only under certain conditions (Ferguson et al., 1987; Imai et al., 2000). This allows comparison of populations feeding on tissue to those in nutrient medium, and study of the adaptations that allow these changes to occur. Additionally, because this system allows living monolayers to be maintained alongside ciliates, extracellular matrix and other host factors that act to modulate the epithelia and to maintain its integrity in the face of ciliate attack, can be studied in great detail. Through these model parasite infection scenarios, a greater understanding of the invasion of host tissues by histophagous ciliates can be studied, and may lead to better treatments of these diseases.

Chapter 5

Inactivation of viral hemorrhagic septicemia virus (VHSV) by the ciliate *Tetrahymena thermophila*

5.1 Introduction

The inactivation of human viruses and bacteriophages by ciliates has been studied only sporadically over the last 60 years. Commonly, the ciliate has been *Tetrahymena thermophila* because this protozoan is a convenient model organism and is widely distributed in freshwater, including in sewage treatment plants (Estaban et al., 1992). For human viruses, the interest has been in the killing of pathogenic viruses in wastewater. *Tetrahymena* spp. were shown to inactivate influenza virus (Groupe and Pugh, 1952; Groupe et al., 1955), adenovirus (Sepp et al., 1992), and poliovirus (Kim and Unno, 1996). Bacteriophage, or phage, inactivation was examined because phage are practical to study and important in microbial ecology. Phages have been used as safe surrogates for human pathogenic viruses in studies on the movement and survival of viruses in the environment (Grabow, 2001; Halvelaar et al., 1991). Phages interact in the microbial loop, which is the flow of nutrients from primary producers to heterotrophs to larger organisms, and phages act to transfer carbon down the food chain by lysing bacteria and causing the release of organic carbon to the water (Wilhelm and Suttle, 1999) and so phage inactivation is another component to understand in this regulation. Phages have been found to be differentially susceptible to the actions of *T. thermophila*. Both the bacteriophages, T4 and Φ X174, were ingested by *T. thermophila*, and the ciliate inactivated T4 but not Φ X174 (Akunyili et al., 2008; Pinheiro et al., 2007; Hennemuth et al., 2008).

Few other viral groups appear to have been studied for their inactivation by ciliates but for several reasons one interesting group is fish viruses. Firstly, the viruses, ciliates, and fish might be expected to interact because they share a common aquatic environment and, as well as viruses, fish can be associated with ciliates. Ciliate/fish relationships can vary from pathogenic to ectocommensal, such as feeding on detritus from living, dying and dead fish

(Basson and Van As, 2006). Secondly, fish viruses and their diseases are economically important because of their impact on aquaculture. Finally, relatively little is known about the inactivation of fish viruses in the environment (Hawley and Garver, 2008; Afonso et al., 2012), especially the roles of microorganisms, but such knowledge is needed in order to understand viral transmission and to control disease outbreaks.

Therefore, *T. thermophila* and viral hemorrhagic septicemia virus (VHSV) have been studied for possible interactions. VHSV has been a long-standing concern for the aquaculture industry, particularly for rainbow trout (*Oncorhynchus mykiss*) in Europe (Skall et al., 2004; 2005; Groocok et al., 2012). The virus belongs to the novirhabdovirus genus, and is a bullet-shaped, enveloped virion that is 180 nm long and 60 nm in diameter. The genome is arranged as a single 11-kb strand of negative-sense ssRNA, encoding 6 genes. In this chapter, ciliates have been found for the first time to inactivate a fish virus, VHSV.

5.2 Materials and Methods

5.2.1 Virus, cell line, and ciliates

Preliminary work on the effect of incubation with *Tetrahymena thermophila* on viral titre was done with several strains of viral hemorrhagic septicemia virus (VHSV) and in all cases titre declined. The formal demonstration of this viral inactivation and all work presented here was done with strain U13653 (type IVb) from Dr. J. Lumsden (Department of Pathobiology, University of Guelph, Guelph, ON) that had originally been isolated from Lake Ontario (Lumsden et al., 2007) and will be designated simply as VHSV.

VHSV was propagated on the cell line, EPC (*epithelioma papulosom cyprini*), which recently has been found to be a fathead minnow cell line (Winton et al., 2010). The cells were routinely grown at 22 °C, and the growth medium was L-15/10 % FBS (fetal bovine serum). The L-15/10 % FBS was Leibovitz's L-15 (Hyclone), with 2 mM L-glutamine (Hyclone), 10 % FBS (Hyclone) and penicillin (100 IU/mL) / streptomycin (100 µg/mL) solution (Sigma-Aldrich). VHSV was produced on confluent monolayers of the cell line EPC in 75 cm² flasks at 14 °C. The medium for viral propagation was the same as the growth

medium except with 2 % FBS and is written as L-15/2 % FBS. Once the virus infection had destroyed the EPC monolayer, the cell lysate together with the medium was centrifuged for 5 min at 3000 x g. The supernatant was passed through a 0.2 µm syringe filter (Pall Corporation, Washington, NY) and frozen at - 80 °C. This solution is referred to as VHSV and its infectivity was expressed as the median tissue culture infectious dose (TCID₅₀), which was determined on EPC as described by Pham et al (2011). Typically this stock solution had a titre of approximately 10⁷-10⁸ TCID₅₀/mL.

Tetrahymena thermophila (strain B1975) was obtained from Dr. D. Lynn (Department of Integrative Biology, University of Guelph, Guelph, ON). The ciliates were maintained at room temperature in protease peptone, yeast extract (PPYE) as described previously (section 2.2.1; Pinheiro et al., 2007; 2008). PPYE consisted of 0.125% (w/v) dextrose (Sigma), 0.5% (w/v) proteose peptone (Becton-Dickinson, Franklin Lakes, NJ) and 0.5% (w/v) yeast extract (Becton-Dickinson) dissolved in MilliQ water. Ciliates were enumerated with a Z2 Coulter Particle Counter and Size Analyzer (Coulter, Luton, UK).

5.2.2 Incubating the ciliates with VHSV

The incubation of the virus with the ciliates was initiated and carried out as follows. The stock VHSV solutions were diluted in L15/10 % FBS to varying degrees, depending on the experiment as described in the figure legends. Mid to late log phase cultures of ciliates in PPYE were centrifuged for 10 min at 1000 x g. The pelleted ciliates were resuspended in L-15/2 % FBS and distributed into 15 mL centrifuge tubes to give either 50,000 or 500,000 ciliates/tube. The ciliates were again collected by centrifugation and the pellets resuspended in a volume, usually 5 mL, of L-15/2 % FBS with VHSV to give densities of approximately 10,000 or 100,000 ciliates/mL. The tubes were incubated for up to 72 h at 14 °C, 22 °C or 30 °C. Heat-killed ciliates were also tested to evaluate whether the ciliates had to be living in order to influence VHSV titre. The ciliates were killed by heating for 3 min at 54 °C, and confirmed to have been killed by microscope observation, and then incubated with VHSV for 24 h in L-15/2 % FBS.

5.2.3 Assessing the infectivity of VHSV after incubation with ciliates

Ciliate/VHSV incubations were terminated in one of three ways to assess the best method for separating ciliates from virus. Following termination, all samples frozen at -20 °C until ready for assay. Ciliates and VHSV in L-15/2 % FBS were first mixed at a ratio of 10 to 1 and maintained at 22 °C for 24 h. Simple termination involved moving the ciliate/VHSV sample directly to -20 °C, with no modifications. Centrifugation for 10 min at 1000 x g, was tested for separating ciliates, removing the VHSV-containing supernatant to -20 °C. Syringe filtration (0.2 µm) was the final method tested for removing ciliates, and filtrate was moved to -20 °C. After all samples were collected, these were thawed and applied to EPC cultures for the detection of VHSV. Viral infectivity of thawed samples was determined using the TCID₅₀ method with scoring of plates occurring after seven days. Data were first expressed as log TCID₅₀/mL, then transformed by subtracting from the ciliate-free control, which was referred to as log reduction factor.

5.2.4 Visualizing VHSV/ciliate interactions

Two microscopic approaches were undertaken to visualize possible interactions between VHSV and the ciliates. One involved labeling the RNA genomes of VHSV with SYBR Gold nucleic acid stain (Invitrogen, Eugene, OR). This stain detects DNA, ssDNA, and RNA (Tuma et al., 1999) and has been used to stain viruses in environmental water samples (Chen et al., 2001) and to label dsDNA (T4) (Pinheiro et al., 2007) and ssRNA (MS2) phages (Pinheiro et al., 2008) in the laboratory. After labeling, samples were examined by epifluorescence microscopy with a Nikon fluorescence microscope. The second approach was immunocytochemical. A mouse monoclonal antibody (IP1H3; Lorenzen et al., 1988) against the G protein of the VHSV envelope was used to stain samples that were subsequently viewed with Zeiss LSM 510 META confocal microscope.

For fluorescent labeling, VHSV was mixed with the SYBR Gold dye solution (10,000 x stock concentrate; 2.5 x working solution) and incubated for 10 min in the dark at room temperature. As a control, a VHSV-free SYBR Gold working solution was incubated for 10 min in the dark. Both solutions were passed through Amicon Ultra-4 100K centrifugal filters

(Millipore Corporation, Billerica, MA) and centrifuged for 10 min at 5,000 x g to remove unbound dye. Three volumes of sterile L-15 with 10 % FBS and antibiotics were passed through each filter, and the concentrate was returned to its original volume in sterile L-15 with 10 % FBS and antibiotics. *Tetrahymena thermophila* (100,000 cells/mL) were maintained in these solutions in the dark at 22 °C. After 24 h the ciliates were fixed in 10% neutral, buffered formalin (1 % final concentration) for 5 min, and observed by fluorescence microscopy (excitation, 450 nm; emission, 520 nm).

For immunocytochemistry, *T. thermophila* (final density 100,000 cells/mL) were first mixed with VHSV for 24 h at 22 °C, isolated by centrifugation, washed, and resuspended in sterile L-15 with 10% FBS and antibiotics. At 0, 15, 30, and 60 minutes after movement to sterile media, ciliates were collected and prepared for immunostaining. Cells were prepared following the coverslip method outlined by Stuart and Cole (2000). The fixative used was 3% paraformaldehyde, 0.25% Triton X-100 and cells were fixed overnight at 4 °C. Ciliate nuclei were stained using DAPI (1 µg/mL; Sigma Aldrich). Coverslips were incubated with mouse mAb directed to the G protein of the VHSV envelope (IP1H3; Lorenzen et al., 1988) at a 1:100 dilution, followed by Alexafluor 488 labeled goat anti-mouse IgG (H+L), at a 1:200 dilution (Molecular Probes, Eugene, OR). Dilutions of antibodies were performed in blocking buffer (1% BSA in PBS). Slides were viewed using a Zeiss LSM 510 META confocal microscope using an argon and 405 nm laser light source. Excitation and emission wavelengths were 340 nm/488 nm for DAPI and 495 nm/519 nm for the immunostaining.

5.2.5 Testing the retention and release of infectious VHSV by the ciliates

The possibility was tested that the ciliates could retain some virus despite being washed and then later release infectious VHSV. The experimental setup for testing this consisted of culture wells (12-well plates, BD Biosciences) in which inserts (BD Biosciences) had been placed to divide each well into an upper chamber and a bottom chamber separated by a barrier with 0.4 µm pores. Previously, ciliates were shown to be unable to pass through pores of this size (Chapter 3). The bottom chambers received EPC cells. These cells were allowed to form monolayers over the growth surface before additions were made to the top chambers.

The top chambers received L-15/2 % FBS either alone or with 1,500 ciliates per chamber. The L-15/2 % FBS in the top chamber was continuous with the L-15/2 % FBS in the bottom chamber. The ciliates added to the top chambers were incubated for 24 h in L-15/2 % FBS either with or without VHSV as described previously. After 24 h, some aliquots of the ciliate/VHSV incubations were added directly to the top chambers (referred to as the unwashed ciliates), whereas for other aliquots, the ciliates were collected by centrifugation and washed (the washed ciliates). The washing was done by centrifuging for 10 min at 1,000 x g, aspirating the supernatant, and resuspending the ciliate pellet in L-15/2 % FBS. This was repeated three times before adding the ciliates to top chambers. The 12-well plates were then incubated at 14 °C and observed daily for up to 7 days for the appearance of CPE in the monolayer of EPC cells in the bottom chamber.

5.2.6 Statistics

All statistic tests were run using GraphPad Prism v.4.0c for Macintosh. Infectivity data were log transformed prior to graphing, and where noted were further normalized to respective control data, by dividing by the control mean, or expressed as a decrease from the initial infectivity of the sample, by subtracting sample data from the mean initial infectivity for each trial replicate. For these calculations the control mean standard deviation was not considered. Specific tests conducted, along with numbers of replicates for each data set are presented in the text and in figure legends, respectively.

5.3 Results

5.3.1 Effect of VHSV on ciliates

The presence of VHSV in the medium had no visible impact on *T. thermophila*, regardless of the incubation temperature. At either 22 or 14 °C, the shape and swimming of the ciliates were the same in L-15/2 % FBS without or with the virus. In both cases, shape and swimming were impaired during the first few hours after the ciliates had been put in L-15/2 % FBS. However, by 24 h with or without virus most ciliates were swimming and capable of phagocytizing fluorescent beads (1 µm). At 14 °C with or without VHSV, the ciliates continued to swim sluggishly after 24 h and very few beads were internalized.

5.3.2 Effect of ciliates on VHSV titre

Incubating VHSV with ciliates reduced the viral titre, regardless of the method used to terminate ciliate VHSV incubations. After 24 h at 22 °C, VHSV/ciliate cultures at a ratio of 1 virus to 10 ciliates were terminated in three different ways, and regardless of the termination method, VHSV was still detected but the titres had been dramatically reduced from the starting titre (Figure 5.1; $P < 0.05$; unpaired t test)). By contrast, when heat-killed *T. thermophila* were incubated with VHSV for 24 h at 22 °C, only a slight decline in viral titre was observed (Figure 5.2). Therefore, only living ciliates brought about a significant reduction in viral titre.

