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Microsporidia in the Animal to Human Food Chain: An International Symposium to Address Chronic Epizootic Disease

Sponsored by the Organisation for Economic Co-operation and Development- Co-operative Research Programme (OECD-CRP) and the Society for Invertebrate Pathology

Session 1: Microsporidia- a general introduction (Grant Stentiford, Chair)

8:30 am Grant Stentiford, Introduction

8:40 Primal Silva, Executive Director, Animal Health Science Directorate, Canada and Member Scientific Advisory Body, OECD – Introduction to the OECD/CRP. Stressors in the global food chain and the importance of pathogens.

8:50 Kristina Rösel, (International Livestock Research Institute, CGIAR Consortium): Parasites in Food Chains

9:10 James Becnel (USDA-ARS, Gainesville FL, USA): Introduction to the Microsporidia

9:30 Patrick Keeling (Canadian Institute for Advanced Research): The Microsporidia: where did they come from and where are they going?

Session 2: Microsporidiosis in humans (Louis Weiss, Chair)

9:50 Louis Weiss (Albert Einstein College of Medicine, NY): Microsporidiosis in humans – an emerging issue? *(Text not available)*

10:10 Elizabeth Didier (Tulane University): Is global immunosuppresion linked to rising burdens of microsporidiosis in human and animal populations?

10:30 *Coffee*

11:00 Bryony Williams (University of Exeter): How do Microsporidia exploit the biochemistry and physiology of the host cell?

Session 3: Microsporidiosis in terrestrial animals (James Becnel, Chair)

11:20 Louis Weiss (Albert Einstein College of Medicine, NY): Microsporidiosis in farmed animals and terrestrial wildlife – their role in zoonoses (*Text not available*)

11:40 Karen Snowden- (Texas A&M): Microsporidiosis in companion animals – their role in zoonoses (Video)

12:00 Susan Bjornson (St. Mary's University): Microsporidia as regulators of insect populations and disease agents in mass-reared insects – a future threat to edible insect cultivation?

12:20 *Lunch*

Session 4: Microsporidiosis in aquatic animals (Grant Stentiford, Chair)

13:30 Michael Kent (Oregon State University): Microsporidiosis in wild fish – an emerging issue?

13:50 Mark A. Freeman (University of Malaya): Wild and cultured fish as potential sources of zoonotic infections in humans

14:10 Grant D. Stentiford (European Union Reference Laboratory for Crustacean Diseases, Cefas, UK): Pathogens of aquatic arthropods – focus on the Enterocytozoonidae

14:30 Yuliya Sokolova (Louisiana State University): Clues for multiple-taxa lifecycles from invertebrate research

14:50 Break

Session 5: Microsporidian role in pollinator health (Leellen Solter, Chair)

15:20 Mark Brown (Royal Holloway, University of London): Is microsporidian infection/disease becoming more common in bumble bees?

15:50 Leellen Solter (Illinois Natural History Survey, University of Illinois): Interactions of Microsporidia with the global honey bee population

Session 6: Future look and final discussion (Symposium Organizers)

16:00 Emily Troemel (University of California, San Diego): Current and future models for microsporidian research

16:30- 17:30 Panel Discussion

Symposium Organizers:

James Becnel, USDA-ARS, Gainesville, FL
Leellen Solter, Illinois Natural History Survey, University of Illinois
Grant Stentiford, Centre for Environment, Fisheries and Aquaculture Science, UK
Louis Weiss, Albert Einstein College of Medicine, NY

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Parasites in Food Chains

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Keywords: burden, foodborne parasitic diseases, globalization, OneHealth

Abstract

While in high-income countries, the majority people die from non-communicable, chronic conditions, nearly 40% of deaths in developing countries are among children under 15 years. Diarrhea is among the top ten leading causes of death and many cases are caused by pathogens transmitted in food and water supplies. This paper introduces major representatives of foodborne parasites and aims to show why they are no longer a public health concern of low-income countries only. Approaches used in assessing and managing the risk of foodborne parasitoses will be presented.

Diseases in complex food production systems

In high-income countries ca. 70% of people die above the age of 70 years, mostly due to non-communicable, chronic conditions such as cardiovascular diseases. Foodborne infections caused illness in 12.5% (4 million) Canadians in 2006, and 16.7% (48 million) US-Americans in 2011. In these countries, the majority of disease cases are caused by unknown agents and the top four of identified pathogens are *Norovirus*, non-typhoidal *Salmonella*, *Clostridium perfringens* and *Campylobacter* species (Thomas et al. 2013; CDC 2011). In low-income countries, nearly 40% of deaths are among children under 15 years. People die mostly of infectious diseases (i.e. lower respiratory infections, HIV/AIDS, malaria, diarrhoea and tuberculosis). Diarrhoea is among the top 10 leading causes of death in lower-middle income countries, killing 1.5 million people in 2012 (Word Health Organisation, 2014). Many of these

deaths are caused by pathogens transmitted to humans in food and water supplies (Gajadhar et al. 2006).

Human food from both plants and animals is produced, processed and marketed in intricately linked systems of primary producers (i.e. corn or cattle), input and service providers (i.e. pesticides, water, veterinary drugs), transporters, processors, wholesalers, retailers, consumers and end-users of by-products (i.e. manure). Foodborne diseases are conditions that are commonly transmitted through ingested food and comprise a broad range of illnesses caused by enteric pathogens, parasites, chemical contaminants and biotoxins which are either naturally present in food (i.e. cyanogens in cassava) or contaminate food at different points in the food production and preparation process (WHO 2007).

Humans harbour about 300 species of helminths and over 70 species of protozoa; many are transmitted by food and water (Ellin 2003). According to the International Classification of Diseases, eight out of the 21 etiological causes of death due to potentially foodborne diseases are caused by parasites (WHO 2007), especially protozoa and cestodes. The complex life cycle involving different development stages inside one or several hosts and/or the environment allows entry of stages infectious to humans at any point of the food chain. Foodborne parasites are acquired by ingesting infectious stages in tissue of infected mammals, fish and even invertebrates as well as contaminated food and water supplies or via contaminated fomites or fingers. Many parasitic diseases have traditionally been considered confined to tropical countries and of little concern to wealthy countries (Krause/Hendrick, eds. 2010) but this perception is slowly changing. *Toxoplasma gondii*, the only parasite in a 2011 ranking of important foodborne pathogens, was identified as the second most important pathogen causing death from foodborne infection in the United States in 2011 (CDC 2011). In 1993 an outbreak with an estimated 403,000 cases of watery diarrhea due to cryptosporidiosis from a single source of contaminated water was reported from Milwaukee in the United States (MacKenzie et al. 2010). Globalized trade (Alarcón de Noya et al. 2010) and travel (Simarro et al. 2012) increases the risk of imported parasitoses from tropical countries.

Selected parasites in food chains

From an initial list of foodborne diseases a list of priority foodborne parasitic diseases was established (WHO 2007; FAO/WHO 2014) including protozoa and nematodes (both foodborne and intestinal) as well as foodborne trematodes and cestodes. This section introduces these groups of foodborne parasites. Microsporidia, the topic of this meeting, are known to enter the

food chain through waterborne routes; but microsporidiosis is also a potential emerging meatborne zoonosis, given that natural hosts of human infective microsporidia can be part of the human food chain. Pleistophora-like microsporidians, may be acquired from raw or lightly cooked fish or crustaceans (Slifko et al. 2000). The potential of pigs as a reservoir for *Enterocytozoon bieneusi* has been discussed in Czech Republic (Sak et al. 2008). Currently, microsporidiosis is not considered a priority foodborne parasitosis.

Intestinal protozoa: The three main representatives of this group are *Giardia*, *Entamoeba* and *Cryptosporidium* spp. with the highest median prevalence of *Entamoeba* and *Giardia* in the Americas, whereas Africa had the highest for *Cryptosporidium* with highest health burdens in children below 15 years (Torgerson et al 2014). The most important source of infection for humans is attributed to contaminated drinking water (Torgerson et al 2014). In low and middle-income countries, approx. 200 million people suffer from symptomatic guiardiasis with about 500,000 new cases reported each year (Togerson and Macpherson 2011), while the number of reported human cases in the USA remained constant around 20,000 per year (Yoder et al. 2012). The epidemiology of zoonotic *Giardia* spp. is still under debate (Torgerson and Macpherson 2011; Savoili et al. 2006).

Cryptosporidiosis is a major cause of diarrhea in humans, globally and is attributed to water, food and contact with infected animals (Slifko et al. 2000). It is a particular problem in immunocompromised people. The major zoonotic species is *C. parvum* and main transmission routes include water and fruit and raw vegetables contaminated with infectious feces from humans or animals; or shellfish such as oysters and mussels (Smith et al. 2007). Even though Africa has the highest burden of cryptosporidiosis, the proportion of zoonotic cryptosporidiosis seems to be highest in high income countries (Xiao and Feng 2008).

Intestinal nematodes: *Ascaris lumbricoides*, a gastrointestinal nematode of humans, enters the food chain through contaminated water and soil. The consumption of raw vegetables and fresh fruit contaminated with soil are major sources of infection. Whether *Ascaris lumbricoides* and *A. suum* are identical and/ or cross-transmissible between humans and pigs (Leles et al. 2012) is still being researched. Acute health problems in humans arise from intestinal obstruction but chronic infections may have a much more important impact as they are associated with (reversible) deficits in growth and physical fitness in children and possibly impaired cognition (Ndimubanzi et al. 2010).

Foodborne protozoa: Toxoplasmosis is a major zoonosis with a global distribution caused by *Toxoplasma gondii*. Humans can become infected by ingesting oocysts (e.g. in contaminated water, food, soil) shed by cats or through the consumption of undercooked meat containing viable bradyzoites. Perhaps more than 50% of the cases of toxoplasmosis can be attributed to the latter transmission route (Torgerson et al. 2014). Maternal infection for the first time during pregnancy can cause foetal or newborn death or congenital abnormalities such as hydrocephalus or chorioretinitis. Non-congenital toxoplasmosis poses a serious threat to immunocompromised patients but has been considered an asymptomatic or mild flu-like illness in otherwise healthy individuals but with lifelong infectivity. Increasingly, this is debated and seropositivity linked to behavioural changes and mental disorders (Flefr et al. 2000; Flegr et al 2002; Lindova et al. 2006; Torey et al. 2007).

Foodborne trematodes: The group consists of *Fasciola*, *Opisthorchis*, and *Clonorchis* spp. Fasciolosis is a true zoonoses with cattle and sheep being the main reservoir for human disease but also pigs, goats, dogs, alpacas, llamas and rats can serve as the definitive host (Torgerson and Macpherson 2011). Snails are recognized as the intermediate host of *F. hepatica* in temperate climate and *F. gigantica* in tropical climates. Transmission to definitive hosts occurs by ingestion of infectious stages in water or on plants. *Opisthorchis* and *Chlonorchis* spp. are parasitoses of mammals eating raw or undercooked freshwater fish. Infections in humans are usually asymptomatic but symptoms increase depending on the infection dose and range from fever, fatigue, rash and gastrointestinal disorders to inflammations of liver and bile duct system, liver abscesses or cirrhosis, pancreatitis with the most serious consequence being cholangiocarcinoma. Approximately 56 million people are infected with foodborne trematodes. Of these, approximately 7.9 million (14%) have severe sequelae with approximately 7,158 deaths per year. The highest health burden (i.e. neoplasms) is caused by *Clonorchis sinensis* and *Opisthorchis viverrini* and occurs in East and Southeast Asia and the Asia Pacific regions; mostly caused by the ingestion of contaminated food (Torgerson et al. 2014).

Foodborne cestodes: Larval stages of the cestodes *Echinococcus* and *Taenia* spp. cause potentially fatal disease of humans. Taeniasis in humans, the final host of *T. solium*, is caused by ingesting parasite cysts from eating undercooked pork and results in mild non-specific gastrointestinal illness (Gajahar et al. 2006). Neurocysticercosis (NCC) in humans is caused when *T. solium* cysts lodge in the brain, a consequence by autoinfection when humans ingest eggs shed by adult worms in human guts. NCC is the cause for 29% of epilepsy patients

(Ndimubanzi et al. 2010)) and transmission by eating undercooked pork is vital for maintaining the parasite's life cycle. Previously considered a disease of developing countries, neurocysticercosis has increasingly been imported to North America from endemic countries (White 2000). Larval echinococcosis in humans is caused if they ingest eggs from contaminated water or raw vegetables. *E. granulosus* causes cyst formation in the liver, lungs or other organ system (cystic echinococcosis) in livestock and humans with dogs as definite hosts and is widely endemic (Budke et al. 2006); while alveolar echinococcosis in humans is caused by the fox tapeworm *E. multilocularis* and causes serious infiltrative growth of metacestodes in the liver with ultimate liver failure as humans are aberrant intermediate hosts (Torgerson et al. 2010). *E. multilocularis* is increasingly found in dogs (Peregrine et al. 2012) and strains previously found in Europe have travelled to Canada (Jenkins et al. 2012).

Foodborne nematodes: Human trichinellosis caused by *Trichinella* spp. is found worldwide except for the Antarctic; it is a direct foodborne zoonosis and is acquired by ingesting infectious larvae with raw or undercooked meat, especially pork from organic or extensive farms, and game meat. While asymptomatic in animals, in humans disease symptoms can range from diarrhoea, periorbital and facial oedema, myalgia, fever, photophobia, headache, conjunctivitis, and skin rash to life-threatening conditions such as myocarditis or meningoencephalitis (Kociecka 2000). While control measures in industrialized countries seem to be disproportional considering the relatively low burden on human health; data on human health burden from potential hotspots in Asia and Africa are still lacking.

The global burden of foodborne parasitic diseases

The full extent and cost of unsafe food, especially the burden from chemical and parasitic contaminants is largely unknown but estimates are improving (WHO 2007). In 2013, one million deaths were caused by parasitic diseases, approximately 60,000 through potentially foodborne parasitic diseases (2013 GBD Collaborators 2015). With more reliable data on the burden of disease, policy makers can prioritize government action and assess the effectiveness of interventions, in both monetary and non-monetary terms. In 2006, the World Health Organization mandated a Foodborne Disease Burden Epidemiology Reference Group (FERG), including a Parasitic Diseases taskforce, which is assessing health burden using the Disability Adjusted Life Year (DALY). DALYs express the years of life lost to premature death and the years lived with disability for varying degrees of severity, making time the common metric for death and disability WHO 2007). The most recent update on the global burden of foodborne

parasitic diseases was compiled by Torgerson and colleagues (Torgerson et al. 2008; Torgerson and Macpherson 2011); an adapted summary is given below (Figure 1) and a comprehensive final report will be published by end of 2015.

Approaches in assessing and managing risk of foodborne parasitic diseases

The International Livestock Research Institute (ILRI) makes use of three approaches in researching parasites in food chains: One Health/Ecohealth concepts, integrated livestock and fish value chain assessments and participatory epidemiology. The One Health and ecohealth concepts acknowledge that the health of humans is connected to the health of animals and the environment, which is particularly true for zoonotic diseases and prompts us to work interdisciplinary with veterinarians, physicians, ecologists, statisticians and economists. We are attempting to understand zoonotic disease transmission patterns and to predict and manage disease in the face global challenges as population growth, the migration of people into new ecological regions, changes in husbandry practices, globalized trade and tourism all increase the frequency of interfaces between parasite reservoirs and hosts which is pariculary complex with parasitic life cyles. The CGIAR Research Program on Livestock and Fish led by ILRI, sees value chain assessment and innovation as a key strategy (ILRI/CIAT/ICARDA/Worldfish 2011). ILRI food safety research under the CGIAR Research Program on Agriculture for Nutrition and Health integrates risk analysis tools for food safety into the program's value chain transformation approach. Participatory epidemiology is a branch of veterinary epidemiology based on the principles and methods of Participatory Rural Appraisal, discussing animal health problems in a given community (Catley 2005). In Veterinary Public Health it has proven to be a fast and relatively cheap way of identifying zoonotic and foodborne risks to public health, a methodology developed by ILRI and partners and coined "participatory risk assessment" (ILRI Safe Food, Fair Food project, 2008-2011) (Makita 2014).

Concluding remarks and future directions

Foodborne parasitic diseases are no longer mainly diseases confined to low-income countries but emerging with globalized migration, trade and tourism. The lack of human health data in developing countries, underreporting, difficulties in source attribution due to the nature of parasitic diseases (time between exposure and clinical signs) make it difficult to comprehend the true extent of the burden and may lead to underestimation and underprioritization of parasitoses. Assessment and management of foodborne parasitoses warrant a One Health and

food chain systems approach, in developing countries supported by means of participatory epidemiology.

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Parasite	Possible global burden (DALYs)	Animal health costs	Trends/ remarks	Reference
intestinal protozoa: *Giardia*, *Entamoeba* and *Cryptosporidium* spp.	? x 10^5-10^6	unknown, but likely to be high		Torgerson & Macpherson 2011
intestinal nematodes: *Ascaris lumbricoides*	1.3 x 10^6	likely high if infective for pigs	highest burden on children 5-15 years	Torgerson et al. 2014; Leles et al. 2012; Murray et al. 2012
foodborne protozoa: *Toxoplasma gondii*	2-8 x 10^6	possibly substantial	new sequaelae are being assigned to toxoplasmosis; highest burden in South America, some Middle Eastern and low income countries	Torgerson & Macpherson 2014; Torgerson & Mastroiacova 2013
foodborne trematodes: *Fasciola*, *Opisthorchis*, *Clonorchis* spp.	>0.5 x 10^6	animal fasciolosis is very high	increasing reports; highest health burden (i.e. neoplasms) is caused by *Clonorchis sinensis* and *Opisthorchis viverrini* and occurs in East and Southeast Asia and the Asia Pacific regions; the major proportion ist caused by the ingestion of contaminated food	Torgerson et al. 2011; Torgerson and Macpherson Fürst et al. 2012
Foodborne nematodes: *Trichinella* spp.	?	control programs are a large financial burden	burden on human health in developing countries lacking	Murrell and Pozio 2011
Foodborne cestodes: *Echinococcus* spp.;	2-5 x 10^7	US$2 x 10^9	burden AE highest in China with rising incidence in Central Asia and Europe	Budke et al. 2006; Torgerson et al. 2010;Torgerson et al. 2011; Torgerson and Macpherson 2014
Taenia solium	2-5 x 10^6	unknown	assumes ca. 30% of epilepsy in low income countries due to NCC	
For comparison:				WHO 2008
HIV	59 x 10^6			
malaria	34 x 10^6			
tuberculosis	34 x 10^6			

Figure 1. Possible magnitude of annual global burden of selected foodborne parasitic diseases (adapted from Torgerson et al., 2011)

Introduction to the Microsporidia

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Keywords: Opportunistic parasites, generalist microsporidia, specialist microsporidia, *Nosema, Tubulinosema, Anncaliia*

Abstract

Microsporidia are strict intracellular parasites with a broad distribution in protists, invertebrates and vertebrates. The cryptic and often chronic nature of infections caused by many microsporidia is a significant problem for all types of beneficial arthropods and vertebrates. This brief introduction provides basic information on the biology of the microsporidia including developmental stages, the spore, and spore survival and transmission. Select examples of life cycles and host interactions for generalist and specialist species of microsporidia are presented to provide a general understanding on the impact microsporidia could have on the production of animals as food sources for man and animals as well as an awareness of any possible safety considerations.

Introduction

Microsporidia have been known as parasites of animals for over 150 years but for the majority of this time they were viewed as a small group of pathogens of concern mainly to beekeepers and those involved in the silkworm industry. There was however a small group of scientists devoted to studies on the taxonomy and biology of microsporidia in the early years that have laid the foundation for recent advancements in the knowledge of this fascinating group of organisms.

Most all invertebrate phyla are hosts for microsporidia as well as many protists including ciliates, myxozoans and gregarines (Vávra and Lukes 2013). All five classes of vertebrates are also hosts for microsporidian species, including fishes, amphibians, reptiles, birds and mammals (Canning and Lom 1986; Snowden 2014; Kent et al. 2014). There are no reports of

microsporidia from plants. Recent phylogenomic analyses have placed the microsporidia together with the Cryptomycota as the earliest branching clade in the fungal kingdom (James et al. 2013). The most common hosts for microsporidia based on current host records are arthropods and then fish with about 800-900 species of microsporidia described out of an overall total of between 1,300-1,500 species. The number of known species probably represents only a fraction of total species of microsporidia, assuming that there is at least one and perhaps multiple species of microsporidia in each animal.

This brief introduction provides basic information on the biology of the microsporidia and on select examples of life cycles and host interactions for generalist and specialist species of microsporidia. The intent is that these examples can provide a general understanding on the impact microsporidia could have on the production of animals as food sources for man and animals as well as an awareness of possible safety considerations.

Biology

Microsporidia are single celled, eukaryotic, spore forming protistian parasites that are distinguished from other protists by a number of unique characteristics. Evidence thus far supports two main clades of microsporidia, the typical (or advanced) and atypical (or primitive) microsporidia. Developmental sequences and cytology have been used to establish the distinctions between the typical and atypical microsporidia as molecular data is not available for the atypical group (Vávra and Larsson 2014). The majority of known microsporidia are of the typical type with about 190 genera and an estimated 1,300 – 1,500 species. This group has simple to complex developmental sequences and life cycles and produces spores of a great diversity of shape. Spores of the typical microsporidia generally have a thick and rigid spore wall, are uninucleate or diplokaryotic and possess a sophisticated injection apparatus (Fig. 1A). Information on the atypical microsporidia is limited but they are distinguished from the typical microsporidia in a number of ways, primarily by being uninucleate, lacking vegetative reproduction, and having simple spores that have reduced organelles such as no polaroplast and a polar filament of special construction (Fig. 1B).

The atypical microsporidia are a composed of two main groups, the chytridiopsids and metchnikovellideans composed of approximately 13 genera and 42 species (Larsson, 2014). This review will focus on the typical microsporidia and additional information can be found in a number of recent reviews (Franzen 2008; Vávra and Lukes 2013; Weiss and Becnel 2014).

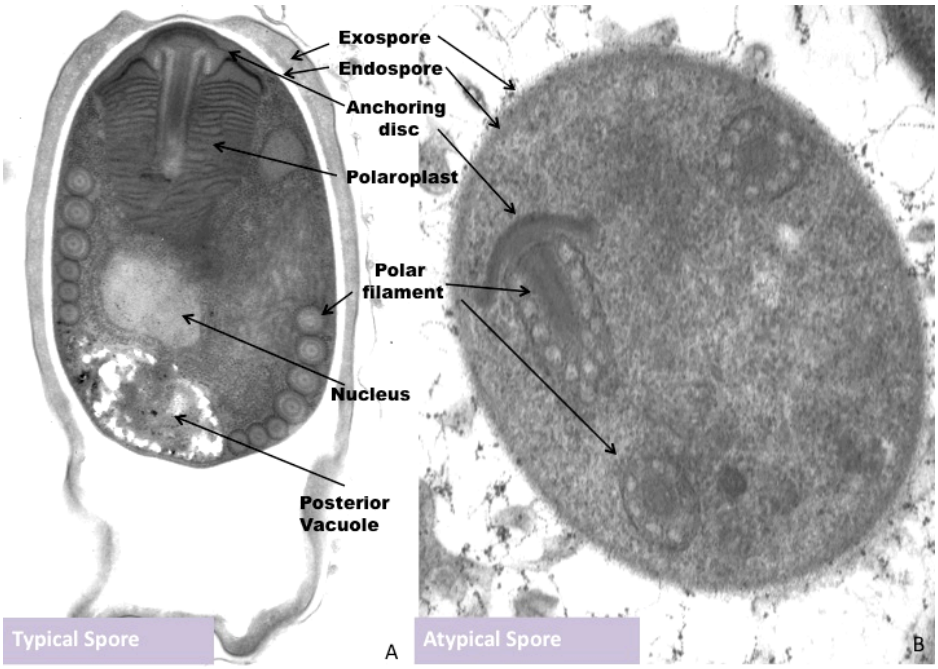

Figure 1

Figure 1. Transmission electron micrographs demonstrating and comparing the features of typical and atypical microsporidian spores. A.) Typical spore demonstrating a rigid spore wall composed of the exospores and endospore, a sophisticated extrusion apparatus composed of the polar filament, polaroplast and the posterior vacuole. B.) Atypical spore demonstrating a greatly reduced spore wall and extrusion apparatus composed of only the polar filament.

Developmental Stages

Pre-sporulation. This phase begins with growth and division of uninucleate or diplokaryotic sporoplasms into meronts that enter a reproductive phase (merogony) to produce sporonts. Reproduction can occur by binary fission, multiple fission or a combination of the two. Binary fission produces two daughter cells that can repeat this process. Multiple fission involves repeated nuclear fissions without cytokinesis to produce multinucleate plasmodia (usually 4-16 but can be variable); the number is usually species or genus specific. These cells can divide into smaller plasmodia (plasmotomy) or into individual cells by multiple fission. At some point, the final products of merogony enter into sporogony and transform into sporonts.

Sporulation. Sporulation is defined as sporogony plus spore morphogenesis. Sporogony is the process of binary or multiple fission of uninucleate or diplokaryotic sporonts that divide directly or with the intervention of a plasmodial stage to produce sporoblasts. Sporogony can take place in direct contact with the host cytoplasm or involve the formation of a parasite derived

sporophorous vesicle or host derived parasitophorous vacuole within which spores are formed. Spore morphogenesis is the transformation of sporoblasts into spores.

The Spore

The most commonly encountered stage of microsporidia is the spore (Fig. 1). Spores are single-celled with one or two nuclei (the diplokaryon) and are most commonly oval or pyriform in shape. They are typically in the 2-8 micron range but can be a small as 1 micron or as large as 30 microns in length. Under light microscopy, spores are highly refractile and usually a vacuole at the posterior end of the spore is visible. The spore has a very complex structure that contains the extrusion apparatus. The spore wall is composed of two layers, an electron-lucent endospore layer that contains chitin and an electron-dense exospore that is often layered. The unique infection apparatus is composed of three main parts: a long, thread-like polar filament, a multilayered polaroplast, which is a highly membranous structure that occupies the anterior half of the spore, and a posterior vacuole. When the spore is in the appropriate host and environment, the spore germinates and the polar filament is everted to become a hollow tube. The sporoplasm travels through this tube and is inoculated into the cytoplasm of the host cell to begin vegetative replication.

Spore Survival and Transmission

Survival of the environmental (extracorporeal) spores of microsporidia is critical to parasite transmission to a new generation of host individuals. Persistence of environmental spores can vary from a few days to years, largely depending on the life cycle of the host. In some systems, there may be one main route of transmission while in others multiple transmission pathways are critical for survival and dispersal of the parasite. For many systems transmission is unknown or poorly understood. In general, spores of microsporidia from terrestrial hosts tend to persist longer in the environment and withstand temperature and environmental extremes. In contrast, spores of microsporidia from aquatic hosts persist for shorter periods of time and are more sensitive to temperature and environmental extremes. An excellent review can be found in Solter et al. (2014).

Microsporidia are known to be transmitted from host to host horizontally and vertically or cell to cell within a host and for some species each of these pathways play important roles. The main portals of entry for microsporidia are oral and ovarian. Horizontal transmission via the oral route occurs when environmental spores are taken in by the host, germinate and initiate infection.

Vertical transmission from an infected female to her progeny can occur in several ways. In some species, spores formed in the ovaries germinate and deposit sporoplasms into the developing eggs that can initiate infection upon hatch. Spores within or on the surface of eggs deposited by an infected female can be fed upon by susceptible hosts and this is therefore vertical spreading of the parasite but transmission is considered horizontal. Within a host transmission from cell to cell (auto-infection) can occur, normally early in the developmental cycle by a spore that usually of a different type than the environmental spore. There are many variations on these main types of transmission for the microsporidia and additional information can be found for invertebrates in Becnel and Andreadis (2014) and for vertebrates in Fayer and Santin-Duran (2014) and Snowden (2014).

Life Cycles and Host Interactions

Species and genera of microsporidia can be divided into either generalist or specialist based on their host ranges and characteristics. Generalists can have broad host ranges, tissue specificities and temperature tolerances and can infect and develop in invertebrates as well as cold and warm blooded vertebrates. Specialists fall into two main groups; 1) species that are restricted to infecting and developing within a narrow range of closely related hosts and 2) species that require an obligate two host system with a definitive host and intermediate host. This type of system has only been found for microsporidia that alternate between mosquitoes and copepods or fish and their arthropod parasites.

Specialists. The life cycles of specialist microsporidia range from relatively simple to the extremely complex. Many species of microsporidia fall into this group based on current information on transmission and host ranges but many more species are poorly known and their status is yet to be determined.

A well studied specialist microsporidium with a simple life cycle is *Nosema apis*. An economically important parasite of the honey bee, *Nosema apis* is representative of microsporidia that possess diplokaryotic stages throughout development and complete their life cycle in one host individual. Adult honey bees ingest binucleate spores that are stimulated to germinate in the gut lumen and deposit the sporoplasm directly into midgut epithelial cells. The binucleate sporoplasm grows in size and matures into the first meront. Approximately 24 hours post-infection the first nuclear division occurs followed by binary fission to initiate the merogonial cycle. Multiplication is typically by binary fission of diplokaryotic cells but can also involve multiple fission of paucinucleate plasmodia. At approximately 48 hours post-infection, some

stages transform into sporonts that divide once by binary fission to form two sporoblasts (de Graaf et al., 1994). This results in a primary binucleate spore characterized by a thin spore wall and short polar filament that germinates spontaneously within the cytoplasm of the epithelial cells. It is believed that this mechanism (autoinfection) serves to spread the parasite to adjacent epithelial cells. Other meronts within the cell continue to multiply and after a number of divisions enter into a second sporulation sequence. Diplokaryotic sporonts divide once to produce two sporoblasts that mature into spores. This second binucleate spore is thick-walled with a long polar filament and is produced in large numbers. The Infected epithelial cells become filled with spores and eventually rupture releasing the binucleate spores into the gut lumen. These spores are voided with the frass and contaminate the bee's environment until ingested by a new host individual.