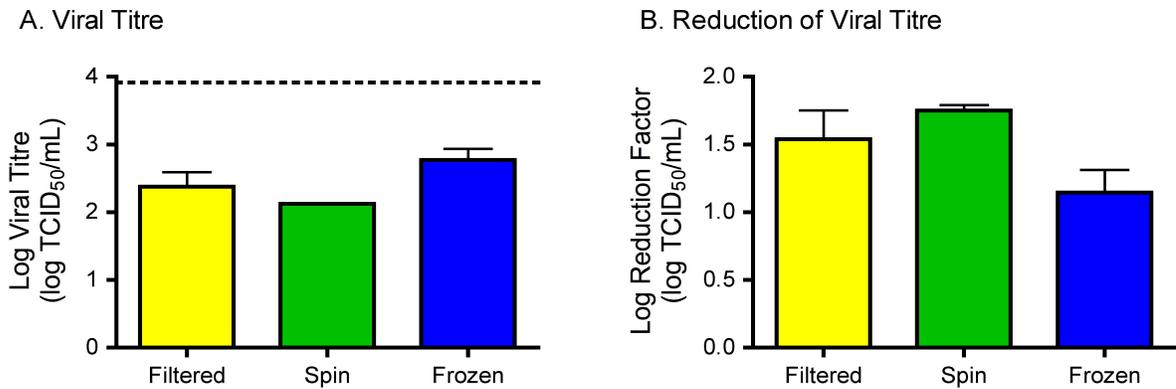


Figure 5.1 Viral titre after ending VHSV/ciliate incubations by different methods

Tetrahymena thermophila were mixed with VHSV at 1:10 virus to ciliate ratio, and maintained at 22 °C for 24 hours. The infectivity values following three methods for collecting ciliates were expressed as measured viral titre at 24 hours (**A**) or expressed as the log reduction factor, from the cell-free control value at 24 hours (**B**). Plotted on the graph is mean \pm SE (n greater than 4). The dotted line represents the mean of the initial viral titre (n = 6). A one-way ANOVA with Bonferroni posttest showed no significant difference between the three separation methods ($p > 0.05$).

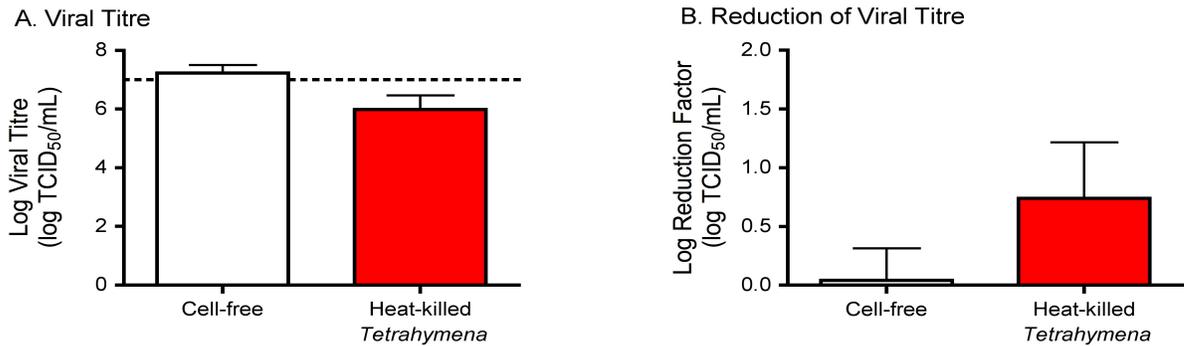


Figure 5.2 Change in viral titre upon incubating VHSV with heat-killed ciliates

Tetrahymena thermophila were killed by heating at 54 °C for 3 min and ultimately mixed with VHSV in L-15/FBS at 22 °C. Remaining viral titre was assayed at 24 h and compared to VHSV maintained in cell-free medium for 24 h (A). The dotted line indicates the mean starting titre of pooled samples from both conditions. For each condition, the reduction in viral titre from initial time to 24 h was calculated and presented (B). Bars represent mean of collected data (\pm SE; n = 5). Neither condition showed a significant change from initial titre over 24 h ($p > 0.05$; unpaired t test).

Of the three methods for terminating incubations of VHSV with living ciliates, the simplest was to place cultures directly into a -20 °C freezer. When these were thawed and applied to EPC monolayers for the detection of VHSV, fragments of ciliates and ciliate bodies were seen, but cytopathic effects (CPE) still developed in EPC. The CPE was due to VHSV and not to ciliate fragments/bodies because when these were obtained from ciliate cultures that had not been exposed to VHSV, no CPE developed. Another way of terminating incubations was to centrifuge cultures to pellet the ciliates and to apply the supernatants to EPC cultures. Finally incubations were terminated by syringe filtering cultures and adding the filtrates to EPC cultures. In both cases CPE subsequently developed in EPC cultures. Regardless of the termination method, the VHSV titre was reduced by incubation with ciliates and to a similar extent ($p > 0.05$; Bonferroni post-tests), approximately 1.5 log units (Figure 5.1). These results suggest that any carryover of ciliate material into the viral assay on EPC does not interfere with the development of CPE and the measurement of viral titre. Therefore in subsequent experiments, freezing was used to end incubations of VHSV and *T. thermophila*.

5.3.3 Effect of incubation time with ciliates on VHSV titre

As the time of incubating VHSV with *T. thermophila* at 22 °C increased, the VHSV titre progressively decreased (Figure 5.3). This was true at two different virus to ciliate ratios: 100 to 1 and 10^5 to 1. The decline in titre with time was similar at both ratios. Over 48 hours at 100 : 1 virus per ciliate (Figure 5.3A), VHSV infectivity significantly decreased by 4.288 ± 0.546 log units from the starting titre in the presence of ciliates ($p < 0.0001$; one-way ANOVA). The decrease in infectivity was not significant by the first measurement (4 h), having decreased by less than 0.1 log units, but by 24 h had decreased 3.024 ± 0.597 log units ($p < 0.0001$; Bonferroni multiple comparisons post-test). A linear regression of the decline in infectivity over time with ciliates resulted in a decline of 0.095 ± 0.016 log (TCID₅₀/mL) h⁻¹ and was significantly different from the regression of the line produced from ciliate-free data ($p = 0.006$; F test). The stability of VHSV at 22 °C without the presence of ciliates was confirmed by a ciliate-free control, run in parallel with the samples over 48 h. The slope of the linear regression of these data was not significantly different from zero ($p >$

0.05; F test). The two slopes were found to be significantly different by regression analysis ($p < 0.05$; ANCOVA).

Over 48 hours at 10^5 : 1 virus per ciliate (Figure 5.3B) the virus titre decreased by 5.927 ± 0.560 log units from the initial titre ($p < 0.0001$; one-way ANOVA). The decrease in titre was significant by the 24 h, having decreased by 2.103 ± 0.642 log units ($p < 0.05$; Bonferroni multiple comparisons post-test). A linear regression of the decline in infectivity over time with ciliates resulted in decline of 0.124 ± 0.021 log (TCID₅₀/mL) h⁻¹ and was significantly different from ciliate-free data ($p < 0.05$; ANCOVA).

5.3.4 Effect of incubation temperature

In contrast to incubating VHSV with ciliates at 22 °C, incubations either with or without the ciliates at 14 °C brought about no change in the viral titre and incubations at 30 °C led to a decline in titre either with or without the ciliate (Figure 5.4). For 14 °C, measured titre of ciliate-free control did not differ significantly from samples containing *Tetrahymena* at any time point ($p > 0.05$; one-way ANOVA with Bonferroni posttest). For 30 °C without ciliates (the control), the titre did not differ significantly from samples containing *Tetrahymena* at any time point ($p > 0.05$; one-way ANOVA with Bonferroni posttest). Previously, temperatures above 26 °C were shown to inactivate VHSV (Pham et al., 2011). For 30 °C with ciliates, the slope was not statistically different from the control ($p = 0.7942$; F test). However, the ciliates enhanced the decline in titre at 24 h but not at 48 h ($p < 0.05$ for change in titre from 0 to 24 h via Bonferroni multiple comparisons post-test; $P < 0.001$ for one-way ANOVA of data collected over 48 hours).

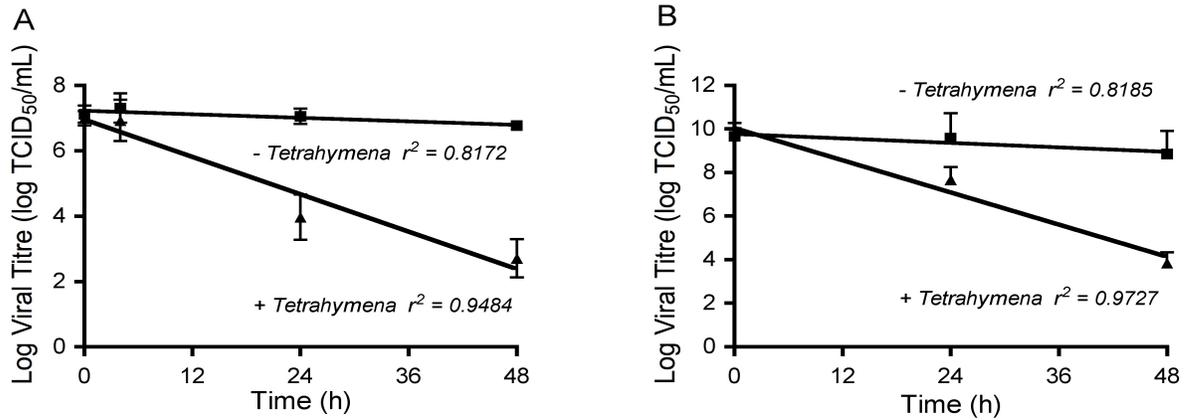


Figure 5.3 Change in viral titre as VHSV/ ciliate incubation time increased

Two densities of *T. thermophila* were mixed with VHSV and the change in infectivity was assayed over time. The higher density was 100,000 cells/mL (100 virus : 1 ciliate), **A**. The second density was 10,000 cells/mL (10⁵ virus: 1 ciliate ratio), **B**. Points on graph are mean ± SE (n greater than 4). Linear regression is plotted on the graph, along with R² value.

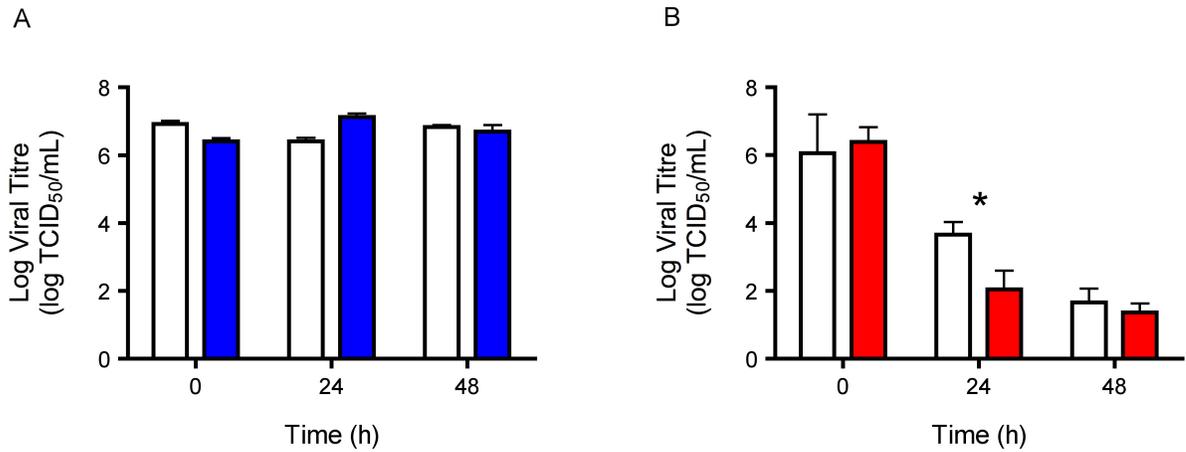


Figure 5.4 Change in viral titre upon incubating VHSV with ciliates at 14 and 30 °C

Two temperatures were tested for their effect on ciliate-mediated reduction of VHSV infectivity. Ciliate - virus mixtures, along with cell-free control, were maintained at either 14 °C, **A**, or 30 °C, **B**. The starting density of *T. thermophila* was 100,000 cells/mL (100 virus : 1 ciliate), and points on the graph are mean ± SE (n greater than 4). Data presented are ciliate-free control (unshaded) and ciliate : virus mixed (shaded). Asterisk indicates significance from cell-free control at that time point ($p < 0.05$; unpaired t test).

5.3.5 Visualizing associations of VHSV with ciliates

VHSV stained with SYBR Gold nucleic acid stain were added to *Tetrahymena* (100 virus : 1 ciliate) for 24 hours and then viewed by epifluorescence microscopy. Fluorescent particles could be seen localized within the cell in compartments that were approximately 5 μm in diameter (Figure 5.5B). These compartments, having the approximate size and shape of food vacuoles, were prominent towards the posterior end of the cell. Although the presence of some fluorescence within the ciliate was universal in the cells viewed, localization included fluorescent vacuoles appearing throughout the cell, including in the anterior. Those ciliates that were given a VHSV-free control solution did not show fluorescence within the cell after 24 hours (Figure 5.5D).

Immunofluorescence staining of *T. thermophila* for VHSV G protein at times after ending incubations with VHSV revealed strong but transitory staining of cilia. *Tetrahymena thermophila* that were not (control) or were exposed to VHSV were examined by confocal microscopy and the results presented in Figures 5.6 to 5.8 and in Appendix C. The control *T. thermophila*, which was VHSV-free ciliates labelled with anti-G, showed no staining (Figures 5.6A1 to 5.6A4). The VHSV-exposed *T. thermophila* stained but the staining pattern changed from 0 to 60 minutes after ending the viral exposure. Immediately afterward (time 0), most *T. thermophila* had diffuse fluorescence at the cell surface, with some *T. thermophila* showing more intense staining around the oral ciliature (Figures 5.6B1 to 5.6B4). Yet for a few *T. thermophila*, the cilia stained brightly and distinctly (Figures 5.6C1 to 5.6C4). At 15 minutes, the diffuse surface staining had largely disappeared and cilia stained intensely (Figure 5.7A1 to 5.7A4). The cilia continued to stain strongly at 30 minutes, with little or no diffuse staining being present (Figure 5.7B1 to 5.7B4). By 60 minutes most *T. thermophila* did not stain, however a few did, and for these, the staining was in small vesicles near the surface and slightly larger vesicles more interiorly positioned (see arrows in Figure 5.8).