Specialist microsporidia with complex life cycles are perhaps best known for the polymorphic species that infect aquatic Diptera, especially mosquitoes. Most species require two successive host generations to complete their life cycle, and at least three genera. *Amblyospora*, *Hyalinocysta* and *Parathelohania*, require obligatory development in an intermediate copepod host (Andreadis 2007). The type species for *Amblyospora* is *Amblyospora californica* in the mosquito *Culex tarsalis* and has a life cycle representative of this group of specialist (Becnel 1992). Different developmental sequences occur in male and female progeny from infected adult mosquitoes. Female progeny from infected adults have benign larval infections that are restricted to oenocytes. In these female adults, binucleate spores are formed after a blood meal and these are responsible for vertical transmission to progeny. Male progeny from infected adults develop fat body infections where diplokaryotic sporonts undergo meiosis producing meiospores that are infectious orally to the copepod intermediate host *Mesocyclops leukarti*. Asexual development of uninucleate stages begins in the ovaries of the intermediate host and ends with the production of uninucleate spores that are infectious orally to a new generation of the mosquito host. In larval mosquitoes, a complex developmental sequence leads to the formation of diplokaryotic sporonts and eventually binucleate spores in female adults to complete the cycle.

The only other group for which an intermediate/alternate host is reported is the fish parasite *Desmozoon lepeophtherii* (junior objective synonym *Paranucleospora theridion*) and the parasitic copepod *Lepeophtheirus salmonis* (Freeman and Sommerville 2009; 2011; Nylund et al. 2010). There are two spore types found in the salmon host, a small, thin walled spore with a short polar filament forms in the cytoplasm of macrophages and epithelial cells and a larger

spore with a thicker wall and longer polar filament forms in the nuclei of epithelia cells of the skin. It is this spore that is suspected to infect the parasitic copepod host which leads to a massive infection to produce spores similar to the intranuclear spores from the fish host.

Generalists. Generalist species tend to have simple developmental cycles that are usually characterized by a single sporulation sequence but some may involve multiple sporulation sequences. A broad host range and the ability of some to infect both invertebrate and vertebrate hosts distinguish these species from the specialists. Generalists are often responsible for opportunistic infections in higher vertebrates. Some notable genera capable of infecting and developing in arthropod and vertebrate hosts are *Anncaliia*, *Tubulinosema*, *Trachipleistophora* and *Encephalitozoon* although there are other genera that have been implicated by molecular data with species in arthropod and vertebrate hosts (exp. *Entercytozoon*, *Endoreticulatus*).

Anncaliia algerae was originally isolated and described from a laboratory colony of the mosquito *Anopheles stephensi* and because of the potential as a microbial control agent extensive host specificity studies were conducted. *Anncaliia algerae* was transmitted per os to species from four different families of insects and two species of trematodes (Brooks 1988). It was also transmitted by injection to species from six orders of insects, a crustacean (Undeen and Maddox 1973) and a mouse (Undeen and Alger 1976). These were some of the first studies to provide conclusive documentation that an invertebrate species of microsporidia was capable of infecting and developing in tissues and hosts distantly related, including vertebrates. The true extent of the host range of *A. algerae* was shown when it was isolated from a corneal lesion in an immunocompetent human (Visvesvara et al. 1999). Since then there have been at least three confirmed reports from humans, one from a skin lesion in a child with acute lymphatic anemia (Kucerova et al. 2004), one from deep muscle in a woman on immunosuppressive drugs (Coyle et al. 2004) and another from vocal cords (Cali et al. 2010). It was suggested by Coyle et al. (2004) that *A. algerae* infection resulted by the crushing of an infected mosquito and mechanically inoculating spores into the skin-bite wound. Transmission studies with a human isolate of *A. algerae* in athynic mice failed to establish infections by intravenous, peroral or intranasal inoculations but ocular delivery of spores produced severe infection in the liver 60 days post exposure (Koudela et al. 2001).

Species in the genus *Trachipleistophora* have a developmental cycle very similar to the mosquito parasite *Vavraia culicis* (also a generalist) and molecular data has confirmed this relationship (Hollister et al. 1996). All stages are uninucleate and groups of 8-32 uninucleate

spores are formed in sporophorous vesicles. *Trachipleistophora* has several species isolated from mammals that have been shown to infect insects when experimentally fed spores. *Trachipleistophora homminis* was originally isolated and described from the skeletal muscle of an AIDS patient (Hollister et al. 1996) and was subsequently shown to infect two species of mosquitoes (Diptera) by feeding spores to larvae (Weidner et al. 1999). *Trachipleistophora extenrec* was isolated and described from muscles of the Madagascan insectivore and spores fed to the Egyptian cotton leafworm (Lepidoptera) produced infections in larvae and adults (Vávra et al. 2011). Transmission studies with *T. homminis* in SCID mice have shown that injection and ocular inoculation can result in systemic infections but no infections were found by peroral inoculation (Koudela et al. 2004). This has led to speculations that infected insects could release spores that could enter a vertebrate host through breaks in the skin or when rubbed on the eye.

There are several other genera where there are phylogenetic relationships between microsporidia isolated from humans and insects. *Tubulinosema* was erected by Franzen et al. (2005a) and includes seven species (*T. acridophagus*, *T. hippodamiae, T. kingi*, *T. loxostegi, T. maroccanus*, *T. pampeana* and *T. ratisbonensis*) with insect hosts in the Orders Coleoptera, Diptera, Lepidoptera, Hymenoptera and Orthoptera. All stages of these parasites are diplokaryotic and develop in direct contact with the host cytoplasm (Plischuk et al. 2015). An isolation of a *Tubulinosema sp.* from a human was found to have 100% identity with the ssrDNA sequence for *T. acridophagus* that was originally isolated and described from a North American grasshopper. The family Tubulinosematidae also includes the two described species of *Anncaliia*, *A. vesicularum* and *A. algerae*, that have also been isolated from humans (Franzen et al. 2005b).

Encephalitozoon cuniculi was first described from rabbits and this and other species of *Encephalitozoon* have been reported from a broad range of mammals. Other than 2 reports of *Encephalitozoon*-like infections in ticks, there have been no reports of species of *Encephalitozoon* in invertebrates. Recently, *Encephalitozoon romaleae* was described from the lubber grasshopper *Romalea microptera* and was morphologically and phylogenetically related to the *Encephalitozoon* clade of species (Lange et al. 2009). *Encephalitozoon romaleae* was transmitted to several other orthopteran hosts, but not all, and did not infect a number of other insects tested (Lange et al. 2009).

Concluding remarks and future directions

The cryptic and often chronic nature of infections caused by many microsporidia is a significant problem for all types of beneficial arthropods and vertebrates. If left unchecked, the parasite can spread throughout the population especially if transmission can occur by both horizontal and vertical pathways. In large-scale production of arthropods both specialist and generalist species could establish and negatively impact both quantity and quality of the product. Early detection for known and unknown microsporidian species is essential to good management as well as having remediation plans in place when a problem is detected.

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Microsporidia in the Tree of Life

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Abstract

Pinning down the position of the microsporidia within the tree of life has been a long and difficult problem due to the difficulty in comparing their divergent cellular structures and molecular sequences with those of other eukaryotes. Early studies based on morphology generally placed them with other spore-forming parasites, and early molecular and ultrastructural data suggested they were an early-diverging eukaryotic lineage. Since the mid-1990s, however, a combination of genome-wide phylogenetics and a broader sampling of eukaryotic microbial diversity have led to the conclusion that the microsporidia are instead members of a large and diverse clade that branches at the base of the Fungi, named Cryptomycota (the name is disputed, but this was the original name for the clade). This clade includes previously characterized parasites of chytrid fungi, *Rozella* (generally accepted to be a fungus) and parasites of algae, aphelids (a group previously thought to be either fungi or some kind of ameoba). These parasites are not very well described, but do share some characteristics of their infection strategy with microsporidia, as well as many interesting differences (e.g. the possession of a flagellum, phagocytosis, and mitochondria with oxidative phosphorylation). At the same time, an early-diverging microsporidian lineage (*Mitosporidium*) has also been described that lacks some of the more derived features of other microsporidia, especially in its possession of a more or less canonical mitochondrion. How the various sub-groups of Cryptomycota relate to one another needs to be

resolved with future exploration and phylogenomics including all relevant groups. Overall, the emerging clarity of the relationships between of microsporidia and other eukaryotes gives us a great deal of insight into how their unusual characteristics evolved. \

Ancient history - getting back to fungi

The early history of research on micrsporidia has been excellently reviewed by Franzen (Franzen 2008), while relatively recent molecular phylogenetic results have also been reviewed more specifically elsewhere (Keeling 2009; Keeling and Fast, 2002; Méténier and Vivarès 2001; Roger 1999; Williams and Keeling, 2003), and so these topics will not be reviewed in detail here except to say they were originally placed in a group formally within the fungi but containing a variety of eukaryotes and bacteria. For most of their history they have been grouped with other spore-forming parasites, until the Archezoa hypothesis suggested they were actually among the first eukaryotes and ancestrally lacked mitochondria (Cavalier-Smith 1983). Early molecular phylogenetic analyses supported this position (Brown and Doolittle 1995; Kamaishi et al. 1996a; Kamaishi et al. 1996b; Vossbrinck et al. 1987; Vossbrinck and Woese 1986), but from the mid-1990s phylogenies based on a broader diversity of genes began to show some affiliation with fungi instead (Brown and Doolittle 1999; Edlind et al. 1996; Fast et al. 1999; Germot et al. 1997; Katinka et al. 2001; Keeling and Doolittle 1996; Keeling et al. 2000). Analysis of the first complete microsporidian genome from *Encephalitozoon cuniculi* (Katinka et al. 2001)ultimately showed the 'microsporidia-deep' position was associated with more divergent genes, suggesting an artifact known as long-branch attraction had been misleading interpretation (Thomarat et al. 2004). At the same time, relict, highly reduced mitochondria known as mitoses were discovered, formally ruling out the Archezoa hypothesis (Germot et al. 1997; Goldberg et al. 2008; Hirt et al. 1997; Peyretaillade et al. 1998; Williams et al. 2008; Williams et al. 2002).

Fungi or sisters to fungi

While molecular phylogenetics has now consistently shown microsporidia to be related to fungi, exactly how has been more difficult to discern until recently. In some studies they branch within the fungi (Gill and Fast 2006; Keeling 2003; Keeling et al. 2000; Lee et al. 2010), while in others they branch outside the group (Tanabe et al. 2002), or with the earliest-diverging members (Capella-Gutierrez et al. 2012; James et al. 2006). Just about every possible position within or as sisters to fungi has been recovered in various studies, emphasizing the difficulty in working with the highly divergent genes of microsporidia. Taxonomically the difference between within vs. sisters to fungi may appear trivial, but it is can have a significant impact on how the evolution

of the group is interpreted. It is possible that this distinction was unresolvable with data only from the then-known microsporidia and fungi; indeed, the resolution to this question ultimately came from exploration of microbial diversity leading to the discovery (so far) of three key lineages that go a great way to solving the problem.

Cryptomycota. A large scale phylogeny of the "fungal tree of life" showed microsporidia branching with a single basal species of algal parasite called *Rozella allomycis* (James et al. 2006). *Rozella* species are parasites of algae or chytrid watermolds that swim to and infect their host via an invasion tube, possibly relying on a posterior vacuole to push them into the host, and possibly ingesting host cytoplasm by phagocytosis (James and Berbee 2012). Environmental surveys of SSU rRNA showed *Rozella* to be the tip of an iceberg, since it fell within a very large and diverse clade that was altogether sisters to the known fungal orders (Jones et al. 2011a; Lara et al. 2010). This group, called Cryptomycota (Jones et al. 2011a; Jones et al. 2011b), was found in many environments, and in situ hybridization showed at least some cryptomycete cells were flagellated and associated with algal cells in nature. As most Cryptomycota are only known from SSU rRNA they are not readily comparable to microsporidia, whose SSU rRNA is too divergent to use reliably in phylogenies. However, phylogenomic studies using the recently completed *Rozella* genome confirmed their close relationship with strong support (James et al. 2013). The genome also showed *Rozella* to share a number of interesting features with microsporidia, including the presence of ATP transporters essential for microsporidian energy metabolism and the absences of mitochondrial complex I, being replaced by NADH dehydrogenase and alternative oxidase (James et al. 2013). More recently, two additional parasites of amoebae, *Paramicrosporidium* and *Nucleophaga* have been shown to fall in two distinct positions within the Cryptomycota in SSU rRNA trees (Corsaro et al. 2014a; Corsaro et al. 2014b). *Paramicrosporidium* is especially interesting since it shares morphological features in common with microsporidia, and branches as sister to microsporidia in SSU rRNA trees (Corsaro et al. 2014b), although the divergence of the microsporidian genes makes this placement very difficult to interpret. A well-supported position derived from phylogenomic analysis will be of great interest, to see if these parasites represent sisters to the microsporidia.

Aphelids. Aphelids are a group of algal parasites first described in the late-1800s. The flagellated infectious stage attaches to an algal cell, penetrates its wall with a tube, and is pushed into the host due to a build up of pressure from a posterior vacuole. The parasite then ingests the host cytoplasm by phagocytosis, forming a large plasmodium, which divides into

many small flagellated cells that leave the host via the invasion tube (Karpov et al. 2014). A taxonomic link to fungi has been suspected, but they have also been classified with different subgroups of amoebae (Karpov et al. 2014). The first molecular data from SSU rRNA from *Aphelidium* and *Ameoboaphelidium* showed that aphelids correspond to one of the environmental clades of Cryptomycota (Karpov et al. 2013; Letcher et al. 2015). Combined analyses using rRNA operon or the rRNA operon plus RNA polymerase subunits RPB1 and RPB2, showed a sister relationship between alphelids and microsporidia (Karpov et al. 2014; Karpov et al. 2013; Letcher et al. 2015). However, the key phylogenomic analysis that includes all three groups, microsporidia, aphelids, and *Rozella*, has not yet been published.

Mitosporidium. The discoveries that showed the relationship between microsporidia, *Rozella*, and aphelids have made great progress in identifying the nature of the ancestral state of microsporidia, looking from outside the group, but progress has also been made towards the same goal looking from within the group. Specifically, the discovery and characterization of *Mitosporidium daphnia* is major advance because it represents a newly found lineage that is intermediate in many respects between other microsporidia and their closest relatives, and unambiguously related to the microsporidia (Haag et al. 2014). *Mitosporidium* is a copepod parasite that shares much in common with other microsporidia, but is distinguished both at the morphological and molecular levels. In morphology it possesses a similar spore morphology and overall infection strategy, as well as a polar tube, but one that is less well developed and pronounced. Most obviously perhaps, *Mitosporidium* retains a mitochondrion with recognizable cristae (i.e., not a mitosome), as well as aerobic respiration with most elements of oxidative phosphorylation (like *Rozella*, it is missing complex I) (Haag et al. 2014). On the molecular level, *Mitosporidium* is distinguished not by a lack of metabolism as are other microsporidia, but rather by missing elements of genome maintenance and expression (Haag et al. 2014). Phylogenomic analysis placed *Mitosporidium* at the base of microsporidia, to the expulsion of *Rozella*, but this analysis did not include aphelids or *Paramicrosporidium*.