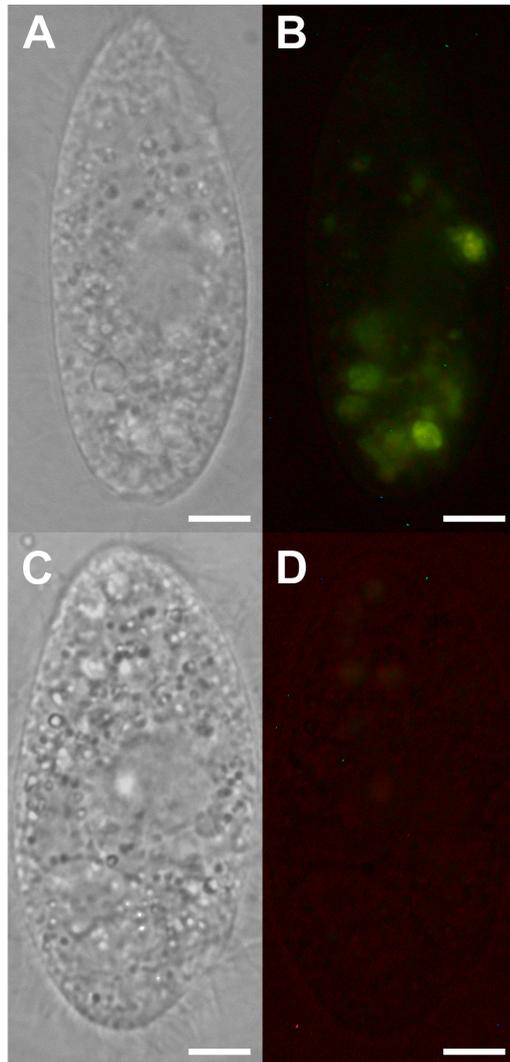


Figure 5.5 Phase-contrast and fluorescence microscopy of ciliates after incubation with SYBR labeled VHSV

The nucleic acid stain SYBR Gold was used to stain VHSV, and allowed viewing concentrations of labeled virus inside the *Tetrahymena* after the two were mixed for 24 hours (A and B). Ciliates were also mixed with a VHSV-free dye solution that was washed in the same manner as the VHSV sample (C and D). Cultures were fixed in 10 % neutral buffered formalin immediately prior to microscopy. Bars are 10 µm in all panels.

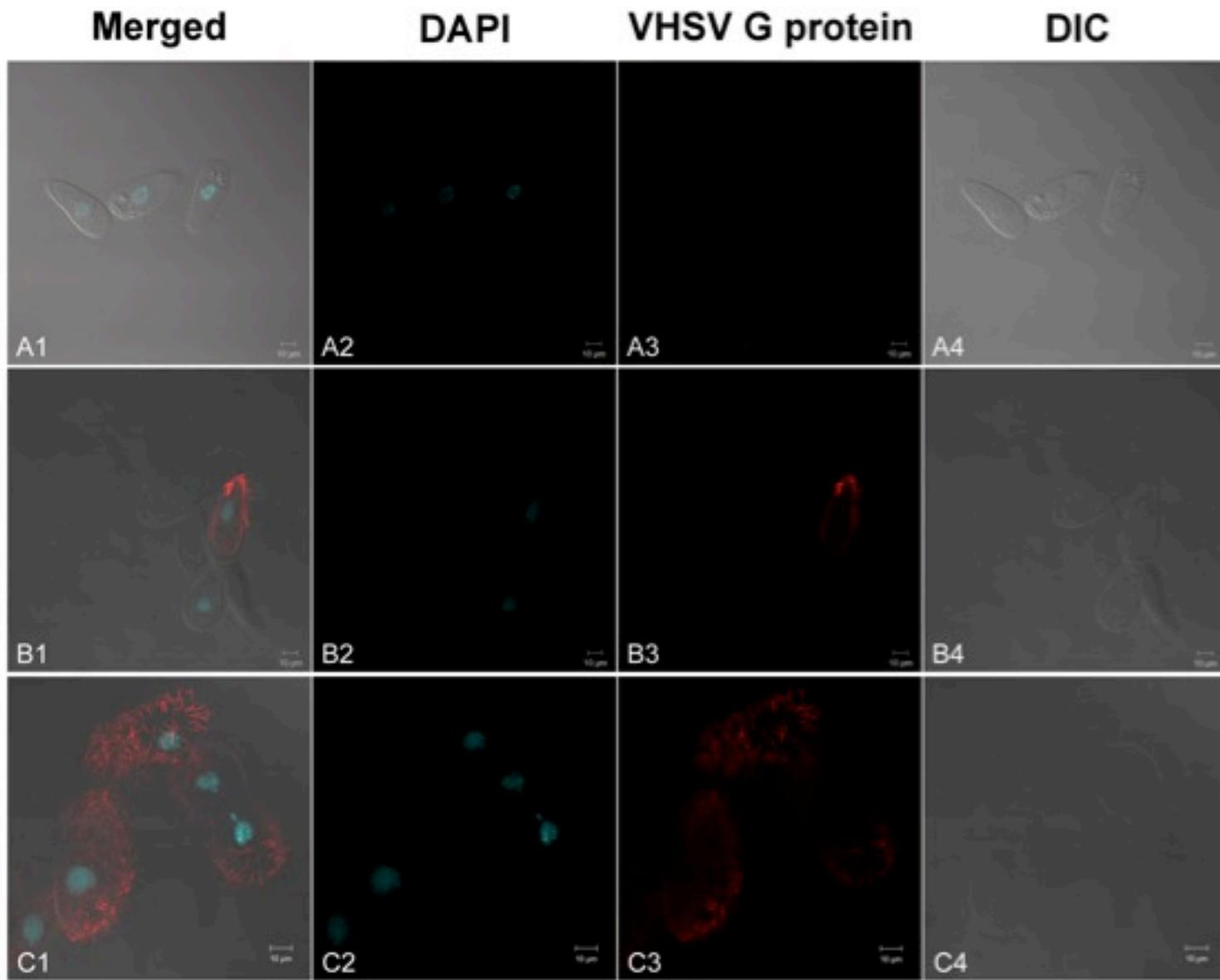


Figure 5.6 Immunofluorescent staining of ciliates for VHSV G protein immediately after ending VHSV/ciliate incubations

Ciliates were pulsed with VHSV for 24 hours before washing, and subsequent immunostaining with mAB directed to VHSV envelope protein G. Control ciliates, not maintained with VHSV are presented as panels **A1,2,3,4**. Ciliates maintained with VHSV were prepared for immunostaining immediately after being moved to sterile medium and two fields of view are presented, the first with intensely stained oral cilia (**B1,2,3,4**), and a second where diffuse staining partially covers the surface of some cells (**C1,2,3,4**). From left to right, confocal images are merged multi-channel image, DAPI nuclear stain, G protein immunostain, and differential interference contrast (DIC) single channel images. Bars are 10 μ m in all panels.

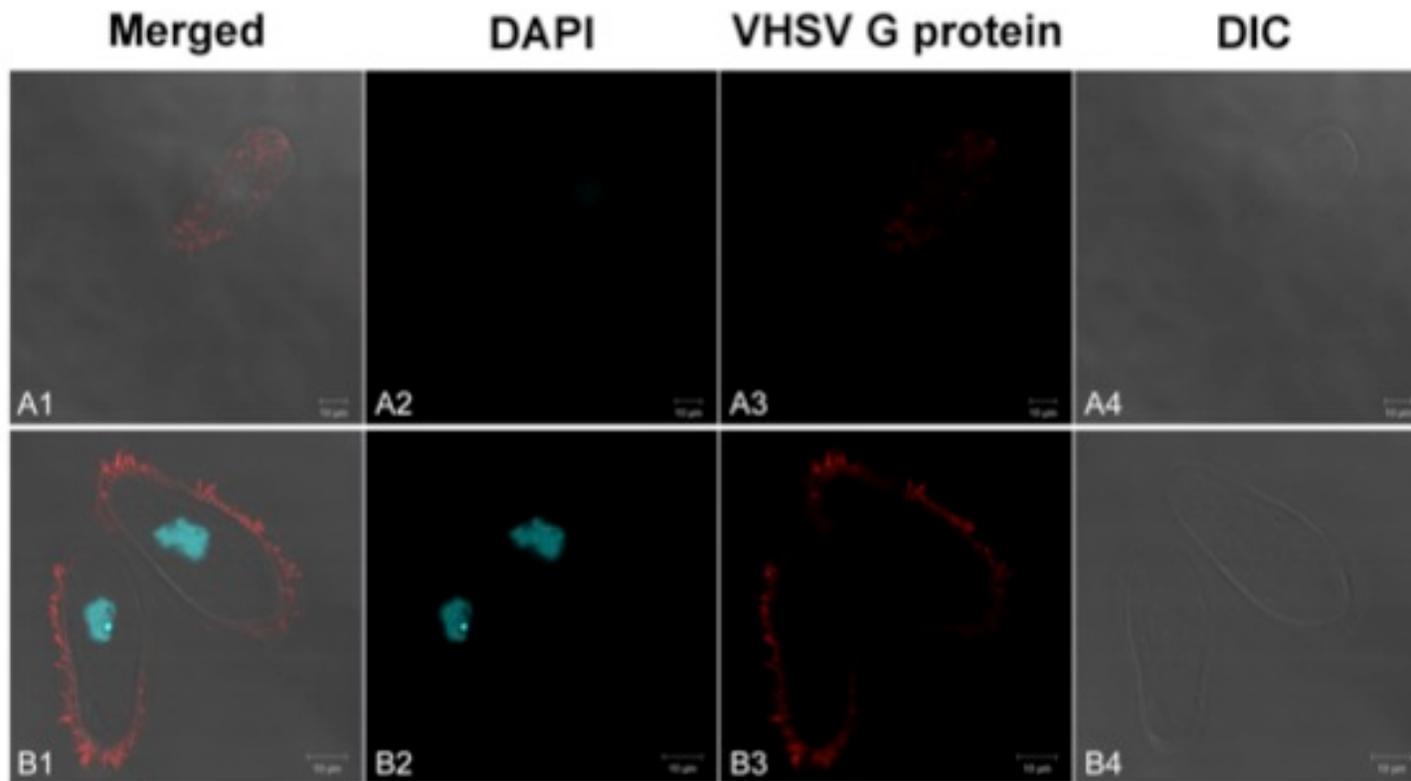


Figure 5.7 Immunofluorescent staining of ciliates for VHSV G protein 15 and 30 min after ending VHSV/ciliate incubations

Ciliates that were moved to sterile medium for 15 min (A1,2,3,4) or 30 min (B1,2,3,4) after VHSV exposure were prepared for immunostaining with mAB directed to VHSV envelope protein G. From left to right, confocal images are merged multi-channel image, DAPI nuclear stain, G protein immunostain, and differential interference contrast (DIC) single channel images. Scale bars are 10 μm in all panels.

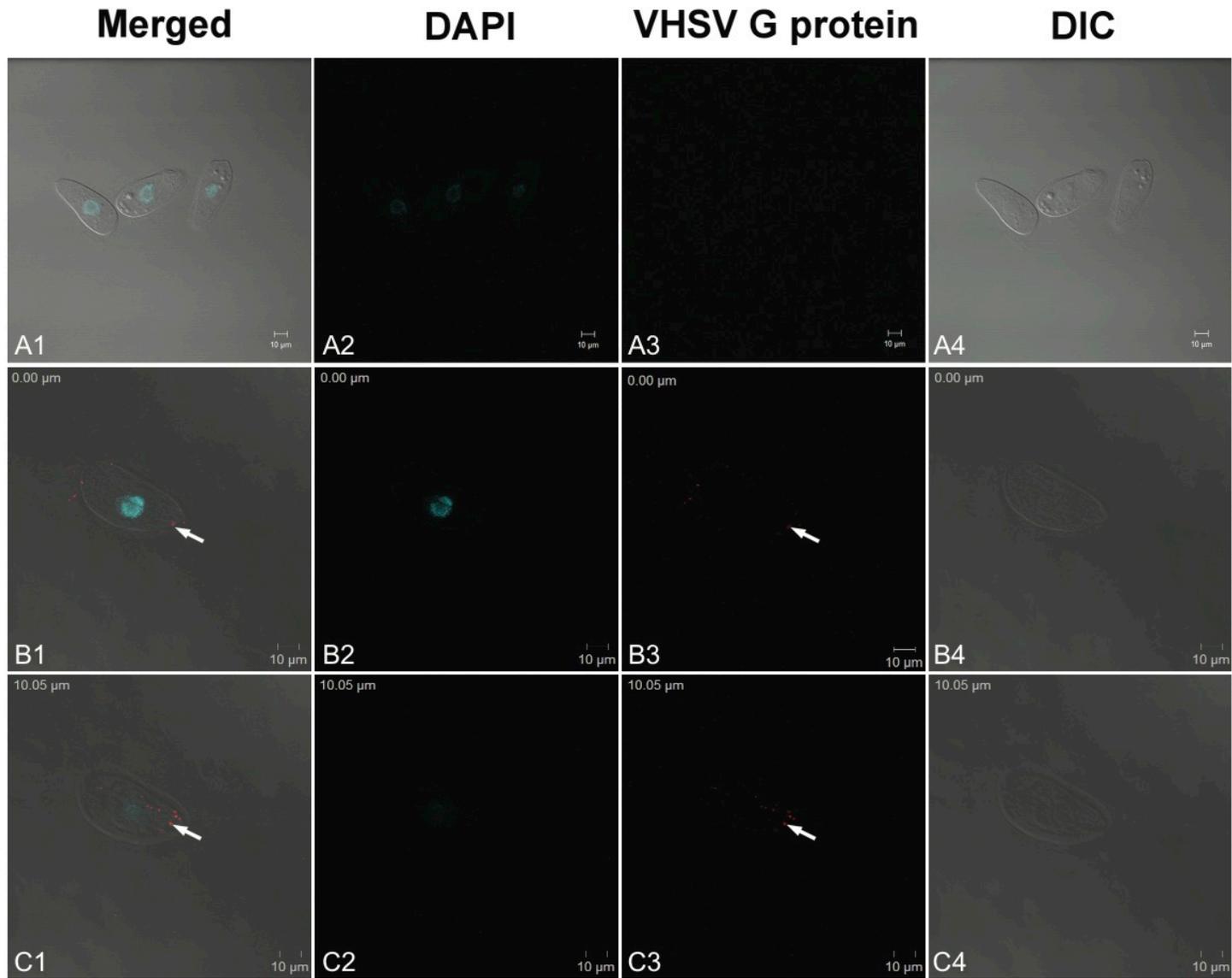


Figure 5.8 Immunofluorescent staining of ciliates for VHSV G protein 60 minutes after ending VHSV/ciliate incubations

Confocal micrographs of ciliates that had been in sterile medium for 60 min after separation from VHSV and washing. A control image of ciliates that were not in contact with VHSV is presented as panels **A1,2,3,4**. Micrographs from two slice depths from a Z-stack of ciliate that had contacted VHSV previously are presented as **B1,2,3,4** (approximate centre of cell; labeled 0 μm), and **C1,2,3,4** (10.05 μm above focal plane of DIC). Arrows indicate discrete localization of G protein-containing vesicles. Confocal micrographs are presented, from left to right, as merged multi-channel image, DAPI nuclear stain, G protein immunostain, and differential interference contrast (DIC) single channel images. Bars are 10 μm in all panels.

5.3.6 Retention and release of VHSV by ciliates

As virions were associated both inside and outside the ciliates, the ability of ciliates to retain and release VHSV was tested in culture wells with inserts of a pore size of 0.4 μm that divided each well into a bottom chamber and a top chamber. When just ciliates were placed in the top chambers, the EPC monolayers in the bottom chambers remained intact. Thus, the ciliates did not release cytotoxic substances that moved through insert pores. When just VHSV was placed in the top chambers, EPC monolayers in the bottom chambers developed CPE and were destroyed. Thus virus could move through the pores separating the top and bottom chambers. Ciliates and VHSV were incubated together at 22 °C for 24 hours, then washed and moved into the top chambers of wells that had EPC monolayers in the bottom chamber. The EPC monolayers remained intact and no CPE developed (Table 5.1). Thus ciliates could be washed free of any infectious VHSV.

Table 5.1 Testing retention and release of VHSV by ciliates in culture wells with top and bottom chambers ^a

| Prior treatments to additions for top chamber | Additions into top chamber | Development of CPE in EPC on surface of bottom chamber |
|---|---|--|
| none | Ciliates | - |
| none | VHSV | + |
| VHSV and ciliate co-incubations for 24 h ^b | Supernatant after centrifugation ^c | + |
| VHSV and ciliate co-incubations for 24 h | Pellet of ciliates washed thrice ^d | - |

^a Top and bottom chamber were separated by culture inserts with pores of 0.4 µm.

^b Ciliates were added at 1,500 cells/mL to produce approximately 10⁵ VHSV : 1 ciliate ratio.

^c Centrifugation at 1,000 x g for 10 min.

^d Ciliates were collected into pellets by centrifugation at 1,000 x g for 10 min and resuspended into L-15/FBS. This was repeated two more times before the ciliates were added into the top chamber.

5.4 Discussion

Viral hemorrhagic septicemia virus (VHSV) is the first fish virus and rhabdovirus to join a list of viruses found to be susceptible to inactivation by ciliates. Previously, non-enveloped icosahedral bacteriophages (T4, T5 and λ) were shown to be inactivated by *Tetrahymena thermophila* (Pinheiro et al., 2007; Hennemuth et al., 2008). *Tetrahymena* also inactivated non-enveloped, icosahedral animal viruses (adenovirus and polio virus) (Sepp et al., 1992; Kim and Unno, 1996). For inactivation of enveloped, helical viruses, the first report was for the influenza virus (Groupe and Pugh, 1952), an orthomyxovirus, and now for the rhabdovirus, VHSV. However, the role of the ciliates in the inactivation of these two types of animal viruses appears to be different. With influenza virus, the inactivation was attributed to a factor present in both killed and living *Tetrahymena* (Groupe and Pugh, 1952). By contrast, with VHSV the viral titre was only significantly reduced by incubation with living *T. thermophila*. Heat-killed ciliates brought about only a slight inactivation. Thus VHSV inactivation appears to be an active process on the part of the ciliates and due to several kinds of interactions.

One way that *T. thermophila* appeared to inactivate VHSV was by internalizing the virus into food vacuoles. Internalization was suggested by the appearance of fluorescence bodies about the size of food vacuoles in the ciliates after their incubation with SYBR Gold labeled VHSV. Similar results were obtained previously with SYBR Gold labeled phages and internalization was attributed to either phagocytosis or macropinocytosis in the cytopharynx of the oral apparatus (Pinheiro et al., 2007; 2008). Phagocytosis generally involves particles $> 0.25 \mu\text{m}$ (Ramoino et al., 2012), whereas macropinocytosis internalizes fluid in vesicles larger than $1 \mu\text{m}$ (Swanson and Watts, 1995). VHSV, which have approximate diameters of 60 nm and lengths of 180 nm, might form some aggregates that are greater than $0.5 \mu\text{m}$ and be phagocytosed. Inside vesicles, the VHSV did not stain with the monoclonal antibody to VHSV G protein. In *Tetrahymena*, the pH of food vacuoles decreases to 4.0 - 3.5 during the first 60 min of phagosome formation (Nilsson, 1977), and low pH is known to change VHSV

G protein conformation (Mas et al., 2004). Therefore, an acid-induced change in G protein conformation possibly prevents the protein from being bound by anti-G antibodies.

VHSV inactivation could occur within the food vacuoles. VHSV is stable for sometime at pH 4 (Dixon et al., 2012) so the low pH alone might not bring about inactivation. However, over a longer period of time in phagosomes VHSV would also be subject to digestion by proteases and RNases from lysosomes. Ultimately enzymatic digestion then could inactivate VHSV. In support of this inactivation pathway is the lack of inactivation at 14 °C, a temperature where phagocytosis by *Tetrahymena* is greatly reduced (Nilsson, 1976), as likely would be the activity of lysosomal enzymes.

Another interaction of the virus and *T. thermophila* was the apparent selective binding of VHSV to cilia, which is a newly recognized phenomenon in ciliate/virus relations. The interaction was visualized by immunofluorescent staining for VHSV G protein and confocal microscopy. Fifteen to thirty minutes after a 24 h incubation of *T. thermophila* with VHSV had ended, cilia stained specifically and strongly. This suggests that the surface of cilia contains component(s) that bind VHSV, and that these binding sites are more abundant in the ciliary membrane than in the cellular membrane making up the rest of the ciliate surface. Phosphatidylserine (PS) is thought to be the receptor for VHSV (Estepa and Coll, 1996) and for rhabdoviruses in general (Albertini et al., 2012) but other receptor molecules, including gangliosides, could be involved (Albertini et al., 2012). A precedent for the cilia membrane being distinctive is found in another protozoan, *Trypanosoma brucei*: the protein and lipid composition of the flagellar membrane was different from the cell surface membrane (Balber, 1990; Tyler et al., 2009). Why the staining of cilia was much less evident immediately after the VHSV exposure had ended was likely due to most virus being washed off during the centrifugation and aspiration steps that terminated exposure to the virus. However, despite the washing steps, some virions would be carried over in small volumes of medium that failed to be aspirated and by being trapped nonspecifically on *T. thermophila*. Over the next 30 minutes after the washing, these virions would bind specifically to cilia, which would exhibit fluoresce upon immunofluorescent staining for VHSV G protein.

At 60 minutes, fluorescence was seen in small vesicles at the periphery and slightly larger vesicles in the interior of a few *T. thermophila*. Fluid phase and receptor-mediated endocytosis has been described at the surface of ciliates (Albertini et al., 2012), especially at the parasomal sac (Elde et al., 2005). These processes lead to the formation of small vesicles that ultimately fuse into food vacuoles (Albertini et al., 2012). Therefore, the smallest fluorescent vesicles could have arisen from VHSV being internalized at the parasomal sac through pinocytosis (fluid phase endocytosis) and fused to form the larger fluorescent vesicles. In turn, the fluorescent vesicles could fuse with food vacuoles where the fluorescence would be lost and the virus inactivated as discussed in the previous paragraph.

At 60 minutes the staining of cilia for VHSV G protein had largely been lost but the mechanism(s) behind this loss is unclear. Possibly the virions that were initially bound to cilia move over time down the cilia to become internalized at the parasomal sacs, which are at the base of cilia. This would make the cilia free of virions to stain. Yet why free virions in the medium do not repopulate the binding sites and keep the cilia fluorescent needs an explanation. One possibility is that the number of free virions remaining in the medium to bind cilia was inadequate to maintain the fluorescent staining of cilia. This is supported by the experiment to test the capacity of *T. thermophila* to retain and release infectious virions. After being incubated with VHSV for 24 h and then being washed, *T. thermophila* did not release into the medium enough VHSV to cause CPE in a monolayer of susceptible fish cells, EPC. An alternative possibility is that the internalization of the virions initially bound to the cilia removed binding sites for VHSV so that cilia were no longer able to bind VHSV and stain for G protein. Regardless of the mechanism, the staining of cilia for G protein was transitory.

Relatively little is known about the possible interactions between VHSV and aquatic organisms other than fish, but the interactions are important to define in order to understand how the virus survives in the environment and the disease is transmitted. In this report the ciliate *T. thermophila* inactivated virus by physically coming into contact with the virus

particle, whereas among some bacterial species virus inactivation is caused by released antiviral substances (Myouga et al., 1995). Among invertebrates, VHSV has been detected in amphipods and leeches but whether these associations indicated replication, inactivation, or a neutral interaction was unknown (Faisal and Schulz, 2009; Faisal and Winters, 2011), although interestingly VHSV has been reported to replicate in insect cells (Lorenzen and Olesen, 1995). For aquatic vertebrates, freshwater turtles might support viral replication and be alternative hosts (Goodwin and Merry, 2011). Based on the results of this study, ciliates should be considered as part of a potential biotic barrier to VHSV transmission through water.

Chapter 6

Activation and transport of an aquareovirus, chum salmon reovirus (CSV), by the ciliates *Tetrahymena thermophila* and *T. canadensis*

6.1 Introduction

Viruses are the most abundant biological entities in water ($10^7/\text{mL}$ to $10^8/\text{mL}$) (Breitbart, 2012; Maranger and Bird, 1995), whereas ciliates are present at numbers five or more orders of magnitude lower. Nonetheless, viruses and ciliates interact because they are constantly and nonspecifically internalizing particles and water. The vast majority of the interactions should be with bacteriophages, which account for most of aquatic viruses (Breitbart, 2012). However, the ciliates could come across animal viruses routinely in some man-made environments and episodically and spatially in natural environments. In sewage and sewage treatment plants, both human viruses and ciliates are abundant and can interact (Curds and Cockburn, 1970; Estaban et al., 1992). Episodically, encounters of ciliates and viruses occur around dying or dead aquatic animals that had been virally infected. Spatially, ciliates and viruses might interact around fish chronically shedding viruses, as some ciliates feed on detritus from living fish (Basson and Van As, 2006).

Phage/ciliate interactions have been studied in the laboratory and appear to vary with the phage. Several phages (T4, T5, MS2, λ and ΦX174) have been incubated together with a few ciliates, *Tetrahymena thermophila*, *T. pyriformis* and *T. silvana* (Akunyili et al., 2008; Hennemuth et al., 2008; Pinheiro et al., 2007, 2008). In all cases, the phages are ingested but what happens next appeared to depend on the phage type. T4, T5 and λ were inactivated (Pinheiro et al., 2007; Hennemuth et al., 2008). For MS2, inactivation did not occur over 6 h (Akunyili et al., 2008) and was incomplete over 48 h of incubation with *T. thermophila* (Pinheiro et al., 2008). Internalized ΦX174 were not inactivated and instead led to the ciliates transporting the phage, which has been referred to as protistophoresis, and providing

protection from UV (Akunyili et al., 2008). The lack of Φ X174 inactivation was attributed to either resistance of the icosahedral capsid to proteases and/or the single-stranded DNA genome to nucleases (Akunyili et al., 2008).

Less is known about the interactions of ciliates with animal viruses but they are important to know in order to understand the survival and transmission of viruses in the environment. *Tetrahymena* spp. have been shown to inactivate several human viruses. These were influenza virus (Groupe and Pugh, 1952; Groupe et al., 1955), adenovirus (Sepp et al., 1992), and poliovirus (Kim and Unno, 1996). Recently a fish virus was studied for the first time (Chapter 4), as *T. thermophila* inactivated the novirhabdovirus, viral hemorrhagic septicemia virus (VHSV). VHSV has a negative-sense single-stranded RNA genome and is enveloped. In this study, interaction of a different type of fish virus, an aquareovirus (AqRV) called Chum salmon reovirus (CSV), with *T. thermophila* and *T. canadensis* was examined. The AqRV have double-stranded RNA genomes and capsids with inner and outer shells (Lupiani et al., 1995). For the first time a new interaction between ciliate and virus is described, activation, and for the first time the capacity of ciliates to transport or ferry a virus is demonstrated.