Concluding remarks and future directions

The most obvious first step given our present knowledge is to construct a phylogenomic tree that includes all known groups of interest: *Rozella*, aphelids, *Paramicrosporidium*, and microsporidia, including *Mitosporidium*. To date, all but *Paramicrosporidium* and aphelids have been analysed at the whole-genome level, but even then always in pairs or individually compared with microsporidia. A genome-wide analysis that includes all known players at this

point will hopefully provide an initial framework on which to begin to plot the order of events in the transition from a relatively 'normal' cryptomycete ancestor to the now highly derived microsporidia. Similarly, characterizing cryptomycetes currently represented only by rRNA sequences is essential to fill out this picture. These have the potential to alter the topology of the tree once more diverse taxa are included in phylogenomic analyses, and some may have biology that is critical to mapping how evolutionary transitions took place. Lastly, as has been shown here repeatedly, purely exploratory work to determine the real diversity of this part of the tree of life is essential if we are going to reconstruct the evolution of major lineages accurately. Each missing branch on the tree represents not only the absences of potentially important clues, but also the opportunity to misinterpret those clues we do have.

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Are Global Immunodeficiency and Immunosuppression Linked to a Rising Burden of Microsporidiosis in Humans?

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Abstract

Opportunistic infections (OI's) in HIV/AIDS laid to rest the debate about whether microsporidia could infect and produce disease in humans. This motivated the development of improved diagnostics methods and resulted in identification of new microsporidia species, increased awareness, and inclusion of microsporidiosis as a differential diagnosis, especially in patients with gastrointestinal complaints. The vast majority of microsporidia infections have been detected in persons with compromised immune systems but, consistent with studies on mammalian laboratory animals and seroprevalence studies in immune-competent humans, it is highly probable that microsporidia can persist to cause chronic infections that may reactivate to produce overt clinical signs in those becoming immune-deficient or undergoing immunosuppressive therapies. The prevalence of OI's, including those due to microsporidia, has dramatically declined with the implementation of combination anti-retroviral therapies (cART) for reducing HIV levels and restoring immune competence, but now microsporidia infections are increasingly identified in persons undergoing therapies to prevent transplant rejection or for treatment for malignancies. Children affected by malnutrition, poor hygiene practices, and co-infection with other parasites also carry higher burdens of microsporidiosis, and microsporidial keratitis as a likely consequence of topical steroid use, is also growing in recognition. The world's population is aging and HIV-infected individuals, including those undergoing cART, exhibit accelerated signs of aging. Thus, it is anticipated that in the near future, microsporidiosis

also will surface in this growing population that is undergoing immune-senescence associated with aging.

Microsporidiosis in humans prior to the HIV/AIDS pandemic

The earliest human infections with microsporidia were reported in children, usually associated with compromised immunity (Sprague, 1974; Fayer and Santin-Duran 2014). Among these earlier infections were two fatal cases of an infant described with *Encephalitozoon chagasi* (nomen nudum) associated with myocarditis and encephalitis in 1937 and a 4-week-old female. Both of these cases were not fully characterized and the latter is now considered to have been due to *Toxoplasma gondii*. The earliest accepted cases of human microsporidiosis included a non-fatal infection due to an *Encephalitozoon sp.* reported in 1959 in a 9-year-old boy experiencing convulsions and an athymic infant who succumbed to a disseminated infection with *Anncaliia* (syns. *Nosema, Brachiola*) *connori* (Sprague 1974; Franzen et al. 2006). Diagnosis of these early cases of microsporidiosis relied almost exclusively upon light microscopic methods and predated the use of more sensitive and specific molecular and biochemical testing, so it is likely that earlier microsporidia infections in humans had been mistaken, under-reported, or erroneously attributed to other causes. The sweeping HIV infection and AIDS pandemic that arose in the middle 1980's, however, brought to light the opportunistic capability of microsporidia to infect humans and produce disease in virtually all organs Orenstein et al. 1997; Desportes et al. 1985). With time, diagnostics methods improved for detecting these small organisms and this increased awareness for including microsporidiosis in differential diagnoses in patients with diarrhea as well as with nonspecific clinical signs. As a result, microsporidia are increasingly identified in populations ranging from immune-competent individuals with persistent infections who express few or no clinical signs to persons with primary and secondary immune-deficiencies who exhibit overt clinical signs of disease (Didier and Weiss 2011).

Primary Immune Deficiency

Primary immune deficiencies (PID) comprise a class of disorders derived from defects in the intrinsic host's immune system that are inherited and fairly rare, estimated at about 50,000 cases in the USA (Chapel et al. 2014). These conditions are typically categorized on the basis of the immune system compartment that is impacted such as humoral immunoglobulin deficiencies, T cell defects in athymic individuals with DiGeorge syndrome, defects in phagocytic cells that impact innate immune responses, defects in the complement pathway, or

combined immune deficiencies. Microsporidia infections in these individuals also have been only rarely reported. The earliest case occurred in an athymic child infected with *A. connori* (Fayer and Santin-Duran 2014; Sprague 1974). Another study of 32 individuals with PID in Poland identified two individuals (6.3%) with microsporidiosis due to *Encephalitozoon* (syn. *Septata*) *intestinalis* and an undetermined species of *Encephalitozoon* (Bednarska et al. 2014)

Secondary Immunodeficiency and Immunosuppression

Secondary immune deficiencies are far more common, are considered acquired, and result from chemotherapy and / or radiation treatments for malignancies, immunosuppressive therapies to prevent transplant rejection, malnutrition and poor sanitation, aging, and infectious diseases, among others (Bonilla 014) The greatest impact on the increased recognition of microsporidiosis in humans resulted from the HIV infection pandemic and ensuing immune-deficiency from declining levels in CD4+ T cells that contributed to AIDS-defining conditions of opportunistic infections (OIs) and malignancies. Microsporidiosis then began to be recognized in solid organ and hematopoietic stem cell transplant recipients undergoing immunosuppressive therapies to prevent rejection as well as in patients treated for neoplastic diseases. In addition, especially young children with immature immunity and malnutrition who are often co-infected with other parasitic infections are increasingly identified with microsporidia infections. Aging and immune-senescence also are potential risk factors for increasing incidence of microsporidiosis due to the growing world's population of elderly persons.

HIV infection and AIDS. A new microsporidian species, *Enterocytozoon bieneusi,* was also the first microsporidian species reported to produce an OI in an HIV-infected individual with AIDS and diarrhea Desportes et al. 1985). Additional new species of microsporidia were subsequently identified and characterized from HIV-infected individuals. These included *Encephalitozoon hellem, E. intestinalis, Anncaliia* (syn. *Brachiola*) *vesicularum, Pleistophora ronneafiei, Trachipleistophora hominis,* and *Trachipleistophora anthropopthera* (Table 1). Species already identified in animals or HIV-negative individuals were later reported in HIV-infected patients, as well, and included, *Encephalitozoon cuniculi,* the type species of the genus *Encephalitozoon*, *and Vittaforma corneae* (syn. *Nosema corneum*) (Fayer and Santin-Duran 2014).

Table 1. Microsporidia Infections Associated with Immunodeficiency and Immunosuppression

	Conditions of Immunodeficiency and Immunosuppression			
Species	**HIV**	**Transplant**	**Cancer**	**Other Conditions**
Anncaliia (syn. *Nosema, Brachiola*) *algerae*		Kidney	+	Rheumatoid arthritis
		Lung		Ocular infection, steroids Crohn's disease, diabetes
Anncaliia (syn. *Nosema*) *connori*				Athymic child
Anncaliia (syn. *Brachiola vesicularum*)	+			
Encephalitozoon cuniculi	+	Kidney		Children
		Bone marrow		Primary immune dificiency Diabetes, heart disease Ocular infection, steroids
Encephalitozoon hellem	+		+	Ocular infection, steroids
Encephalitozoon intestinalis	+	Bone marrow	+	Children
				Ocular infections, steroids
Encephalitozoon spp. (undetermined)		Pancreas/kidney	+	Diabetes
		Kidney		Ocular infection, steroids
Enterocytozoon bieneusi	+	Kidney	+	Children
		Liver Heart/lung Kidney/liver		
Microsporidium ceylonensis, M. africanum				Ocular infection, trauma
Nosema ocularum				Ocular infection

Pleistophora ronneafiei	+			
Trachipleistophora anthropopthera	+			Ocular infection, steroids
Tubulinosema hominis	+			Ocular infection, steroids
Tubulinosema acridophagus		Bone marrow	+	
Vittaforma corneae	+			Ocular infection, steroids, trauma Children
Unidentified species			+	Sjogren's disease, ocular infection, immunosuppressives

Prior to the implementation of cART, microsporidiosis was reported with an average prevalence of approximately 15%, ranging from 5 – 85%, depending on the population sampled and methodology that was applied (Fayer and Santin-Duran 2014). Microsporidia infections were considered an end-stage AIDS-defining OI in persons with less than 100 CD4+ T cells per ul blood. After the implementation of cART, the prevalence of microsporidia infections dramatically declined, presumably due to improvements in immune competence, but infections still occur in those unable to access cART. In addition, the population of persons with HIV is aging due to the increased longevity afforded by cART but these individuals often develop HIV-associated non-AIDS (HANA) conditions that mimic diseases observed in the elderly and occur at an earlier age than in non-HIV-infected adults (i.e. undergo accelerated aging). In addition, newly-diagnosed HIV infections in persons over age 50 are increasing and these individuals tend to be diagnosed later during the course of infection (High et al. 2012). Thus, a growing concern is that older individuals with new HIV infection or those with long-term cART and experiencing HANA conditions with accelerated immune senescence may become vulnerable to OIs, including from the microsporidia. Furthermore, serological surveys suggest that microsporidia persist to cause latent infections that could reactivate with aging or with subsequent use of chemotherapy or immunosuppressive treatments Kucerova-Pospisilova and Ditrich 1998; Sak et al. 2011; van Gool et al. 1997).

Organ transplantation and immune suppression. Immunosuppressive agents are administered to organ transplant recipients to help prevent rejection and many of these drugs

(e.g. cyclosporine, tacrolimus, prednisolone, corticosteroids) also reduce T cell-mediated immune responses. Microsporidia infections in solid organ and hematopoietic stem cell transplants first began to be reported in the middle 1990's (Gumbo et al. 1999) Among the earlier reports, *E. bieneusi* was identified in recipients of a heart and a heart/lung transplant while undetermined species were detected in two recipients of a liver transplant and a bone marrow transplant. *E. bieneusi* and the *Encephalitozoon* spp., especially *E. cuniculi,* appeared to be the most common species infecting transplant recipients (Lanternier et al. 2009), similar to the species identified in HIV-infected patients. In addition, a fourth (new) genotype of *E. cuniculi* was identified in a renal transplant patient (Talbani et al. 2010). Diarrhea was the principle clinical sign in transplant recipients carrying *E. bieneusi* infection, whereas systemic infections with or without diarrhea were mostly reported with *Encephalitozoon* species.

Anncaliia algerae has not been reported in HIV-infected patients to date but was recognized as a cause of myositis in a solid organ transplant recipient (lung) with Crohn's disease and diabetes as well as in individuals immunosuppressed by treatments for rheumatoid arthritis and malignancy (Watts et al. 2014). Interestingly, this species commonly infects mosquitoes and demonstrates the vector-borne capacity for the microsporidia. Another new species not yet seen in HIV-infected individuals is *Tubulinosema acridophagus* that was reported in two immune-deficient individuals, including a multiple myeloma patient who received a stem cell transplant Meissner et al. 2012).

Few studies have attempted to estimate the prevalence of microsporidiosis in transplant recipients. One study from Poland reported a prevalence of 16.7% (8 of 48) in transplant patients primarily due to *Encephalitozoon* infections Bednarska et al. 2014) and this prevalence was similar to the estimated 15% microsporidia prevalence in HIV patients prior to the implementation of cART. The majority of independent case reports identified *E. bieneusi* in transplant recipients (Galvan et al. 2011), similar to *E. bieneusi* being the most common species in HIV-infected AIDS patients (Fayer and Santin-Duran 2014). More studies are needed, however, to better estimate the prevalence and incidence of microsporidiosis in organ transplant recipients. The number of organ transplants in the USA has increased from 15,756 in 1991 to 28,954 in 2013, while the number of individuals on the waiting list has jumped from 23,198 to 121,272 and the number of donors also has risen from 6,953 to 14,257 (DHHS, 2014) suggesting that microsporidia infections will increase proportionally with the increasing numbers of transplants. Furthermore, general consensus is that microsporidia infections in transplant recipients are acquired after becoming immunosuppressed, but infections also may be

transmitted from the transplant donor to the recipient(s). For example, *E. cuniculi* recently was detected in three patients who received organ transplants from a single donor who was retrospectively determined to be seropositive for the same genotype (Hocevar et al., 2014). Seroprevalence data in otherwise healthy individuals ranged from 1.3% to 33% in blood donors, pregnant women, and persons with occupational exposure to animals (Ghosh et al., 2014) that has further raised concerns about possible future transmissions of microsporidia from donors to recipients and a need for serologic screening of donors (Hocevar et al. 2014).

Malignancy and radiation / chemotherapy. Microsporidiosis also is being identified in patients receiving chemotherapy and/or radiation for treatment of neoplastic or malignant diseases. Neoplastic disease is often accompanied by other conditions of immune compromise such older age or diabetes. Among the earliest reports of microsporidiosis associated with malignancy was a pulmonary infection due to an undetermined species in a patient with chronic myeloid leukemia who had received a bone marrow transplant Kelkar et al. 1997. An 18-month-old child with acute myelogenous leukemia developed microsporidial endopthalmitis that responded to albendazole (i.e. thus, possibly an *Encephaliotozoon* infection) and this case appears to be among the earliest microsporidia infections in a patient with malignancy without organ transplantation immunosuppressive therapy (Yoken et al. 2002). More recent reports identified *E. intestinalis , E. hellem, E. cuniculi, E. bieneusi* or mixed infections in individuals with acute myelomonocytic leukemia, multiple myeloma, or other forms of cancer, including pediatric patients Chabchoub et al. 2009; Hamamci et al. 2015; Jimenez-Gonzalez et al. 2012; Sivgin et a.l 2013). A new microsporidian species, *T. acridophagus*, was identified as a cause of renal disease in a 67 year-old women with non-Hodgkin's lymphoma undergoing chemotherapy who subsequently developed chronic lymphocytic leukemia (Choudhary et al. 2011) and *A. algerae* was identified in the false vocal cords of an older gentleman with chronic lymphocytic leukemia (Cali et al. 2010).

As for transplantation cases, few prevalence studies have been conducted for microsporidiosis in patients with cancer. A surveillance study of patients in Malaysia with gastrointestinal symptoms and microsporidiosis reported that while 27.5% (25 of 91) of these patients were considered immune-competent, 30.7% (28 of 91) had malignancies, 7.7% (7 of 91) had diabetes, and 9.9% (9 of 91) had both diabetes and a malignancy Norhayati et al. 2008). The most common malignancies in the microsporidia-infected individuals were acute lymphocytic leukemia, acute myeloid leukemia, and non-Hodgkin's lymphoma. In another study *E. intestinalis* infection was detected in 17% (3 of 18) hepatocellular carcinoma patients with

diarrhea (Yakoob et al. 2012) and in a surveillance study of Malaysian cancer patients, 21.9% (68 of 311) patients were positive for microsporidia due to *E. intestinalis* and *E. hellem* (Lono et al. 2008).