6.2 Materials and Methods

6.2.1 Virus, cell line, and ciliates

Stock cultures of Chum salmon reovirus (CSV) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). This aquareovirus was isolated from chum salmon ovarian fluid on the chinook salmon (*Oncorhynchus tshawytscha*) embryonic cell line CHSE-214 and deposited in ATCC by Dr. James Winton (Winton et al., 1981).

Three fish cell lines were used. The cell line CHSE-214 was used to propagate CSV as described previously (DeWitte-Orr and Bols, 2007). For specific experiments done at temperatures of 26 °C and above, use was made of the zebrafish embryo cell line, ZEB2J (Xing et al., 2008), and the epithelial fathead minnow cell line, EPC (Winton et al., 2010). As well, a few infection trials were constructed with a rainbow trout brain cell line, RTbrain that

previously had been shown to be unsusceptible to CSV (Bryson and Bols, unpublished). The cell lines were routinely grown at 18 °C, except ZEB2J at 26 °C. The growth medium was L-15 with 10 %, referred to as L-15/ 10 % FBS, and this consisted of Leibovitz's L-15, with 2 mM L-glutamine from Hyclone (Sigma-Aldrich, St. Louis, MO), fetal bovine serum (FBS; 10 % (v/v); Sigma-Aldrich) and penicillin (100 IU /mL) / streptomycin (100 µg /mL) solution (Sigma-Aldrich). For CSV propagation, stock CSV was added, at a 10⁻¹ dilution, to confluent monolayers of CHSE-214 in 75 cm² flasks containing L-15 with 2 % FBS. Virus was harvested when cytopathic effects had destroyed the monolayer, generally 4-7 days post-infection, by centrifugation of culture medium (5 min at 3000 x g). This supernatant was passed through a 0.2 µm syringe filter (Pall Corporation, Washington, NY) and frozen at - 20 °C prior to use for experimentation. This solution is referred to as CSV and its infectivity was expressed as the median tissue culture infectious dose (TCID₅₀), which was determined on CHSE-214 as described by DeWitt-Orr and Bols (2007). Typically this stock solution had a titre of approximately 10⁵-10⁶ TCID₅₀/mL.

Tetrahymena canadensis and two strains of *Tetrahymena thermophila*, B1975 and NP1, were used. *Tetrahymena canadensis* and *T. thermophila* (B1975) were obtained from Dr. D. Lynn (Department of Integrative Biology, University of Guelph, Guelph, ON). *Tetrahymena thermophila* NP1 was acquired from the *Tetrahymena* Stock Center (Cornell University, Ithaca, NY). The ciliates were maintained in proteose peptone yeast extract (PPYE) and enumerated with a Z2 Coulter Particle Counter and Size Analyzer (Beckman Coulter, Brea, CA) as described previously (Pinheiro et al., 2007; 2008; section 2.2.1). The PPYE consisted of 0.125 % (w/v) dextrose (Sigma-Aldrich), 0.5 % (w/v) proteose peptone (Becton-Dickinson, Franklin Lakes, NJ) and 0.5% (w/v) yeast extract (Becton-Dickinson) dissolved in MilliQ water. Cultures were routinely grown at 22 °C. However, NP1 is a temperature conditional mutant incapable of phagocytosis when grown at 30 °C (Orias and Pollock, 1975). Therefore, for experiments to exploit this deficiency, the ciliates were grown for at least 18 h at 30 °C prior to use.

6.2.2 Incubating the ciliates with CSV

The incubation of the virus with the ciliates was initiated and carried out as follows. The stock CSV solutions were diluted in L15/10 % FBS to varying degrees, depending on the experiment and described in the figure legends. The ciliates from mid to late log phase in PPYE were collected into pellets by centrifugation for 10 min at 1,000 x g. The pellets were resuspended in L-15/10 % FBS and distributed into 15 mL centrifuge tubes at either 50,000 or 500,000 ciliates/tube. The ciliates were again collected by centrifugation and the pellets resuspended in a volume, usually 5 mL, of L-15/10 % FBS with CSV to give different ciliate densities. For *T. thermophila* B1975, the densities were approximately 10,000 or 100,000 ciliates/mL; for *T. thermophila* NP1 the density was 10,000 ciliates/mL; for *T. canadensis* the density was 5,000 ciliates/mL. For *T. thermophila* B1975, the tubes were incubated for up to 96 h at 4 °C, 14 °C, 22 °C or 30 °C. For *T. thermophila* NP1, the tubes were incubated for up to 72 h at 22 °C or 30 °C. For *T. canadensis* the tubes were incubated only at 22 °C and for 48 h. In all cases samples were removed and filtered with a 0.2 µm syringe filter (Pall Corporation) before being frozen at -20 °C.

Dead ciliates were also tested in order to evaluate whether the ciliates had to be living in order to influence CSV titre. The ciliates were either heat-killed as described previously (Chapter 5) or killed by adding 1 ml of 10 % neutral buffered formalin (NBF) into 9 mL of culture. After 10 minutes in 1 % NBF, the ciliates were collected as a pellet by centrifugation at 1,000 x g for 10 min and washed three times with L-15/10 % FBS. Dead ciliates were resuspended in L-15/10 % FBS and incubated at 22 °C for 48 h with CSV at densities of 10,000 cells/mL (low density) or 100,000 cells/mL (high density) as described above for living ciliates. Ciliate-free controls were run concurrently for each density, and were treated similar to the ciliate-containing counterparts. At the end of incubations, samples were filtered with a 0.2 µm syringe filter (Pall Corporation) and frozen at -20 °C. Subsequently, samples were thawed, diluted to varying degrees in L-15/10 % FBS, and added to fish cell lines.

6.2.3 Assessing the infectivity of CSV after incubation with ciliates

The endpoint for judging an effect on CSV was the development of the characteristic cytopathic effect (CPE) of this virus in fish cell lines after introducing thawed samples into cultures. The CPE is the fusion of fish cells into syncytia to form large multinucleated cells that ultimately lyse (DeWitte-Orr and Bols, 2007; Winton et al., 1981). Four fish cell lines CHSE-214, RTbrain, EPC and ZEB2J were used. Susceptibility to CSV or to ciliate-treated CSV was tested at 18 °C, 26 °C and 30 °C for ZEB2J, 18 °C and 30 °C for EPC, and 18 °C for RTbrain. For these cell lines, susceptibility was judged by the presence of syncytia by the end of the trial period. Quantifying an effect on CSV titre was done with CHSE-214 at 18 °C beginning immediately upon adding ciliates to the virus, and viral infectivity was determined using the TCID₅₀ method as described previously (DeWitte-Orr and Bols, 2007). Data were first expressed as TCID₅₀/mL and then log transformed. In some cases, the results were expressed as the change in log value from the starting titre or ciliate-free control.

6.2.4 Ciliate-conditioned cell culture medium

To determine whether ciliates influence CSV through the release of factors that act on the virus, ciliate-conditioned medium was prepared and tested. *Tetrahymena thermophila* B1975 were added to L-15/10 % FBS at a density of 100,000 cells/mL and maintained for 24 h at 22 °C, to allow the ciliates to release enzymes and exudates. Ciliates were removed from the medium using a 0.2 µm syringe filter. CSV was added to this conditioned medium and maintained at 22 °C for 48 h. As a control, CSV was added to L-15/10 % FBS and maintained concurrently under the same conditions. At various times during this incubation, the viral titres were determined and expressed as described in section 6.3.1.

6.2.5 Visualizing CSV/ciliate interactions

CSV were fluorescently labeled using SYBR Gold nucleic acid stain (Invitrogen, Eugene, OR) by mixing with dye solution (10,000 x stock concentrate; 2.5x working solution) and allowing the mixture to incubate for 10 min in the dark at room temperature. This labeled virus solution was then passed through an Amicon Ultra-4 100K centrifugal filter (Millipore Corporation, Billerica, MA) and centrifuged for 10 min at 5,000 x g to remove unbound dye.

Three volumes of sterile L-15/10 % FBS were passed through the filter as a wash solution, and the concentrate was returned to its original volume with sterile L-15/10 % FBS. Labeled CSV were added to *T. thermophila* B1975 (100,000 cells /mL), maintained in the dark at 22 °C for either 6 h, or 24 h. Ciliates were then collected and fixed using 10 % NBF. Ciliates were viewed immediately using a fluorescence microscope equipped with a Nikon B-2A filter (excitation, 450 nm; emission, 520 nm).

6.2.6 Testing the retention and release of infectious CSV by the ciliates

Tetrahymena canadensis was given the opportunity to acquire, retain and release CSV. The endpoint for judging the outcome was the development of the characteristic CPE after the introduction of ciliates into CHSE-214 cultures. The CPE was the fusion of CHSE-214 into syncytia to form large multinucleated cells that ultimately rolled up off the growth surface and lysed (DeWitte-Orr and Bols, 2007; Winton et al., 1981). The conditions for the ciliates to acquire, retain and release CSV were as follows.

For acquisition, cultures of CSV-infected CHSE-214 in L-15/ 2 % FBS were first set up. Confluent CHSE-214 monolayers in 75 cm² cell culture flasks were infected with 1:10 dilution of CSV stock and incubated at 18 °C for 3 days. At 3 days the monolayer was still intact, although a few areas were developing small syncytia, and was carefully rinsed with three volumes of phosphate-buffered saline. A total of 15,000 ciliates in 10 mL of L15/ 10 % FBS was carefully added to each flask, which were incubated at 18 °C. After 18 h, 1 mL aliquots of the medium were placed into 15 mL centrifuge tubes to end the formal acquisition period and begin a protocol to separate any passively adsorbed CSV from the ciliates. The protocol was centrifugation through a step gradient of polysucrose (Histopaque-1119; Sigma-Aldrich) which in previous experiments appeared to remove ciliates of any surface-bound bacteriophages (Pinheiro et al., 2007; 2008).

The retention challenge began by centrifuging the ciliates through a step gradient of Histopaque-1119 as diagramed for bacteriophages in Figure 2 of Pinheiro et al., (2007). After 1 ml of the ciliate/CSV co-culture suspension was placed into a 15 mL centrifuge tube, 1 ml of Histopaque was layered under it using a sterile Pasteur pipette. The test tube was

centrifuged at 400 x g for 10 min at room temperature. This gave a bottom layer with a pellet of ciliates and a top layer of medium and CSV. The medium and some of the Histopaque was removed by aspiration. The pellet of ciliates was then resuspended in L-15/10 % FBS and washed. Washing consisted of three consecutive cycles of centrifugation at 1000 x g for 10 minutes, followed by aspiration, and resuspension. After the final resuspension, the ciliates in L15/ 10 % FBS were added to CHSE-214 cultures in 12-well tissue culture plates, to begin the release phase of the experiment.

The release step was done in wells that contained confluent monolayers of CHSE-214. The wells received approximately 1,500 ciliates in 1 mL of L15/10 % FBS were incubated at 18 °C, and observed daily by phase contrast microscopy for up to 7 days. During this time *T. canadensis* swam continuously above the monolayer. The formation of syncytia in the monolayer was the indicator that release had occurred.

Several controls were set up. One was to introduce into CHSE-214 cultures ciliates that had not been exposed to CSV-infected CHSE-214 beforehand. Another was to introduce into CHSE-214 cultures ciliates that had been killed by heating at 54 °C for 3 min before being exposed to CSV-infected CHSE-214. In both cases the ciliates were then processed as described above and added to CHSE-214 cultures, which were monitored for the development of an infection by the appearance of the characteristic CPE.

6.2.7 Statistics

Infectivity data, as TCID₅₀/mL, were log transformed prior to graphing. Where noted, data were expressed with respect to control data, by dividing by the control mean, or expressed as a decrease from the initial infectivity of the sample, by subtracting sample data from mean of initial infectivity for each trial replicate. For these calculations, the control mean standard deviation was not considered. All statistic tests were run using GraphPad Prism v.4.0c for Macintosh, using log transformed data. Specific tests conducted, along with numbers of replicates for each data set are presented in the text and in figure legends, respectively. For linear regression and analysis of covariance (ANCOVA) between multiple regressions lines, the same software package was used.

6.3 Results

6.3.1 Effect of CSV on ciliates

The presence of the CSV in the medium had no visible impact on the ciliates, whether the incubation temperature was 4, 14, 18, 22, 26 or 30 °C. Although the shape was abnormal and swimming was impaired during the first few hours after the ciliates had been put in L-15/ 10 % FBS, this was true without or with the virus. By 24 h with or without CSV, most ciliates were swimming and capable of phagocytosing fluorescent beads (1 µm). However, fluorescent beads were not internalized by *T. thermophila* B1975 at 4 °C or by *T. thermophila* NP1 at 30 °C.

6.3.2 Effect of *T. thermophila* B1975 on CSV infectivity

After incubation with ciliates, CSV behaved like untreated CSV (control) and caused in the same adherent fish cell lines at the same temperatures the characteristic cytopathic effect (CPE) (Table 6.1). The CPE was the fusion of cells to form syncytia or giant cells that eventually lysed (Figure 6.1). Without or with prior exposure to ciliates at 22 °C for at least 24 h, CSV caused the development of CPE over 4 to 7 days at 18 °C in cultures of CHSE-214, ZEB2J, and EPC, but not in RTbrain (Table 6.1). At 26 °C, untreated CSV also caused CPE in ZEB2J. Incubating the fish cell lines at the higher temperature of 30 °C blocked the development of CPE in ZEB2J and EPC, but was not tried with CHSE-214 and RTbrain because ultimately these salmonid cells die at such a temperature (Bols et al., 1992). When incubated with ciliates at 30 °C prior to being added to fish cell cultures, CSV caused cpe in EPC at 18 °C, and ZEB2J at 18 °C and 26 °C, but neither cell line at 30 °C.

Table 6.1 Effect of prior incubation with ciliates on infectivity of CSV ^a

| Prior incubation of CSV with <i>T. thermophila</i> | Fish cell line with incubation temperature | Development of syncytia at 7 days post infection |
|--|--|--|
| none | CHSE-214 at 18 °C | + |
| 24 - 48h at 22 °C | CHSE-214 at 18 °C | + |
| 24 - 48h at 30 °C | CHSE-214 at 18 °C | + |
| none | RTbrain at 18 °C | - |
| 24 - 48h at 22 °C | RTbrain at 18 °C | - |
| none | EPC at 18 °C | + |
| none | EPC at 26 °C | + |
| none | EPC at 30 °C | - |
| 24 - 48h at 22 °C | EPC at 18 °C | + |
| 24 - 48h at 22 °C | EPC at 30 °C | - |
| 24 - 48 h at 30 °C | EPC at 18 °C | + |
| 24 - 48 h at 30 °C | EPC at 30 °C | - |
| none | ZEB2J at 18 °C | + |
| none | ZEB2J at 26 °C | + |
| none | ZEB2J at 30 °C | - |
| 24 - 48h at 22 °C | ZEB2J at 18 °C | + |
| 24 - 48h at 30 °C | ZEB2J at 18 °C | + |
| 24- 48 h at 30 °C | ZEB2J at 26 °C | + |
| 24 - 48h at 30 °C | ZEB2J at 30 °C | - |

^a The starting titre of CSV solutions added to cell lines, without prior incubation with *Tetrahymena*, was approximately 10⁴-10⁵ TCID₅₀/mL, however the titre was not reassessed after incubation with ciliates.

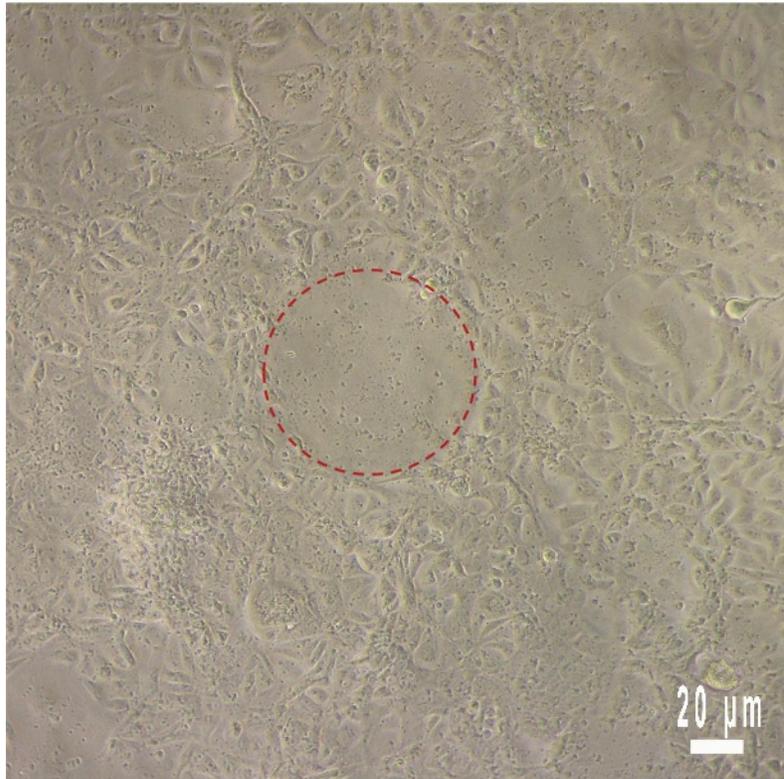


Figure 6.1 Characteristic cytopathic effects (CPE) of CSV on the cell line CHSE-214

A confluent monolayer of CHSE-214 cells was infected with CSV-containing cell lysates. After 2 days at 22 °C the first sign of CPE were apparent. For CSV, these are the formation of circular syncytia (dashed circle), which will continue to grow in size and ultimately lyse, destroying areas of the monolayer.

For quantifying the extent to which the incubation with ciliates influenced the titre of CSV, the CSV was incubated with *T. thermophila* B1975 at 22 °C and the titre derived on CHSE-214 at 18 °C. Surprisingly, incubation with the ciliates increased the viral titre (Figure 6.2). At the lower starting density (10,000 cells/mL), ciliates significantly increased the infectivity of the CSV in suspension, measured as titre, over the length of the incubation period, 96 h ($p < 0.0001$; one-way ANOVA). This increase reached a maximal point after 72 h, when the CSV titre was more than 3.5 log greater than the initial titre. When ciliates were added at an initial high density (100,000 cells/mL), CSV was again activated, increasing in titre 3.93 ± 0.92 (\pm SE; $n = 6$) log from initial titre after 96 h ($p < 0.05$; one-way ANOVA). At this density, the ciliates had a maximal activation after 72 h when titre was increased by 5.13 ± 0.89 (\pm SE; $n = 4$) log from the starting titre. When compared to ciliate-free CSV controls, the presence of ciliates significantly increased titre as early as 48 h for low density samples ($p < 0.001$; one-way ANOVA with Bonferroni Multiple Comparisons test), and 24 h for high density samples ($p < 0.001$; one-way ANOVA with Bonferroni Multiple Comparisons test). Ciliate-free CSV control data were run in parallel to ciliate-containing samples and no significant variation was found across these data ($p > 0.05$; one-way ANOVA), indicating no change in the CSV titre over the 96 h.

The enhancement of CSV infectivity was not mediated through the medium alone and required living ciliates (Figure 6.3). When CSV was incubated for 48 h in medium that had been conditioned by the growth of *T. thermophila* B1975 for 24 h, infectivity was not increased significantly. Compared to CSV maintained in medium that had not been conditioned, no change in viral titre was found ($p > 0.05$; one-way ANOVA). Also, ciliates that had been killed by either heat or formalin prior to being incubated with CSV did not subsequently enhance CSV infectivity (Figure 6.3).

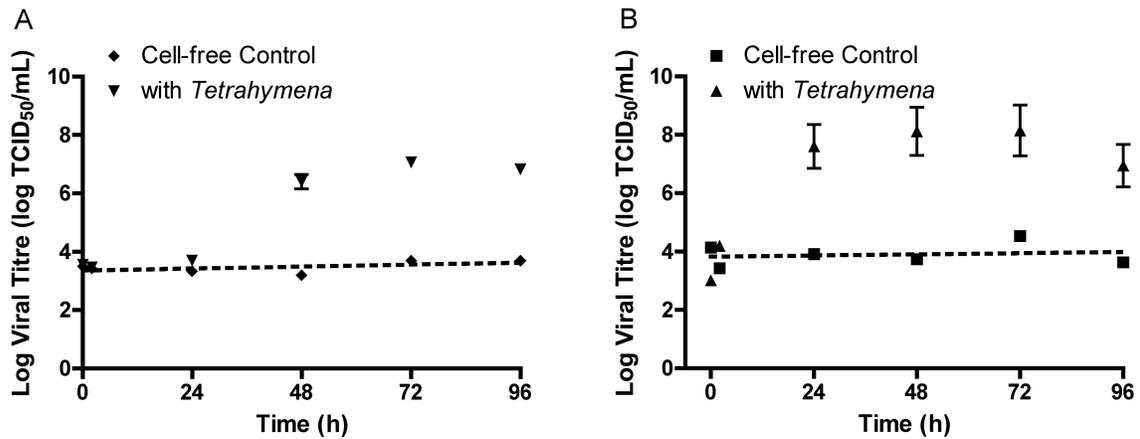


Figure 6.2 Incubating CSV at 22 °C with ciliates increases the CSV titre

Cell lysates that arose from destruction of CHSE-214 cultures by a CSV infection and thus had CSV were incubated at 22 °C either alone or with 10,000 (A) or 100,000 (B) *T. thermophila*/mL for varying times prior to being titred on CHSE-214. Points on graph are mean \pm SE of log transformed TCID₅₀/mL values (n greater than 3). Linear regression is plotted on the graph for CSV control data for illustrative purposes. One-way ANOVA of ciliate-free control data yielded no significant variation across either data set ($p > 0.05$).

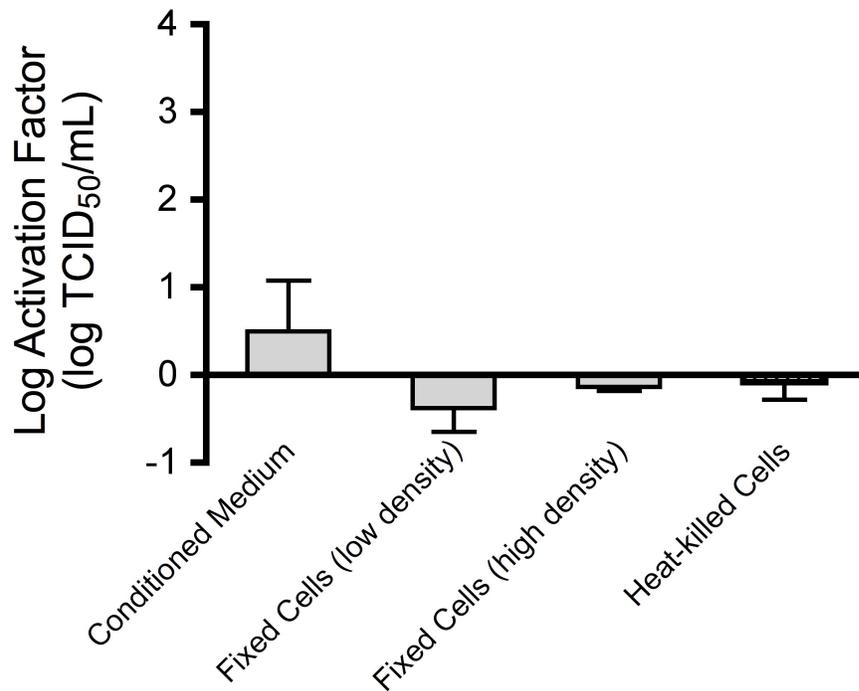


Figure 6.3 Incubating CSV at 22 °C with ciliate-conditioned medium or dead ciliates fails to increase the CSV titre

Cell lysates that arose from destruction of CHSE-214 cultures by a CSV infection and thus had CSV were incubated at 22 °C with ciliate-conditioned medium or dead ciliates for 48 h. Formalin-fixed ciliates were added at either a low density (10,000 cells/mL) or high density (100,000 cells/mL). Heat-killed ciliates were added at 100,000 cells/mL. The mean (n = 6) viral titre from the ciliate-free control after 48 h was subtracted from the titre measured under each condition, and this was the log activation factor. Standard deviation of the control titre was discarded for this transformation. Bars on graph are mean \pm SD (n = 6).

Like incubations at 22 °C, incubations at 30 °C with *T. thermophila* B1975 also increased the viral titre (Figure 6.4) but incubation at 14 °C caused much less enhancement and incubation at 4 °C brought about no viral activation (Figure 6.5). In each of these experiments, the titres were assessed in CHSE-214 at 18 °C. When maintained with ciliates at 30 °C, CSV titre increased significantly ($p < 0.0001$; one-way ANOVA), reaching an increase of 3.79 ± 0.56 log (\pm SE) from the starting titre at 72 h. Maintained at 4 °C, CSV titre changed by 0.32 ± 0.08 (\pm SE; $n = 6$) of the initial value and this was not significantly different from the no-ciliate control, which changed only 0.25 ± 0.10 (\pm SE; $n = 3$) of the initial titre ($p > 0.05$; Bonferroni Multiple Comparisons test). Therefore at both 22 and 30 °C *T. thermophila* B1975 enhanced the infectivity of CSV for susceptible fish cells at 18 °C, but not at 4 °C. As *T. thermophila* B1975 did not undergo phagocytosis at 4 °C, the activation of CSV was studied with the temperature sensitive mutant NP1, which does not phagocytose at 30 °C.

6.3.3 Effect of *T. thermophila* NP1 on CSV infectivity

When *T. thermophila* NP1 were grown for 18 h at 30 °C, a temperature where they would not phagocytose, and then incubated with CSV for 72 h at 30 °C, the ciliates failed to enhance the infectivity of CSV (Figure 6.4). The CSV titre did not increase over the 72 h ($p > 0.05$; one-way ANOVA). Linear regression of NP1 data (not shown) yielded a slope that did not differ from zero ($p > 0.05$; F test), and when compared to that of a ciliate-free CSV control data, neither slope nor elevation differed significantly between the two data sets ($p > 0.05$ for both slope and elevation; ANCOVA). By contrast when NP1 was grown for 18 h at 22 °C, where they are able to phagocytose, and then incubated with CSV for 72 h at 22 °C (data not shown), the ciliates increased infectivity by 3.85 ± 0.66 log (\pm SE; $n = 6$). As mentioned earlier, the wild type, *T. thermophila* B1975, enhanced CSV infectivity at both 22 °C and 30 °C (Figures 6.2 and 6.4). Therefore, phagocytosis seems to be required to achieve CSV activation.

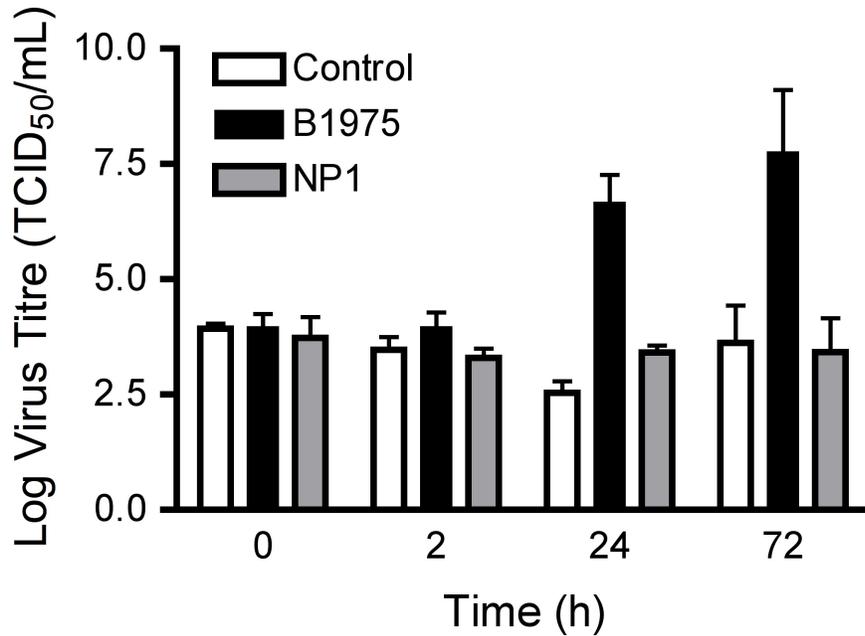


Figure 6.4 Incubating CSV at 30 °C with *T. thermophila* B1975, but not NP1, increases CSV titre

Cell lysates that arose from destruction of CHSE-214 cultures by a CSV infection and thus had CSV were incubated at 30 °C for up to 72 h either alone (open bars) or with a starting density of 10,000 ciliates/mL of either B1975 (dark bars) or NP1 (shaded bars) strains.