Topical steroid use and microsporidial keratitis. Generalized immune suppression as well as the topical use of steroids also contributed to a greater observation of ocular infections produced by microsporidia. The earliest reports of ocular microsporidia infections identified *Microsporidium ceylonensis, Microsporidium africanum, Nosema ocularum*, a *Nosema sp.,* and *V. corneae* that produced stromal keratitis and were often linked to trauma and/or topical application of steroids (Sharma et al. 2014). Two relatively newer species, *T. anthropopthera* and *T. hominis* also produced stromal keratitis. In general, these cases of stromal keratitis did not occur in the HIV-infected population, with the exception of the individual reported with *T. anthrophthera* who also had been administered topical steroids Pariyakanok and Jungwutiwes 2005). Microsporidia infections associated with keratoconjunctivitis however have been recognized more often than with stromal keratitis (Garg 2013; Sharma et al. 2011) and were more common in HIV-infected individuals, especially prior to the use of cART. *E. hellem* was a new species first reported as a cause of keratoconjunctivitis in AIDS patients (Didier et al. 1991). Since then, *E. hellem, E. cuniculi, E. intestinalis, Nosema spp.,* and *V. cornea* also have been found in both HIV- and non-HIV-infected individuals with keratoconjunctivitis (Sharma et al. 2014). Keratitis due to an undetermined microsporidian species also was diagnosed in a patient with Sjogren's (autoimmune) disease who had been treated with local and systemic immunosuppressive drugs (Fernandes and Sharma 2013). Many of the ocular microsporidia infections were associated with systemic infection as well as topical steroid treatments that were believed to contribute to greater susceptibility or exacerbation of the ocular disease. To date, no cases of ocular infections with *E. bieneusi* have been reported (Sharma et al. 2014).

Children with immature immunity, malnutrition, and/or parasitic co-infection. The higher susceptibility of children to microsporidia infections, especially below 6 years of age, appears to result from a myriad of causes including immaturity of the immune system, inadequate hygiene practices due to younger age, and malnutrition with an associated predisposition to infection with parasites that impact immune responsiveness. In a surveillance of HIV-negative individuals in Spain, the prevalence of microsporidiosis was significantly higher in children (10 of 55 or 18.2%) than in adults (15 of 240 or 6.3%) (Lobo et al. 2012). A review on the epidemiology of *E. bieneusi* specifically, represented prevalence rates ranging from 4.4% - 22.5% in HIV-negative children (Matos et al. 2012). Of 72 Roma children living in Slovakia, 22 (30%) were positive for

microsporidia with *E. cuniculi* being more prevalent than *E. bieneusi* (Halanova et al. 2013). In Malaysian children, *V. corneae however,* was more prevalent than *E. bieneusi*

Aging. In 2010, approximately 8% of the world's population was 65 years of age or older and this is expected to grow to 16% by 2050 (Suzman and Beard 2011) Aging is associated with an increase in chronic inflammatory diseases associated with immune-senescence, as well as declining numbers of naïve T cells that make it more difficult to respond effectively to new infections. Only one prevalence study has been published that focused on microsporidiosis in HIV-negative elderly individuals with a mean age of 73.5 years old, who had reported to a geriatric out-patient clinic (Lores et al. 2002). Of the 60 patients, 47 reported symptoms of diarrhea and 8 (13% overall or 17% of those with diarrhea) were infected with *E. bieneusi.* Many of those immunosuppressed as a result of receiving solid organ or bone marrow transplants or from treatments for malignancies were older which likely contributed to susceptibility to microsporidiosis, as well. While little information exists relating aging as a risk for microsporidia, it is expected that immune-senescence associated with the increasing age of the world's population will lead to greater recognition of microsporidia in the elderly.

Concluding remarks and future directions

Immunodeficiency produced by HIV infection and the ensuing AIDS pandemic exposed a number of organisms with pathogenic capabilities in humans, such as the microsporidia. The implementation of cART has completely changed the landscape of HIV infection from an acute and usually lethal condition resulting from OIs and malignancies into a chronic infection that has afforded greater longevity with a drastic decline in the prevalence of OIs, including those caused by microsporidia. With the improvements in diagnostics and awareness, microsporidia continue to be recognized in other groups of humans, however, such as those undergoing immunosuppressive treatments or experiencing immune-deficiency conditions. Furthermore, concerns are growing for a possible reactivation of persistent microsporidia infections based on seroprevalence data that would predict an increase in incidence of clinically relevant microsporidiosis in the aging population, in persons infected with HIV who are experiencing accelerated aging, as well as in transplant recipients and older patients with malignancies.

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How do Microsporidia Exploit the Biochemistry and Physiology of the Host Cell?

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Abstract

Understanding how parasites interact and exploit their hosts is a challenging but exciting field of biology. As the eukaryotes with the smallest number of predicted proteins, microsporidia are the primary model for understanding eukaryotic metabolic streamlining. They also represent minimal model systems for understanding how parasites have evolved and how they manipulate their hosts. However they cannot be manipulated genetically, which makes investigating protein function by forward of reverse genetics impossible. This has left the field with somewhat of a glut of genomic data with few tools to make the most of it. This talk will discuss our current knowledge of microsporidian host exploitation based on comparative genomics and transcriptomics. It will also look at microsporidia as energy parasites and the progress being made to demonstrate how microsporidia maximise exploitation of host nucleotides.

Introduction

Microsporidia have been shown to have a broad range of affects of their hosts, some of them obvious at the morphological level. These include: the hypertrophy of host cells, and at the most extreme, the formation of xenomas in the fish infecting microsporidia (Lom and Dykova 2005). Other microsporidia result in the feminisation of populations (Ironside and Alexander 2015), and others have been suggested to secrete juvenile hormone to maintain the insect hosts in a juvenile state, though this has recently been disputed (Down et al. 2008; Karlhofer et al. 2012). There are also many observations that microsporidia surround themselves with the

mitochondria of the host (Scanlon et al. 2004). Whilst all these observations represent fascinating biological phenomena, due to the difficulty with working with microsporidia, the molecular and cellular basis for many of these manipulations remains unknown.

What has genomics told us about parasite exploitation of the host?

One approach to understand how a parasite interacts with its host is to understand its metabolic requirements and what it needs to take from the host. This can predicted from metabolic reconstructions based on the biochemical pathways encoded by a parasite's genome. The first microsporidian genome project to be fully sequenced was that of *Encephalitozoon cuniculi* (Katinka et al. 2001). The annotated proteins were mapped into known metabolic pathways, giving a basic overview of the metabolites generated and consumed by this minimal parasite. This also allowed identification of some incomplete pathways and gaps in metabolism (Katinka et al. 2001). Some of these gaps could be accounted for by the high rate of sequence change in microsporidia genes, meaning that their function cannot be easily inferred from sequence similarity to other proteins. Others represent gaps in microsporidian metabolism and metabolites that microsporidia require to function but cannot produce themselves. That is to say, it identifies some of the metabolites that microsporidia may need to import from their hosts.

What was evident from this genome project that microsporidia genomes encode a restricted repertoire of genes for *de novo* synthesis of many of the basic building blocks required by the microsporidia. There are a limited number of genes associated with components of amino acid biosynthesis pathways. Genes for the *de novo* biosynthesis of purine and pyrimidine nucleotides are absent, though the pathways to perform nucleotide interconversions are present and genes encoding a fatty acid synthase complex are also absent. All genes required for the tricarboxylic acid cycle, fatty acid b-oxidation, the respiratory electron-transport chain and the F0 F1-ATPase complex are absent. This means that energy is either acquired from the host or generated by substrate level phosphorylation. Now proteomic work has shown glycolysis to be predominantly active in the spore stage and likely provides the ATP for the presumably energetic processes of polar tube firing and germination. This implies that ATP import occurs in the intracellular stage and glycolysis is relied upon when the microsporidian is surrounded by the thick cell wall and has no access to host ATP (Heinz et al. 2012).

Over the last 15 years, many more microsporidia genomes have been sequenced and there are now 26 microsporidian genomes available on the MicrosporidiaDB/EuPathDB database (Checked August 2015) (Aurrecoechea et al. 2011). What comparative genomics has shown is

that the microsporidian metabolic repertoire shows some variation across the phylum. This will inevitably translate to differing requirements on the host. It is apparent that microsporidia jettisoned many core eukaryotic genes early in their evolutionary history and that microsporidia retain a similar 'core' proteome across the group (Figure 1).

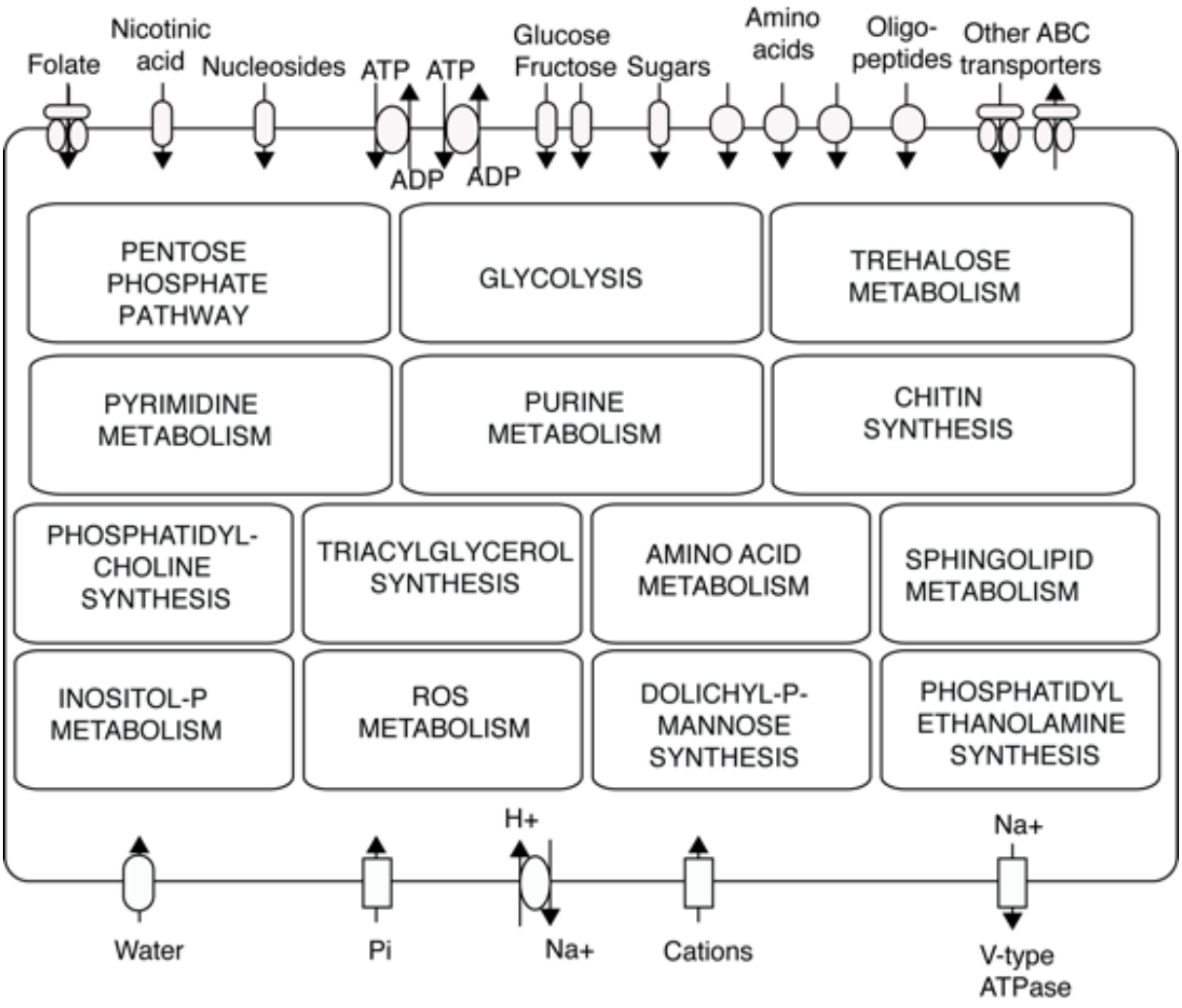

Figure 1. A generalised reduced microsporidian metabolism adapted from (Katinka et al. 2001; Nakjang et al. 2013)

There are however different patterns of retention and loss of certain pathways. For example, the complement of energy producing pathways varies across the phylum. Whilst *E. cuniculi* has a reduced mitochondrion, no mitochondrial genome and no electron transport proteins, the most basal studied microsporidian, *Mitosporidium daphniae* has retained a mitochondrion with a genome and the genes coding for parts of the electron transport chain, though no complex I (Haag et al. 2014). At the other extreme other species have gone beyond just losing the

electron transport chain and seem to have started whittling down the glycolytic pathway. Work by Keeling *et al.* deeply samples the *E. bieneusi* genome using different technologies and confidently showed that the glycolytic pathway has lost several components and is unlikely to be functional (Keeling et al. 2010). Components of the glycolytic pathway have also been shown to be lost in close relatives of *E. bieneusi* (Unpublished data). However and interestingly, it seems that different components of the pathway have been lost in different taxa. This suggests that there may have been a loss of some selective pressure to retain glycolysis in these lineages allowing the pathway to degenerate. Curiously this group infects a variety of hosts and tissues and even cellular environment: Some species live exclusively in the nucleus of cells of the crab hepatopancreas. For this reason there is no obvious common factor allowing the loss of the pathway. It may be that these species are using alternative ATP generating pathways. Several alternatives to glycolysis exist in the prokaryotes, but we have not currently found any of these pathways in our data. Therefore how these organisms survive and germinate without an obvious source of ATP is a mystery.

Comparative genomics has also been used to pinpoint microsporidian genes and gene families that have undergone gene duplication and expansion against the general trend of genomic reduction and streamlining (Nakjang et al. 2013). It has also been used highlight genes and genes families across the diversity of microsporidia that tend to have secretion signals that could potentially send a parasite protein out into the host environment (Nakjang et al. 2013). Genes in both these categories may be important in host parasite interactions. One gene that has undergone gene duplication and expansion in multiple microsporidian lineages is the hexokinase gene, leading to more that one hexokinase gene in many microsporidian genomes (Nakjang et al. 2013). This gene family also tends encodes a secretion signal (Nakjang et al. 2013). This finding of a secretion signal in these proteins had previously provoked the idea that microsporidia may be sending enzymes out into the host environment to boost host metabolism (Cuomo et al. 2012). This was supported by experimental evidence to show that these secretion signals, predicted by bioinformatics, were functional when expressed in *S. cerevisiae*. In the host, the hexokinase may promote host anabolic metabolism increasing the availability of amino acids, lipids, and nucleotides for the pathogen (Cuomo et al. 2012).

What has transcriptomics told us about parasite manipulation of the host?