Linear regressions (not shown) of ciliate-free control and NP1 data sets were compared via ANCOVA and found to have similar slope and elevation ($p = 0.9581$, $F = 50.472$). Data on graph are mean \pm SD of log transformed TCID₅₀/mL values ($n = 6$).

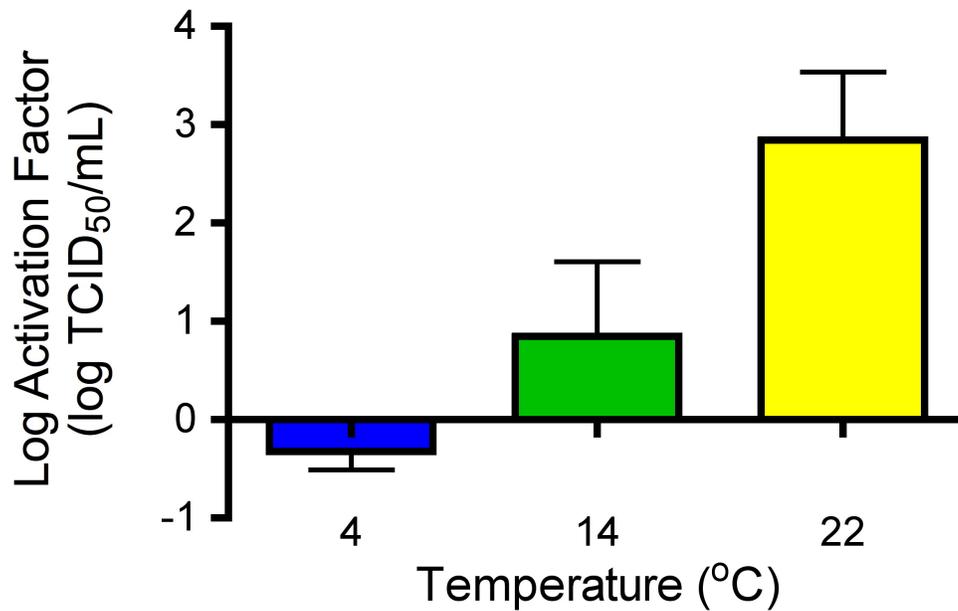


Figure 6.5 Incubating CSV at 4 °C with *T. thermophila* B1975 fails to increase the CSV titre

Cell lysates that arose from destruction of CHSE-214 cultures by a CSV infection and thus had CSV were incubated for 48 h either without (ciliate-free control) or with a starting density of 10,000 ciliates/mL *T. thermophila* B1975 at 4, 14 or 22 °C. Viral titres at each temperature are expressed as change in log value from the respective initial titre, or log activation factor. Error bars indicate mean \pm SD (n = 6).

6.3.4 Visualizing the internalization of CSV by ciliates

When *T. thermophila* B1975 were incubated with CSV that had been labeled with SYBR Gold and then viewed by epifluorescence microscopy, fluorescent bodies or vesicles were seen inside the ciliates (Figure 6.6). These had the approximate size and shape of food vacuoles and were evident by 6 h and became more numerous and intensely fluorescent by 24 h. The vesicles were prominent towards the posterior end of the cell. Ciliates that had been incubated for 24 h in a CSV-free control solution did not show fluorescence (Figure 6.6).

6.3.5 Retention and release of CSV by ciliates

As at least one bacteriophage has been reported to be released after internalization (Akunyili et al., 2008). Here, CSV virions appeared to be inside the ciliates, and tests were made of the ability of ciliates to acquire CSV from infected cultures and to pass the virus to uninfected cultures after the ciliates had being thoroughly washed. These experiments were done with *T. canadensis* rather than *T. thermophila* because unlike *T. thermophila*, these ciliates by themselves did not destroy fish cell monolayers (Chapter 4). For *T. canadensis* to be used for these experiments, it could not inactivate CSV but had to have either no effect on, or activate the virus.

Like *T. thermophila*, *T. canadensis* enhanced CSV infectivity. *T. canadensis* was incubated with CSV for 48 h and the viral titre was compared to initial titre (Figure 6.7). Ciliates were added at a lower density (5,000 cells/mL) than *T. thermophila*, but still showed a significant increase in CSV titre when in contact with ciliates of 1.31 ± 0.20 log units (\pm SE; n = 6) greater than the initial titre ($p < 0.001$; unpaired t test).

Notable CPE developed in CHSE-214 cultures after the addition of *T. canadensis* that previously had swum over CSV-infected CHSE-214 monolayers for 18 h and passed through a step gradient of polysucrose (Histopaque). By two days, syncytia covered approximately 75 % of the CHSE-214 monolayer and by four days, the monolayer had been destroyed (Figure 6.8). By contrast, ciliates alone or heat-killed ciliates that had been incubated with CSV-

infected CHSE-214 monolayers for 18 h did not cause the development of CPE and the monolayers remained intact. Therefore, *T. canadensis* were able to acquire CSV from infected CHSE-214 cultures, retain the CSV through centrifugation, and release infectious CSV into other CHSE-214 cultures.

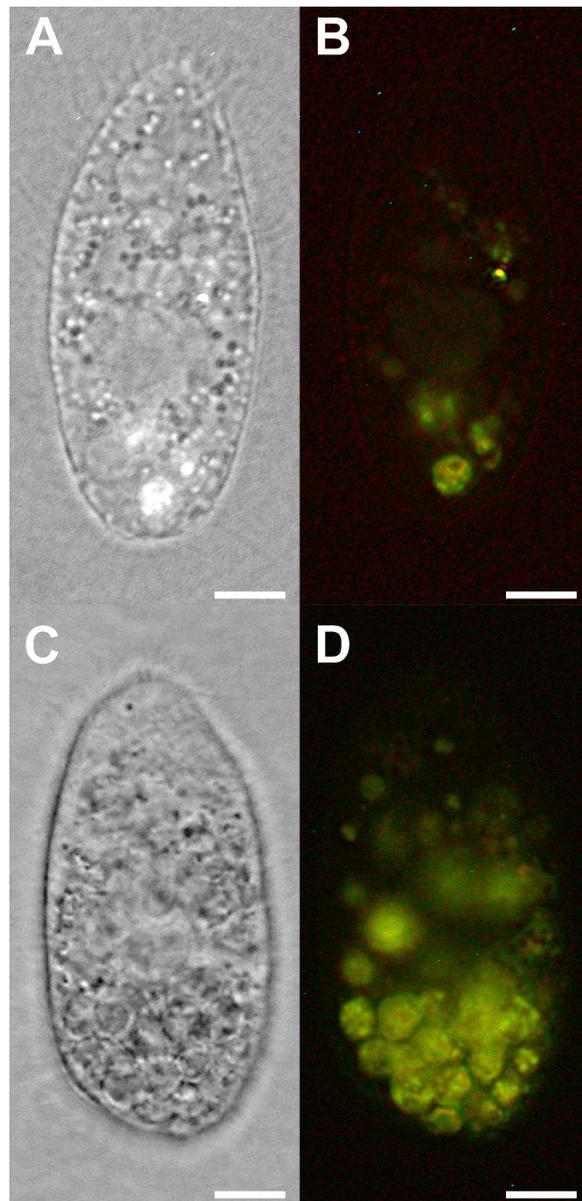


Figure 6.6 Visualizing SYBR Gold labeled CSV in ciliates

CSV were stained with the nucleic acid stain SYBR Gold and incubated at 22 °C with *T. thermophila* B1975. At 6 h (A, B) and 24 h (C, D) ciliates were fixed in 10 % NBF and examined by phase contrast (A, C) and fluorescence (B, D) microscopy. Scale bars are 10 μm .

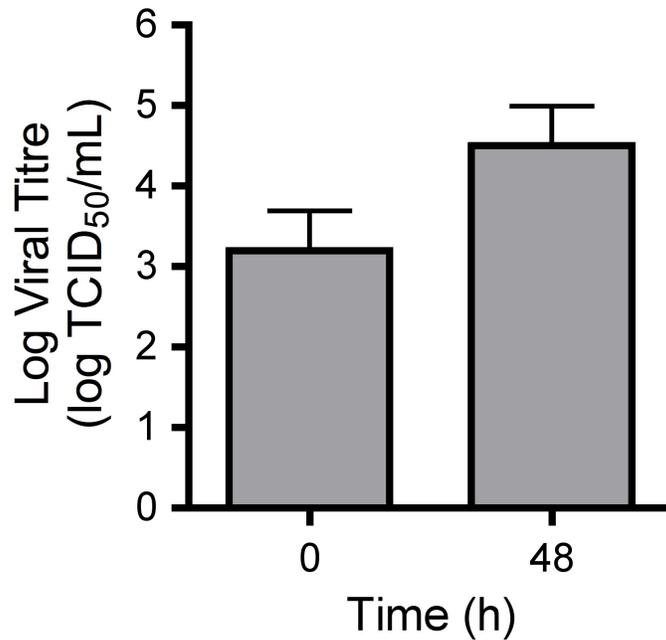


Figure 6.7 Incubating CSV with low numbers of *T. canadensis* increases the CSV titre

Cell lysates collected from CHSE-214 cultures infected with CSV were incubated at 22 °C with 1,500 *T. canadensis*/mL for 48 h before being titred on CHSE-214. Bars are mean \pm SD of log transformed TCID₅₀/mL values (n = 6). An unpaired t test compared the change in titre from 0 to 48 h, and deemed the increase significant (p < 0.01).

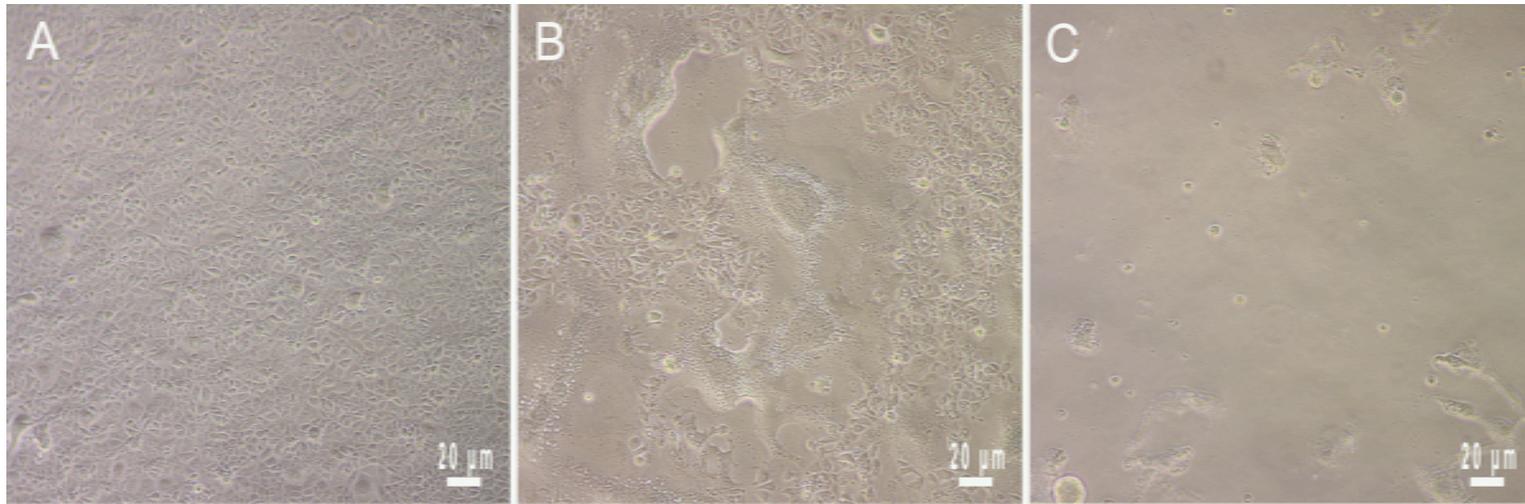


Figure 6.8 Evidence that *T. canadensis* acquires, retains and releases infectious CSV

Tetrahymena canadensis were allowed to swim over CSV-infected monolayers of CHSE-214 for 18 h, collected and spun through a step-gradient of Histopaque, and transferred to CHSE-214 cell monolayers. The confluent monolayer is pictured at the time of ciliate addition (A), 2 days later, showing many syncytia (B) and 4 days later, showing complete destruction of the monolayer (C).

6.4 Discussion

Chum salmon reovirus (CSV) is the first example of a virus have its titre increased, or being activated, by the presence of ciliates. Previously, *Tetrahymena* spp. were shown to decrease the viral titre, or inactivate, non-enveloped, icosahedral bacteriophages (T4, T5 and λ) (Pinheiro et al., 2007; Hennemuth et al., 2008), non-enveloped, icosahedral animal viruses (adenovirus and polio virus) (Sepp et al., 1992; Kim and Unno, 1996), and two enveloped, helical viruses, the influenza virus (Groupe and Pugh, 1952), and the fish rhabdovirus, viral hemorrhagic septicemia virus (VHSV) (Chapter 5). CSV resisted inactivation, and the only other example of a virus resisting inactivation by ciliates was the bacteriophage, Φ X174 (Akunyill et al., 2008). Additionally, living but not dead, ciliates enhanced CSV infectivity, or in other words, activated CSV. Although no convincing examples of an animal virus being produced in ciliates exist, the possibility of CSV replicating in the ciliates is difficult to rule out completely. But for at least two reasons the CSV enhancement of infectivity was not likely due to CSV production in *Tetrahymena*. Firstly, *Tetrahymena* that were incubated with CSV showed no sign of deterioration, which might be interpreted as a cytopathic effect and indication of viral replication. Secondly, *T. thermophila* incubated with the virus at 30 °C enhanced CSV infectivity, but CSV did not replicate at 30 °C in the fish cell line ZEB2J, even though these cells grow well at 30 °C (Xing et al., 2008). The simplest explanation for these results is that replication and enhancement are different processes and that ciliates carry out only enhancement of CSV infectivity.

Tetrahymena thermophila appeared to enhance CSV infectivity by first internalizing and then externalizing the virus. After incubation with SYBR Gold-labeled CSV, the ciliates acquired fluorescence bodies about the size of food vacuoles, an observation which suggests CSV does become internalized. Previously, SYBR Gold labeled phages and VHSV had been shown to accumulate into food vacuoles (Pinheiro et al., 2007; 2008; Chapter 5). Internalization has been attributed to phagocytosis or macropinocytosis in the cytopharynx of the oral apparatus (Pinheiro et al., 2007; 2008). In the case of VHSV, internalization also might occur by pinocytosis at the ciliate surface, which could still deliver the virus to food vacuoles (Chapter 5). Similar internalization routes could be operating for CSV. The

necessity for internalization in order to activate CSV is suggested by two observations. Firstly, the enhancement of infectivity occurred at temperatures that supported phagocytosis, 22 and 30 °C, and not at temperatures where phagocytosis would be reduced or absent, 14 and 4 °C (Nilsson, 1976). Secondly the *T. thermophila* mutant NP1, which at 30 to 37 °C does not have a normal oral apparatus and is incapable of phagocytosis (Orias and Pollock, 1975), was unable to enhance CSV infectivity at 30 °C. By contrast, at this temperature wild type *T. thermophila* did enhance infectivity. For infectivity enhancement, externalization would have to take place in order for CSV to cause CPE in the susceptible fish cell line, CHSE-214, and thus be detected. *Tetrahymena* have been shown to externalize viable bacteria (Berk et al., 2008; Brandl et al., 2005; Ghaffari et al., 2008; Koubar et al., 2011) and infectious Φ X174, a bacteriophage (Akunyili et al. 2008). Thus a precedent exists for a virus to be internalized and externalized in an infectious form but an increase in infectivity is novel.

Proteases can enhance reovirus infectivity. This was first described approximately 50 years ago for members of the rotavirus family (Spendlove and Scheffer, 1965), although infectivity enhancement by proteases was not subsequently found for other mammalian reoviruses (Joklik, 1972). By contrast, short treatments with trypsin or chymotrypsin enhanced the infectivity of representative members of most aquareovirus groups (McPhillips et al., 1998). Aquareoviruses have multiple capsid layers and seven structural proteins, including the outermost capsid proteins VP7 and VP5 (Subramanian et al., 1994). The capsid proteins VP7 and VP5 have been described respectively as the "protection" and "penetration" proteins and during endocytosis by susceptible cells the cathepsin family of cysteine proteases act on them to allow infection (Trask et al., 2010). Treating virions in a test tube with proteases led to the digestion of VP7, concurrent cleavage of VP5, and enhanced infectivity (Nason et al., 2000; Fang et al., 2008). Protease treatments also can lead to the complete disassembly of the outer capsid and generate morphological forms that are termed infectious subviral particles (ISVPs) (Borsa et al., 1973). ISVPs for aquareovirus have increased infectivity over native full virions (Fang et al., 2008; McPhillips et al., 1998).

The increase in CSV infectivity by *Tetrahymena* likely can be attributed to the proteolytic cleavage of outer capsid components as virions passed through the phagosomal-lysosomal system. The proteome of the *Tetrahymena* phagosome has recently been studied, revealing cathepsin proteases with strong matches to cathepsin L and B (Jacobs et al., 2006). An older literature showed that *Tetrahymena* had a variety of proteolytic enzymes (Straus et al., 1992), including lysosomal proteases inside the organelle and released into the medium (Muller et al., 1966). However medium conditioned by *Tetrahymena*, and expected to contain released lysosomal enzymes, failed to enhance CSV infectivity. Therefore, the simplest explanation for the enhancement of CSV infectivity is that the ciliates digest the outer capsids through their phagosomal-lysosomal proteases and then release into the medium these modified but more infectious virions.

Prolonged trypsin treatments (2 h) inactivated aquareoviruses (Nason et al., 2000), but with *Tetrahymena* the enhancement of CSV infectivity was maintained during incubations for up to 72 h. This has several possible explanations. The actions of proteolytic enzymes within ciliates might be more restrictive in their actions than purified enzymes. Other processes, such as viral aggregation, might occur during the transit of virions through the ciliates that after the initial proteolytic activation impede further proteolytic degradation. Finally, the transit time for CSV through the ciliates might have been sufficient for activation but the externalization of virions prevented further proteolytic degradation and inactivation.

The release of a more infectious form of CSV by the ciliates could either promote or hinder viral disease transmission. Transmission would be promoted if the more infectious form of CSV were able to infect fish hosts more effectively. However, the ciliate-altered virions might not be able to extend the range of fish susceptible to CSV because the released virions infected fish cell lines normally susceptible to CSV but not an unsusceptible one. Transmission would be hindered if the ciliate-treated CSV was more vulnerable to inactivation in the environment, such as by ultraviolet light. The possible impacts of ciliates on CSV environmental survival and infectivity in vivo need investigation in the future.

In addition to activating CSV, *Tetrahymena* could promote viral disease transmission by retaining and later releasing infectious CSV. Retention and release was illustrated by the fact that after incubation with CSV and several cycles of washing, *Tetrahymena* could be transferred to a culture of a susceptible fish cell line, CHSE-214, and cause the characteristic CPE of CSV. The retention and release capabilities could help in the shedding of virions from CSV infected fish, living or dead, by transporting and/or protecting the virions. The very motile ciliates would be able to transport CSV away from shedding fish. In the case of bacteriophages, the potential to move phages in water is termed protistophoresis and might be especially significant in still water (Akunyili et al., 2008). During transport inside the ciliates, CSV virions might be protected from environmental inactivation, such as through light or UV, and thus the ciliates might aid CSV persistence in the environment. Together, transport and protection would be expected to increase the chances of the virus encountering fish to infect. These phenomena would be not true for all fish viruses, as the ciliates inactivated VHSV (Chapter 5), and the extent to which they apply to other aquareoviruses and other viral classes will be interesting to determine in the future. For virally infected fish shedding two or more viruses of different classes, the ciliates could be inhibiting the transmission of one and enhancing the transmission of another.

Chapter 7

General Conclusions and Future Research

With the ability to efficiently culture *Tetrahymena* species alongside animal cells or animal viruses using in vitro culture, insights into how ciliates may interact with other organisms have been investigated in this thesis. The presence of fetal bovine serum (FBS) greatly facilitated the transfer of *Tetrahymena* to animal cell culture media, and could provide all required nutrients for proliferation. When in contact with animal cells in culture, some tetrahymenas could destroy a monolayer while some survived without causing damage. Lastly, *T. thermophila* were found to decrease the titre, or inactivate one type of fish virus, and to increase the titre, or activate another. These results lead to new questions as to the role of *Tetrahymena* species in affecting fish health, and some future directions are outlined below.

For some ciliates that parasitize fish, such as *Ichthyophthirius multifiliis*, cultivation in vitro is not yet possible and this is a major hurdle for the development of viable ciliate stocks, and treatment of infected fish. For *Tetrahymena*, when FBS was present in the media, ciliates adapted to animal culture conditions and proliferated, regardless of other nutrients present. Future work may consider to investigate these molecules found in serum and produce novel defined media additives allowing ciliates to adapt to high osmolality, or promote proliferation of fastidious ciliates. Serum contains many potential factors contributing to this, and these would include osmolytes such as K^+ , Cl^- , or inositol; hormones such as insulin; or α 2-macroglobulin. Defined supplements such as these may allow progress in developing standard culture practices for hard to culture parasites, such as *I. multifiliis*, that could deeply impact the aquaculture industry.

The ciliate/animal cell monolayer system described here presents an opportune research model to study histophagy. In fish succumbing to tetrahymenosis, the gills are commonly the

site of infections, and a gill cell/ciliate system would allow investigation of possible drug treatments to either block ciliate penetration, or anti-protozoals. The inability of *T. canadensis* to cause cell destruction suggests species-specific differences in cell biology or gene expression that allows some tetrahymenas to carry-out histophagy but not others. Investigating these differences could lead to novel drug targets for treating tetrahymenosis. Finally, the current protocols for genetic modification of *T. thermophila* available in the literature (Aldag et al., 2011; Chalker, 2012) suggests their use as a live vaccine, through the modification of antigenic proteins on the cilia to express antigens from known fish parasites. Here, the inability of *T. canadensis* to cause tissue damage in live fish, if in vitro results apply, would be a benefit. Antigen-presenting *T. canadensis* may hold potential to be added to small-scale aquaculture without the danger of opportunistic parasitism by the ciliates.

When maintained together with *T. thermophila*, VHSV was found to be inactivated over time, while CSV was activated and viral titre increased - an unprecedented ciliate/virus interaction. In this way ciliates may directly influence the persistence of animal viruses in natural waters, or protect the viruses from factors such as UV radiation. Activation especially poses a health risk, and other virus types may be similarly activated. A number of human viruses related to aquareovirus similarly produce highly infective subviral particles upon proteolytic treatment, namely mammalian orthoreovirus and rotavirus. These viruses may be found in drinking water supplies or wastewater treatment plants, where viruses come into contact with large numbers of grazing ciliates. Survival of free-living ciliates on vegetables (Gourabathini et al., 2008) and long-term within poultry broiler houses (Baré et al., 2011), despite disinfection practices, raises food safety concerns over the potential for ciliates to act as viral *Trojan horses*. Progress using fluorescence in-situ hybridization to study intracellular symbiotic bacteria in free-living ciliates (reviewed in Görtz, 2011) could aid in designing rapid techniques to survey viruses being carried/protected by ciliates. In this way, a more detailed understanding of how ciliates interact with animal viruses in the wild may be obtained.

The role of ciliates in affecting the health of our fisheries may involve direct or indirect associations between fish and protists. In either case, the impact of grazing ciliates will

involve a variety of microbe-metazoan and microbe-microbe interactions that portray the richness of the natural environment and the necessity for an interdisciplinary approach when investigating microbial ecology. Findings from this project will have application in the management of natural and man-made fisheries and understanding the spread of disease in these environments, as well as potential implications in wastewater treatment and the spread of water-borne viral diseases in general.

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General Conclusions and Future Research References

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Appendix A: Phases of the destruction of the Chinook salmon cell line, CHSE-214 monolayer by *Tetrahymena thermophila*

This appendix consists of a video file that presents the progression through the typical major phases in the destruction of an animal cell monolayer, by the ciliate *T. thermophila* (strain B1975). The animal cell line was CHSE-214, and the progression of damage occurred similarly in each cell line, although the timings differed. Ciliates were added at a low density (1,500 cells/mL) to confluent wells of a 12-well tissue culture plate, and the medium was L-15 with 10 % FBS (see Chapter 3 for detailed protocol). The video begins when the first sign of damage to the monolayer was visible (< 24 h after co-incubation), and continues through to the ultimate destruction of the monolayer (72 h after co-incubation). Time and magnification are presented as video legends. High magnification of ciliates in concentrated aggregates of feeding cells is presented mid-way through the video; the behaviour of feeding ciliates is notable.

Video files are available on a CD that can be obtained from the Department of Biology upon request, and are located in the *Appendices* folder. The file name for Appendix A is “A_Tthermophila_grazing.AVI”.

Appendix B: Behaviour of *Tetrahymena canadensis* when in contact with animal cell monolayers in culture

This appendix consists of a video file that presents the swimming behaviour of *T. canadensis* when in culture with the Chinook salmon cell line, CHSE-214. Ciliates were added at a low density (1,500 cells/mL) to confluent wells of a 12-well tissue culture plate, and the medium was L-15 with 10 % FBS (see Chapter 4 for detailed protocol). In the video, the ciliates can be seen swimming vigorously throughout the medium, with no visible attraction to the surface of the monolayer. This behaviour is apparent throughout the duration of the co-culture; the presented video shows cells at 9 days post-inoculation of the ciliates. Magnification is noted in the video legend. Note that this behaviour was indicative of the behaviour of *T. canadensis* in all cell lines tested.

Video files are available on a CD that can be obtained from the Department of Biology upon request, and are located in the *Appendices* folder. The file name for Appendix B is “B1_Tcanadensis_swimming.AVI”.

Appendix C: Immunostaining of VHSV envelope protein G when in contact with *Tetrahymena thermophila*

This appendix consists of two video files that are comprised of a Z-stack series from confocal microscopy of immunocytochemistry. Using antibodies to envelope protein G of VHSV, *T. thermophila* were immunostained at two time points after removal from a ciliate-virus mixture (see Chapter 5 for detailed protocol). The videos are *T. thermophila* that had been mixed with VHSV in L-15 with 10 % FBS for 24 h, then separated and washed, and finally moved to fresh L-15 with 10 % FBS for 30 mins (video **C1**; Z-stack slice depth 0.42 μm), or 60 mins (video **C2**; Z-stack slice depth 0.56 μm). Red staining denotes indirect-immunostaining of G protein (495 nm excitation / 519 nm emission), and blue staining denote DAPI nuclear stain (340 nm excitation / 488 nm emission). Scale bars and slice depth are labeled in the video.

Video files are available on a CD that can be obtained from the Department of Biology upon request, and are located in the *Appendices* folder. The file name for Appendix C1 is “C1_VHSV_zstack30min.AVI” , and for Appendix C2 is “C2_VHSV_zstack60min.AVI”.