Another approach to understanding the influence of microsporidia on the host environment is to study changes in the host at the transcriptome level. Several microsporidian and microsporidia-

host parasite transcriptomes have now been sequenced (Aufauvre et al. 2014; Bakowski et al. 2014; Desjardins et al. 2015; Yue et al. 2015). Although the signal from these can potentially be complicated by spurious transcription patterns in the microsporidia (Williams et al. 2005), these can give us a broad overview of microsporidia and host genes that are up or down regulated upon microsporidian infection. Several transcriptome studies also track these gene level changes across an infection time course. It some cases, these transcriptome studies have told us about how the host defends itself against microsporidian infection. For example in the *N. parisii/C. elegans* system, an upregulation of the ubiquitin-mediated response genes is seen, which targets pathogen cells for ubiquitylation (Bakowski et al. 2014). In both *E. aedis*/*A. aegypti* and *V. culicis*/*A. quadrimaculatus* there is an induction of antimicrobial peptides and in *N. ceranae*/*A. mellifera* infection, the generation of an oxidative stress response (Desjardins et al. 2015). However, other upregulated pathways may have potential benefits for the pathogen. These may represent pathways that are upregulated by the host as a response to infection, for example to compensate losses incurred as a result of the infection, or they may represent parasite-mediated changes in metabolite levels. As in the example of hexokinase described above, it may be that the microsporidia are secreting enzymes into the host system to boost metabolism and increase the availability of metabolic resources. There is no strong consensus from transcriptomes on which pathways are more highly expressed in infected hosts, but one set of pathways that are upregulated in both the *N. bombycis*/*B. mori* system and the *T. hominis/*Rabbit kidney cell system are those associated with enhanced synthesis of nucleotides and proteins ((Yue et al. 2015), unpublished data). This is consistent with the idea that microsporidia may be manipulating host metabolism to increase production of useful substrates. Another interesting observation from transcriptomics is that in the silkworm, the microsporidia maybe disrupting the juvenile hormone levels. Using a silkworm microarray system to study gene expression at 2, 4, 6 and 8 days post-infection with *N. bombycis*, it was shown that many components of the juvenile hormone synthesis pathway were upregulated. This led to the suggestion that the microsporidian increases juvenile hormone levels in order to retard larval development providing the pathogen with time and nutrients to fuel reproduction (Yue et al. 2015). Interestingly increased juvenile hormone levels have also be suggested or observed in other microsporidia host systems. This was first suggested in the 1960s to occur in the *Nosema whitei/*Red flour beetle system (Fisher and Sanborn 1962). Elevated juvenile hormone levels were also observed in early development in the *Vairimorpha necatrix/* tomato moth system, where again, it is thought to prolong the host larval state in order to maximise spore yield, and in *Nosema ceranae* infected honeybees where it may lead to premature foraging Down et al. 2008;

Goblirsch et al. 2013). However other studies suggest that these disruptions of the juvenile hormones may sometimes be the consequence of impaired fat body function (the primary site for production for the juvenile hormone degrading enzyme JH-esterase) rather that the effect of parasite manipulation of the host (Karlhofer et al. 2012).

How can we combine genomic and molecular techniques to understand host exploitation?

Perhaps one of the most interesting aspects of microsporidian biology is the fact that, along with intracellular prokaryotes like Chlamydia and Rickettsia, they are energy parasites that tap into energy supply of their host. Across the phylum, microsporidia show varying degrees of loss of energy generating biochemical pathways from *Mitosporidium daphniae*, which retains a mitochondrial genome and elements of the electron transport chain to *Enterocytozoon bieneusi*, which has not only lost all mitochondrial energy producing pathways, but has also lost most of the components of glycolysis (Haag et al. 2014; Keeling et al. 2010). This makes them highly reliant on the host for ATP. This was an idea that was supported by early biochemical work that showed that *Amerson michaelis and Spraguea lophii* sporoplasms could be transiently maintained outside the cell in a medium supplemented with ATP (Weidner and Trager 1973). Furthermore microsporidia have long been known to surround themselves with host mitochondria, presumably to facilitate host ATP uptake.

The mechanism for this ATP uptake was revealed in the first genomic studies of microsporidia. An early small-scale genome survey of the microsporidian *Spraguea lophii*, showed the presence of genes encoding ADP/ATP translocases similar to those found in *Chlamydia* and *Rickettsia* (Hinkle et al. 1997). These were also subsequently identified in the genome of *Encephalitozoon cuniculi* and all successively sequenced genomes up until that of *Mitosporodium daphniae* (Katinka et al. 2001). The phylogenetic distribution of the protein in these two pathogen groups implies that the Microsporidia acquired these via lateral gene transfer from either the Rickettsias or the Chlamydias. Comparative genomic work by Nakjang *et al.* showed that these proteins have undergone gene duplication after the divergence of microsporidia from other fungi, bucking the general trend for protein loss and genome reduction in microsporidia generally (Nakjang et al. 2013). This means that many microsporidia have multiple copies of this protein. Whilst *Mitosporidium daphniae* has none, all other studied microsporidia have at least two copies and many have four (Figure 2).

Functionality of these proteins was first demonstrated by Tsaousis *et al.* working with *E. cuniculi*, who showed that indeed these proteins were able to transport ATP across a membrane. Interestingly, they also showed that whilst three of the four proteins localised to the *E. cuniculi* membrane, one localised to the reduced mitochondrion, working to import ATP into the organelle (Tsaousis et al. 2008). Further work by the Embley lab characterised these proteins in another species of microsporidian: *Trachipleistophora hominis* (Heinz et al. 2014). Here they explored the diversity of substrates that these transporters took across the host membrane. They showed that along with ATP, these could also uptake GTP, ADP, GDP, but not purines. This fits well with the biochemistry of *T. hominis* as predicted from its genome sequence, which suggest that *T. hominis* is incapable on *de novo* purine biosynthesis (Heinz et al. 2012; Weidner and Trager 1973).

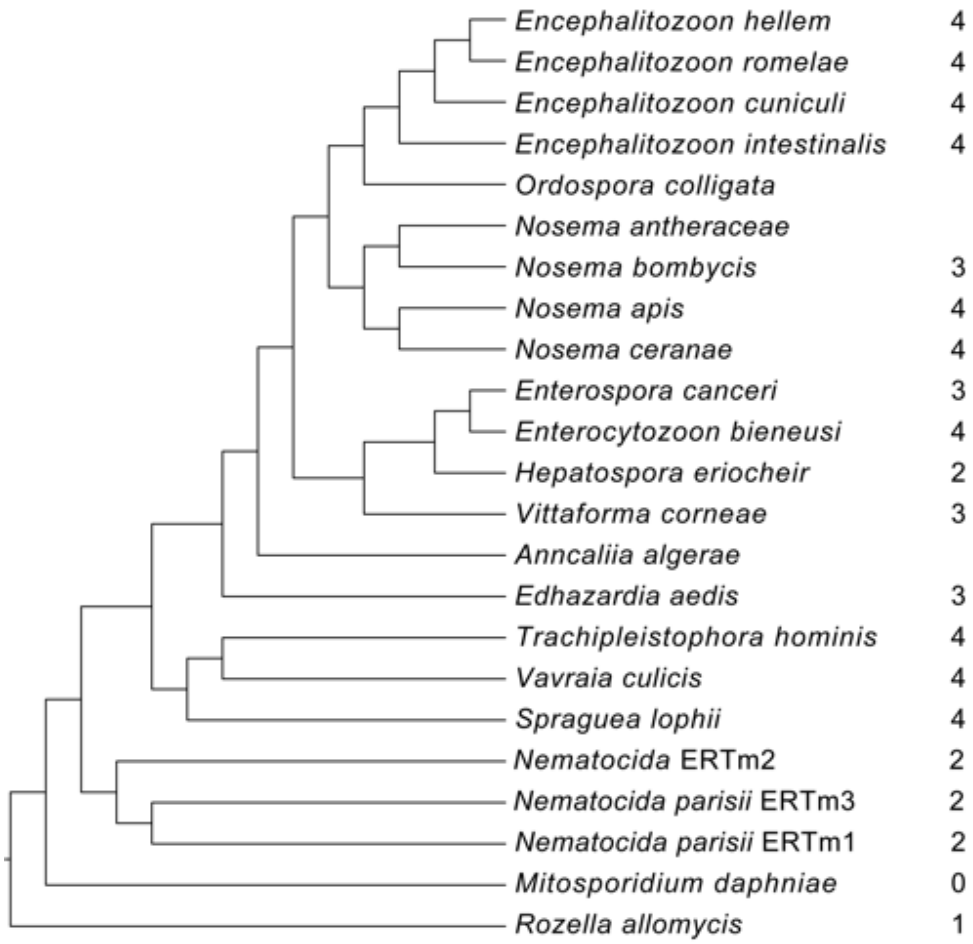

Figure 2. Numbers of laterally acquired ATP/ADP translocases in detected in the genomes of different species of microsporidia

Recent work has also sought to elucidate how microsporidia interact with host mitochondria. The relationship between *E. hellem* had been studied in 2004 and it had been shown that the relationship between the microsporidian and the host mitochondria was not dependent on an intact microtubule network (Scanlon et al. 2004): Recent work by Hacker *et al* has added more support to the idea that the interaction is not based on the manipulation of microtubules Studying *E. cuniculi*, they showed that the mitochondria surrounding its parasitophorous vesicle was just one mitochondrion thick, showing a sharp drop off. This is suggestive of a direct interaction between mitochondria and the microsporidian. This interaction was affected by the addition of protease. Taken together, this suggests a protein-mediated direct tethering on mitochondria to the microsporidia. Additionally, they found that the VDAC mitochondrial channel was concentrated on the microsporidian side of the host mitochondria (Hacker et al. 2014). Whilst the exact nature of the protein based interaction between the pathogen and host still remains unknown, this case study shows how traditional biochemistry, genomics, protein characterisation can work together to resolve outstanding questions in microsporidian biology.

Concluding remarks and future directions

High-throughput molecular methodologies such as genomics, transcriptomics, proteomics and metabolomics can give clues to what microsporidia must take from their hosts and the types of changes that occur during microsporidian infection. From this type of data it is possible to make predictions about how microsporidia are manipulating their hosts. However, the lack of transformation system in microsporidia continues to hamper progress in characterising host parasite interactions. Several attempts at generating a microsporidian transformation system are rumoured to have been attempted, though none have been published as they have presumably been unsuccessful. Some success has been reported in knocking down microsporidia numbers in honeybees by RNAi of NTT proteins but this have not led to a knock-down system in host-parasite cell culture system that would be useful for protein characterisiation. More success may be achieved using tractable host systems and exploring the effect of parasites on these more amenable hosts. This is the type of research has been initiated using the *C. elegans*/*N. parisii* system and development or discovery of more tractable systems may be the key to unravelling the molecular basis of microsporidian exploitation of hosts.

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Microsporidiosis in Companion Animals - Their Role in Zoonoses

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Abstract

There are four microsporidian species of relative importance for both the veterinary and the human medical fields. *Encephalitozoon cuniculi* and *E. hellem* are most frequently identified in mammalian and avian hosts, respectively, and are occasionally accidentally the cause of human disease. In contrast, *E. intestinalis* and *Enterocytozoon bieneusi* are considered human pathogens that are sometimes identified in a variety of domestic and wild animals in epidemiologic surveys, but are not considered important animal pathogens. It is likely that anthropozoonotic (animal to human) and zooanthroponotic (human to animal) transmission of these parasites occur. However, the relative importance of zoonotic transmission events is still unclear when exploring the way that these parasites survive and disseminate in various hosts.

Introduction

There are four microsporidian species of relative importance in both the veterinary and the human medical fields. *Encephalitozoon cuniculi* was first identified as a cause of neurological disease in rabbits in the early 1920's (Wright and Craighead 1922). More recently, two additional *Encephalitozoon* species, *E. hellem* and *E. intestinalis* (originally named *Septata intestinalis*), along with *Enterocytozoon bieneusi* were identified first in human hosts (Desportes et al. 1985; Didier et al. 1991; Cali et al. 1993). All four of these organisms have been reported in both domestic animals and in humans, but the likelihood of direct or indirect zoonotic

transmission for each parasite species and the relative importance of human - animal contact require further research efforts. Scientific literature and clinical experiences suggest that *E. cuniculi* is most important from the veterinary perspective with a variety of natural hosts. Similarly *E. hellem* uses birds as their natural hosts, and humans are occasional accidental hosts for these two parasite species. In contrast, data indicate that *E. intestinalis* and *Enterocytozoon bieneusi* use humans as their natural hosts and that domestic animals are occasional accidental hosts (Fig 1).

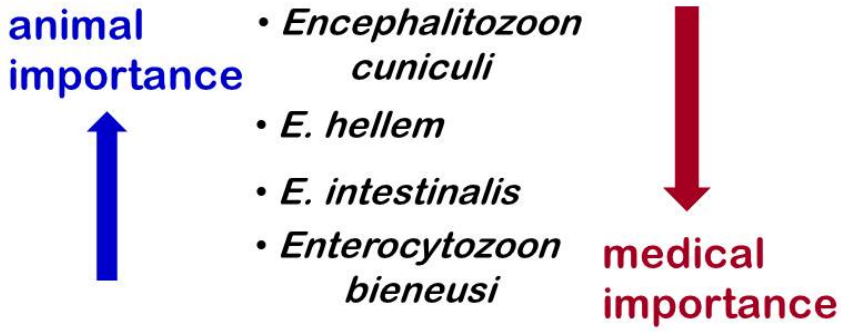

Figure 1. Schematic diagram indicating the relative veterinary and medical importance of four microsporidian species.

Encephalitozoon cuniculi in companion animals

Rabbits and rodents are the most common hosts for *E. cuniculi*, but the parasite has occasionally been identified in a wide range of mammalian hosts including humans (Snowden 2014). Focusing on domestic rabbits first, this organism has largely been eliminated from purpose bred research animal colonies by implementing a serologic test & cull approach. However, the parasite continues to be common in pet and meat rabbits. Most infections are asymptomatic, but sometimes animals develop neurologic disease, often called "wry neck" (Latney et al. 2014). Additionally renal and ocular problems, particularly cataracts and uveitis are occasionally reported in rabbits.

Compiling epidemiologic survey data from serologic testing for *E. cuniculi* reactivity in these pet rabbit populations, a surprisingly high percentage of clinically normal rabbits are antibody positive using a variety of diagnostic methods and in a wide range of geographic locations. As examples, seropositive rates of 22.6%, 42%, 43%, 50.7% and 75.4% were reported in Korea,

Egypt, Germany, Netherlands and Italy respectively in selected recent studies (JinCheol et al. 2014; Abu-Akkada et al 2015; Hein et al. 2014; Newmayerova et al. 2014; Lonardi et al. 2013). These data indicate that this parasite is an important and generally under-recognized pathogen in domestic rabbits.

Over the past 50 years, *E. cuniculi* infections have been reported in laboratory rats, multiple mouse species, hamsters and guinea pigs [5]. By using modern laboratory animal management techniques of serologic surveillance and culling, the parasite is generally eliminated in research rodent colonies. However, there is very little data on parasite prevalence in the 'pocket pet' commercial industry where rats, mice, hamsters and guinea pigs are available as pets. In an infectious pathogen survey of house mice (*Mus musculus*) purchased from 6 pet shops in Germany, 3 of 6 mice from a single location were seropositive for *E. cuniculi* (Dammann et al. 2011). Obviously there is a need for parasite surveillance in pet rodent hosts to determine if there is an unrecognized risk of human expose through this type of household pet.

Similar to rabbits and rodents, dogs are also natural hosts for *E. cuniculi*. These infections in dogs and rabbits occur through the ingestion of the environmentally resistant spores or through transplacental transmission from dam to fetus [5]. Adult dogs are typically asymptomatic or rarely develop chronic progressive renal disease, while puppies may develop fatal neurologic disease. Sporadic canine infections have been reported in older veterinary literature (1950's-70's) (Snowden 2014). In a more recent case series report on canine encephalitozoonosis, several dozen cases of fatal neurologic disease were documented in puppies using histology and molecular confirmation of the parasite identity as *E. cuniculi* genotype 3 (Snowden et al. 2009). Additionally, spore shedding was detected in asymptomatic adult dogs in several kennel settings. In a pattern associated with many microsporidian infections, when host animals are overcrowded and stressed and environmental sanitation declines, disease outbreaks occur. All of these canine cases had associations with high volume breeding kennels, and some would be classified as 'puppy mills'.

Reviewing epidemiologic studies in dogs, there are about a dozen serologic surveys for *E. cuniculi* antibodies in a variety of canine populations with most seroprevalence rates falling within the 15 to 35% seropositive range Snowden 2014). Data suggest that canine encephalitozoonosis is an underdiagnosed problem that needs to be elevated on the veterinary radar.

There have been a small number of human infections reported in scientific literature, where *E. cuniculi* causes systemic, sometimes fatal, disease in immunocompromised patients (Weiss

2014). The source of human exposure and infection is not easily identified in these cases; therefore, the question of the relative importance of zoonotic transmission of the parasite arises. Based on molecular comparisons of the ITS region of the ribosomal RNA gene from multiple *E. cuniculi* isolates, three different parasite strains have been identified Didier et al. 1995). Parasite isolates from human cases have been characterized as both genotype 1, the 'rabbit' strain, and genotype 3, the 'dog' strain (Deplazes et al. 1996; Didier et al. 1996; Snowden et al. 1999). Evidence of direct transmission between pets and humans is not reported in scientific literature. However, spore shedding has been documented in asymptomatic rabbits and dogs, as well as a small variety of bird hosts. Therefore, data suggest that direct or indirect transmission of *E. cuniculi* spores from pets to humans could be possible and is likely. Additional studies are needed to document longitudinal spore shedding in pet animals and to assess the relative risk of human exposure from those animals or from other environmental sources.

Encephalitozoon hellem in birds

The microsporidian species, *Encephalitozoon hellem* was first isolated from human cases of ocular disease, and both respiratory and eye diseases have been described with increasing frequency in humans (Didier et al. 1991; Weiss 2014). A few years after the parasite was found in people, it was identified as a cause of fatalities in budgerigar (*Melopsittacus undulatus*) chicks (Black et al. 1997). Subsequently, *E. hellem* infections have been detected in a wide variety of pet and wild birds of diverse species ranging from tiny hummingbirds and finches to ostriches [5]. Most reports focusing on pet birds include the psittacines, members of the parrot family. Budgerigars (parakeets), cockatiels (*Nymphicus hollandicus*), multiple species of lovebirds (*Agapornis* spp.), Eclectus parrots (*Eclectus roratus*) and cockatoos (*Cacatua* spp.) as well as several species of finches have been documented as hosts for *E. hellem.* Similar to *E. cuniculi* infections in rabbits and dogs, occasional enteric, renal or systemic fatal disease has been reported in *E. hellem* infections in avian hosts. However, most infections are asymptomatic, and spore shedding in asymptomatic birds has been reported multiple times (Barton et al. 2004; Lee et al. 2011).

Comparisons of avian and human isolates of the parasite were identical using molecular analysis of ribosomal RNA genes (Snowden et al. 2000). Most avian veterinarians consider *E. hellem* as a bird parasite that occasionally causes avian disease as well as accidentally infecting humans who may or may not be immunosuppressed. In two separate scenarios this author has worked with a human ophthalmologist to diagnose *E. hellem* as a cause of corneal

disease in healthy young adult people who worked at commercial pet bird breeding farms. It is likely that the humans were exposed to parasite spores in their eyes through the aerosolization of organisms shed in bird droppings. Indirect zoonotic transmission of *E. hellem* was strongly suggested in both of these cases. Unfortunately permission was not granted to publish either of the cases.

There are several epidemiologic surveys for microsporidian parasites in wild bird populations, particularly pigeons. However, limited information is available regarding parasite prevalence in pet bird populations. In a survey of about 200 lovebirds from 8 breeding facilities in Texas, *E. hellem* spore shedding was documented in 25% of the birds with similar prevalences between juveniles and adults (Barton et al. 2004). Interestingly, birds infected with the immunosuppressive beak and feather disease virus were three times more likely to shed spores than virus negative birds. In a South Korean survey of captive-bred pet parrots, 7 of 51 birds were positive by molecular analyses (Lee et al. 2011). In a molecular survey of a variety of captive exotic birds in the Czech Republic, 18 of 287 birds were positive for E. hellem (Kasickova et al. 2009).

Encephalitozoon intestinalis in companion animals

Our third microsporidian species, *Encephalitozoon intestinalis*, is well recognized as a cause of enteric disease in immunocompromised humans, and is increasingly recognized as a cause of diarrhea in immunocompetent people (Weiss 2014). In contrast to the previous two *Encephalitozoon* species, *E. intestinalis* has been reported sporadically in only a few clinical cases in wild and domestic animals, and most of these animals had close association with humans. There have been a number of surveys for microsporidia in a variety of animal hosts, but *E. intestinalis* has been identified in only a few companion animals in several of those studies. In a serologic survey of 5 animal host species in Slovakia, 6% of 111 dogs were serologically positive for anti-*E. intestinalis* antibodies (Malcekova et al. 2010). In a molecular study conducted in Iran, 5 of 100 dogs and 0 of 40 cats were fecal PCR positive for *E. intestinalis* (Jamshidi et al. 2012). In one interesting case report, an HIV infected person with chronic diarrhea caused by *E. intestinalis* owned a pet cat that also shed spores (Vleasquez et al. 2012). Evidence from this and other cases involving wildlife suggest that the parasite can be transmitted zoonotically. It is likely that the transmission is human to animal (zooanthroponosis) in these cases.

Enterocytozoon bieneusi in companion animals

Our fourth microsporidian species*, Enterocytozoon bieneusi,* is the most problematic parasite of this group in humans as a diarrhea-associated pathogen (Weiss 2014). It is challenging to work with this organism experimentally since there are a confusing number of genetically varied strains, the parasite is not established well in *in vitro* culture, and experimental animal infections are problematic.

Many epidemiologic surveys have been conducted in an eclectic variety of domestic, exotic and wild mammals and avian hosts using molecular methods to detect *E. bieneusi* in fecal samples (Snowden 2014). Data suggest that this parasite is found in a wide variety of domestic animals but there are limited reports in companion animals. In a recent study in Thailand, 0 of 206 (0%) dog fecal samples and 25 of 80 (31.3%) of cat fecal samples were positive for several genotypes of *E. bieneusi* using nested PCR (Mori et al. 2013). In contrast, in a Chinese study using different molecular techniques, 15.5% of 348 dogs and 11.5% of 96 cats were positive with 18 and 8 different genotypes being identified in dogs and cats respectively (Karim et al. 2014). In an unusual study in Peru (where guinea pigs are often raised for food), a 2 year-old child was diagnosed with *E. bieneusi* infection that was characterized as an unusual genotype (Cama et al. 2007). A survey of dogs, chickens, cats and guinea pigs in the household showed that feces from 7 of 8 guinea pigs were positive for the same parasite genotype. Extending the survey, 3 of 59 guinea pigs from 2 of 20 neighboring houses were positive for the same parasite strain, but none of the other 388 children in the study were diagnosed with the same parasite genotype. These data certainly suggest the zoonotic transmission of the parasite between the guinea pigs and the child.

From a veterinary perspective, *E. bieneusi* infection in the range of animal hosts is not considered an important pathogen. However, since there are so many genetic strains that vary among human and animal host populations and among geographic locations, the likelihood of animal to human transmission is still unknown and the relative importance of zoonotic potential is still generally uncertain.

Concluding remarks and future directions

Pondering the zoonotic potential of *Encephalitozoon* species and *E. bieneusi*, there are multiple studies that detect microsporidian spores in the environment including soil, surface water and wastewater. The source of these spores is unclear, and the importance of environmental exposure to *E. bieneusi* or *Encephalitozoon* spores with regard to transmission between

humans or animals is still unclear. It is likely that both anthropozoonosis (animal to human) and zooanthroponosis (human to animal) transmission events occur. Further research is needed to clarify the importance of these zoonotic transmission routes when compared to non-zoonotic animal to animal or human to human patterns of transmission. Microsporidia remain intriguing pathogens which continue to present research challenges.

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Microsporidia as Regulators of Insect Populations and Disease Agents in Mass-Reared Insects – A Future Threat to Edible Insect Cultivation?

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Introduction

An increase in the global human population, accompanied by finite arable land and pasture needed for the production of animal livestock, has increased our demand for sustainable sources of food (Premalatha et al. 2011). Insects provide balanced nutrition and are an underexploited food resource for human food and animal feed. Whether insects are reared as food for human consumption, or for their biological control potential in agriculture, insect mass-rearing systems are designed to provide optimal conditions for insect growth. However, rearing conditions may cause overcrowding, temporary starvation and cannibalism, and such stressful conditions often enhance the prevalence of microsporidia (Kluge & Caldwell 1992). Microsporidia are known to cause chronic disease that results in sub-lethal debilitating effects, including reduced longevity and fecundity, developmental delays and abnormalities, and increased mortality (Siegel et al. 1986; Geden et al. 1992; 1995; Saito & Bjørnson 2008). Microsporidia are effective pathogens in mass-rearings because they produce a multitude of resistant, transmissible spores that are often transmitted both vertically (parent to offspring) and horizontally (among offspring). Because infected individuals often remain asymptomatic, diagnosis usually depends on observations of poor productivity followed by microscopic examination of stained specimens for the presence of microsporidian spores.

Microsporidia and insects used for human food

Insects have been part of human diet for more than 3000 years. Today, insects remain a popular food source in some areas of Asia, Australia, Europe and America (see Chen et al. 2009; Ramos-Elorduy 2009). An estimated 1500-2000 insect species are consumed globally, and some are considered a delicacy (see Premalatha et al. 2011). Despite being high in protein and nutrients, insects remain a relatively underexploited food resource for both human and animal feed in the western world (Rumpold & Schlüter, 2013; Chen et al. 2009).

Insects provide balanced nutrition and can be consumed whole or processed (see Rumpold & Schlüter 2013; Klunder et al. 2013). The inclusion of insect protein in animal feed seems intuitive because insects are a natural food source for poultry and fish (Rumpold & Schlüter 2013). Insect production is more efficient than conventional livestock because insects have a high feed conversion efficiency, consume less water, and insect production has minimal impact on the environment. Insects reproduce faster than conventional livestock, have shorter lifecycles, require less space for rearing, and many insect species may be reared on organic waste (see Ramos-Elorduy 2009; Premalatha et al. 2011; Klunder et al. 2013; Rumpold & Schlüter 2013).

Insects have been reared successfully for millennia and microsporidia have a well-known association with insects that are mass-reared for human benefit. For example, the silkworm, *Bombyx mori* L., has been reared domestically since about 700 BC (Davidson 2012) and has been associated with diseases for centuries. In 1845, pébrine disease in silkworm grown in culture in France had a profound, negative impact on the silk industry. Infected silkworm larvae eat less and have developmental problems that may result in mortality. Larvae that develop into adults may be deformed and exhibit reduced fecundity (James & Li 2012). Studied by Pasteur, the disease organism was later identified as the microsporidium *Nosema bombycis* (Davidson 2012).

Prized for their production of silk, silkworm pupae are often consumed once the silk has been harvested, providing an important source of protein. Although the Pasteur method (a technique to screen and propagate healthy individuals from mixed infections) is an effective control measure for microsporidia in silkworm rearings (Davidson 2012), *N. bombycis* remains the most important pathogen in global silkworm production (James & Li 2012). Microsporidia infect many other insects, including some species that are collected or reared as food for humans. However, there is little information on the effects of microsporidia on insects that are reared specifically for food, or the impact of microsporidia on the nutritional quality of insects.

Microsporidia and insects used in food production for biological pest control

In addition to being consumed by humans, many insect species are important for pest control in agriculture, providing a viable alternative to chemical pest control. Although many are field-collected, several predator and parasitoid species are mass-produced in commercial insectaries. Quality control is important to ensure that disease-free predators and parasitoids are released because microsporidia may cause ongoing, deleterious effects on the performance of future generations in the field. These pathogens may be transmitted among related hosts (Saito & Bjørnson 2008); and infected individuals, once released, are capable of disseminating microsporidia into environments where they did not exist previously (Kluge & Caldwell 1992).

Microsporidia infect more than 30 species of field-collected and mass-reared beneficial insects including parasitoids, predatory insects and mites, phytophagous insects used for weed control, and beneficial nematodes. As pathogens, microsporidia tend to produce a suite of predictable effects, including decreased food consumption, prolonged development, physical deformations, reduced fecundity and longevity, and death (see Bjørnson & Oi 2014).

Microsporidia may directly impact parasitoids or predators, having serious deleterious effects on mass-rearings while also affecting the performance of naturalized populations. For example, *Muscidifurax raptor* is a parasitoid found naturally on dairy farms where they provide effective house and stable fly control. *M. raptor* is also mass-reared for inundative release, and parasitoids from commercial insectaries may be infected with the microsporidium *N. muscidifuracis*. Although the pathogen infects indigenous *M. raptor* (1.1-10.7% prevalence), pathogen prevalence is higher on farms where commercially reared *M. raptor* are released (up to 84%; Geden et al. 1995). *N. muscidifuracis* is transmitted vertically (100% efficiency; Zchori-Fein et al. 1992) and the prevalence of *N. muscidifuracis* increases when parasitoids are overcrowded (Geden et al. 1992).

When compared to uninfected *M. raptor*, the development of infected individuals is prolonged, and those that emerge have shorter life spans and produce fewer progeny. Parasitoid infection results in reduced fly control (Geden et al. 1992; 1995). Uninfected *M. raptor* colonies are re-established through the Pasteur method (Zchori-Fein et al. 1992) and heat treatments reduce pathogen prevalence but do not eradicate the pathogen. Albendazole, fumagillin, and gamma radiation are ineffective for control (Geden et al. 1995; Boohene et al. 2003). Rigorous quality control is needed in *M. raptor* rearings to ensure that only microsporidia-free *M. raptor* are released.

In addition to reducing the fitness of mass-reared insects, microsporidia may have an even greater impact on insect species that are used as food for beneficial insects in mass-rearings. *Macrocentrus ancylivorus* is an important parasitoid of the oriental fruit moth, *Grapholitha molesta*. In 1943, experimental production of *M. ancylivorus* involved the parasitoid being reared on the potato tuber moth, *Gnorimoschema operculella* as an alternative host (Allen & Brunson 1947). Some *M. ancylivorus* adults became infected with microsporidia, having whitish, swollen or malformed ventral abdomens (Allen & Brunson 1945; Allen 1954). Infected individuals had a shortened life span and the majority of infected parasitoid larvae were unable to complete development, resulting in fewer parasitoid offspring (Allen & Brunson 1945; 1947; McCoy 1947; Allen 1954). Microsporidia also infect all developmental stages of the potato tuber moth. Infected *G. operculella* hosts are asymptomatic but the pathogen has adverse effects on development and reproduction (McCoy 1947; Allen 1954). Microsporidia control was achieved through the Pasteur method, heat treatments of *G. operculella* eggs, dry heat sterilization of inanimate objects, and with the use of diluted formaldehyde solutions as a general disinfectant (Allen & Brunson 1947; Allen 1954).

Microsporidia as regulators of agricultural pest insect populations

Microsporidia may also play an important role in the regulation of agricultural pest populations. *Nosema pyrausta*, a pathogen of the European corn borer, *Ostrinia nubilalis*, occurs in corn-growing areas in the United States and periodic epizootics help regulate *O. nubilalis* populations (Hill & Gary 1979; Andreadis 1986). *N. pyrausta* causes debilitating effects in *O. nubilalis*, including reduced fecundity, reduced adult longevity, and increased mortality in young and diapausing *O. nubilalis* larvae. Overwintering mortality can be high in infected larvae when environmental conditions are stressful (Siegel et al. 1986; Sajap & Lewis 1992; Lewis et al. 2009). *N. pyrausta* transmission is vertical in first generation *O. nubilalis* larvae, whereas horizontal transmission becomes an important mode of pathogen transmission for second generation larvae (Andreadis 1986).

Future challenges with the mass-production of insects as food

Based on the cryptic nature of microsporidia, the debilitating effects that they cause, and the difficulties associated with their control, these pathogens have potential to become problematic in mass-rearings of insects that are used for human food. Microsporidia tend to become prevalent in mass-rearings because of stressful conditions that periodically occur (overcrowding, temporary starvation, cannibalism). When infected individuals are asymptomatic, microsporidia

may remain unnoticed until poor productivity is observed. The best strategy for managing microsporidia is to exclude these pathogens from mass-rearings, rather than to attempt to eradicate them once they have become established. Field-collected individuals should be quarantined and screened for pathogens before they are added to existing colonies. Once colonies are established, routine screening of individuals is needed to ensure that colonies remain microsporidia-free. If mass-rearings become infected with microsporidia, the Pasteur method is the most effective means of eliminating the pathogen. Other control measures (heat treatments and chemical controls) have been used with limited success.

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Microsporidiosis in Wild Fish – an Emerging Issue?
(Microsporidia: Very Interesting Pathogens in Very Interesting Hosts)

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Abstract

Microsporidia of fishes are increasing in importance from various aspects. First, there is a continued interest in pathological changes in fish as biomarkers of pollution exposures and lesions caused by microsporidia have resulted in some misdiagnoses of toxicopathic changes. One example is *Ichthyosporidium weissii*, which was initially misdiagnosed as a gondal tumor. Second, there has been an increased concern about microsporidia in wild fishes as sources of infections in finfish aquaculture, which has been expanded significantly in recent years. This prompted our investigations on the host range and source of *Loma salmonae* for marine netpen reared salmon. Third, *Pseudoloma neurophilia* and *Pleistophora hyphessobryconis* are two microsporidia in laboratory zebrafish that have become more important with the dramatic increase in use of this fish as a biomedical model. Infections of wild zebrafish and other aquarium fishes is of interest for understanding the source and pathogenesis of these infections.

Introduction

Over 100 species in about 16 genera have been described from fishes (Kent et al. 2014). Several have been reported to cause significant disease in both wild and cultured fish. Here we review three scenarios demonstrating emerging issues with microsporidia relating to fish. Fish have been used as sentinels for contaminant exposure for decades (Steiniford et al. 2003). A common end point for these studies has been the presence of neoplasms, as well as other

lesions consistent with toxicant exposure. This has resulted in misdiagnosis of some parasite-induced lesions in fish, and one example of this is *Ichthyosporidium weissi*. Aquaculture is expanding dramatically around the world, and one method of rearing fish that has significantly contributed to this is the farming of salmon in open water, marine netpens. These captive fish are exposed to many parasites, including several microsporidia, and we describe here the sources of infection and host distribution of *Loma salmonae* and other *Loma* species in salmonids. Biomedical research is another area that is seeing a dramatic increase in the use of fish, led by the zebrafish *Danio rerio* (Phillps and Westerfield 2015). Two microsporidia have been documented in zebrafish in research laboratories, and here we discuss their host distribution and impacts on research.

Xenomas, pseudotumors, and pollution

Observation of neoplasms in naturally occurring populations of fishes has been used for decades as an indicator of exposure to carcinogenic pollution (Stentiford et al. 2003). Over 30 years ago Harshbarger (1984) reviewed lesions that may be misdiagnosed as neoplasia. Wild fishes, including healthy fish from pristine waters, are naturally infected with a wide variety of parasites. Hence, many of the “pseudotumors” described by Harshbarger (1984) were caused by parasites. Wolf et al. (2015) provides a more recent review of the subject, showing that the problem with misdiagnosis of fish lesions, including confusing parasite infections with toxicopathologic changes is still common. One of the most documented misdiagnoses was "X-cell tumors" in flafish skin and pseudobranchs of cod gills. Orginally thought to be skin tumors, the putative neoplastic cells are actually protozoan parasites (Miwa and Kamaishi 2009; Freeman 2009).

About 6 years ago, dramatic hypertrophy of the gonads, particularly ovaries in arrow gobies, was observed in Morro Bay, CA. These were initially identified as neoplasms, which resulted in a significant research effort funded to identify carcinogens that may be the cause and other toxicopathic effects on these fish. We examined the affected fish and, in contrast to neoplasms, all of the putative tumors (Figure 1) that we observed were actually large, coalescing microsporidian xenomas which contained polymorphic spores consistent with members of the genus *Ichthyosporidium.* Using both ribosomal DNA and morphologic analysis we concluded that this was an undescribed species, and described it as *Ichthyosporidium weissi* (Sanders et al. 2012a). Hence, this is another example of misdiagnosis of a lesion orgininally thought to be caused an anthropogenic compound. Adding to the the potential for misdiagnoses, in recent years several large surveys relating fish lesions to ecocsystem health have not included

histology. One example is the US EPA's Environmental Monitoring & Assessment Program, which often only includes macroscopic changes in their surveys (http://www.epa.gov/emap)

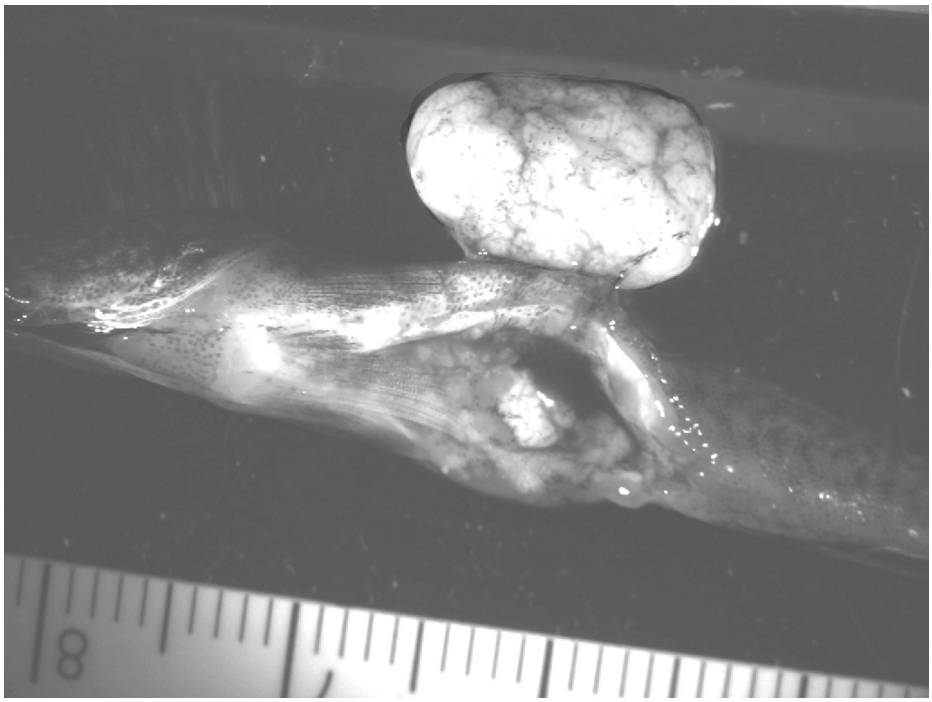

Figure 1. Female arrow goby. White mass represents coaslescing xenomas of *Ichthyosporidium weissii*.

Emerging microsporidian diseases in pen-reared salmon, and their wild fish reservoirs

In the last two decades marine netpen culture of salmonid fishes has expanded dramatically. *Loma salmonae* is well known as a serious pathogen in Pacific salmon species (members of the genus *Oncorhynchus*) (see reviews by Kent and Speare 2005 and Speare and Lovy 2012). The parasite causes morbidity associated with severe chronic inflammation of the gills. Fish farmers were concerned about potential sources and reservoirs for the infection as salmonids reared in marine netpens occur in coastal water with abundant wild fishes, and some of these fishes are actually attracted to the pens. The spore and xenoma morphology of *Loma* species are quite similar, and hence it has been difficult to ascertain if the *Loma* species seen in a variety of fishes were an assemblage of many morphologically similar species or one species with relatively broad host specificity. First we examined shiner perch (*Cymatogaster aggregata*) as this is one of the most common fishes around netpens. This fish was commonly infected with a

Loma sp., but we determined that it was a species distinct from *L. salmonae* based mainly on rDNA analysis (Shaw et al. 1997).

We then extended our study to other common marine fishes in British Columbia; walleye pollock, ling cod, Pacific tom cod, and sablefish (Brown et al. 2010a). This study included spore morphology, parasite development, rDNA and elongation factor 1-alpha (EF-1α) gene. All the data indicated that members of the genus *Loma* are very host specific; with each host having its own species. They were also different from *L. morhua* from Atlantic cod and *L. branchialis* from Atlantic Ocean haddock. Hence new species were erected for the *Loma* species from these marine fishes from British Columbia. The narrow host specificity of *Loma* was also supported by laboratory transmission studies. Members of the genus *Oncorhynchus* (pink and chum Salmon) and brook trout (*Salvelinus fontinalis*) were susceptible to *Loma salmonae* from Chinook salmon, but Atlantic salmon (*Salmo salar*) and Arctic char (*Salvelinus alpinus*) were not (Shaw et al. 2000). Moreover, non-salmonids such as shiner perch, goldfish, sticklebacks, and guppies, were not susceptible to *L. salmonae* from Chinook salmon.

Brown et al. (2010b) then conducted more detailed comparisons of *Loma salmonae* from Pacific salmonids from various geographic locations using the same molecular methods. This analysis indicated that *Loma* from strictly freshwater hosts and enviroments were distinguished from those from anadromous salmonids (i.e., those that include a marine phase in their life cycles). Also, *L. salmonae* from coho salmon from Chile was closely related to *L. salmonae* from anadromous salmon in North America, suggesting that it was a recent import to Chile as coho salmon and other Pacific salmon species are not native to South America.

Microsporidia in zebrafish

The zebrafish, *Danio rerio*, is now an important laboratory model for toxicology, developmental biology, cancer, and infectious disease research (Phillps and Wester 2014). The Zebrafish Model Organism Databae (ZFIN) web site (http://zfin.org) now lists approximately 800 laboratories that employ zebrafish, and a 2014 search of the NIH RePORTER website using the term “zebrafish” revealed a list of 735 grants using this model. Zebrafish actually surpassed *Drosphilia* in PubMed listings in 2011. Hence, with the dramatic increase in the use of fishes as biomedical models, lead by the zebrafish, their pathogens, which include microsporidia, have risen in importance. There are two species of Microsporidia which are known to naturally infect the zebrafish: *Pseudoloma neurophilia* and *Pleistophora hyphessobryconis* (Sanders et al. 2012b; Sanders and Kent 2014). *Pseudoloma neurophilia* was described by Matthews et al. (2001) in the ventral spinal cord, hindbrain, and skeletal muscle of fish housed at the Zebrafish

International Resource Center (ZIRC). Since then, awareness of *Pseudoloma neurophilia* infections in laboratory zebrafish has grown dramatically. Infections of zebrafish by *P. neurophilia* have been reported in more than half of zebrafish facilities examined through the ZIRC diagnostic service (Murray et al. 2011). Clinical infections result in spinal deformaties and emaciation. Most infected fish are subclinical, but there is a concern that such infections are a potential source of non-protocol induced variation in *in vivo* experiments with zebrafish. (Kent et al. 2012).

As the name implies, *P. neurophilia* targets the neural tissue of adult fish, generally centered around the spinal cord and hindbrain. It infects regions that are associated with motor function and probably anxiety (Spagnoli et al. 2015a). Consistent with this, Spagnoli et al. (2015b) showed that subclinically infected fish behave differently than uninfected zebrafish in their response to net capture and habituation to mechanized startle response. Interestingly, the infected fish showed reduced habituation and avoided capture more than control fish.

Until very recently, all zebrafish used in research were derived from lines of fish maintained in the pet fish industry for decades. This prompted the question: Is *P. neurophilia* a parasite of wild zebrafish in India, or did it come from cross tranmission with another aquarium fish as zebrafish in this setting are exposed to numerous fishes from around the world? Using simple, natural transmission studies, we found that *P. neurophilia* can infect many aquarium and laboratory fishes, such as fathead minnow, platys, and medaka. In contrast, the parasite has not been reported in wild caught zebrafish (Smith et al. 2011). Our observations of the lack of the infection in a few wild caught fish support this this finding.

Pleistophora hyphessobryconis

In contrast to the high prevalence of *P. neurophilia* in laboratory zebrafish, *Pleistophora hyphessobryconis* is only rarely observed. Commonly known as “neon tetra disease” for its type host, the neon tetra, *Paracheirodon innesi*, this myocyte-infecting microsporidium is widespread in the aquarium trade. *P. hyphessobryconis* infects a wide range of fishes in several families and has been reported from many species of aquarium fishes including zebrafish (Sanders et al. 2010). Infections of zebrafish by *P. hyphessobryconis* generally produce latent infections that become acute, resulting in clinical signs and often mortality after experimental or incidental immunosuppression (Sanders et al. 2010). Interestingly, Dr. Sumit Homechaudhuri, University of Calcutta, recently provided us with a few wild-caught zebrafish that were held in capivitiy for a three months in India with no other fish. Using histology, we observed microsporidia consistent with *P. hyphessobryconis* in 2 of 5 of these fish (Fig. 2). The general paradigm has been that

this parasite came into the aquarium industry through wild tetras (family Charaidae) from South America. This microsporidium was observed in wild minnows (*Phoxinus phoxinus*) in Hungary, but it was thought to have been transferred to these fish from aquarium fish (Lom and Dyková 1992). Our observation of the putative infection in wild-caught zebrafish from India suggests that the infection may occur in wild zebrafish.

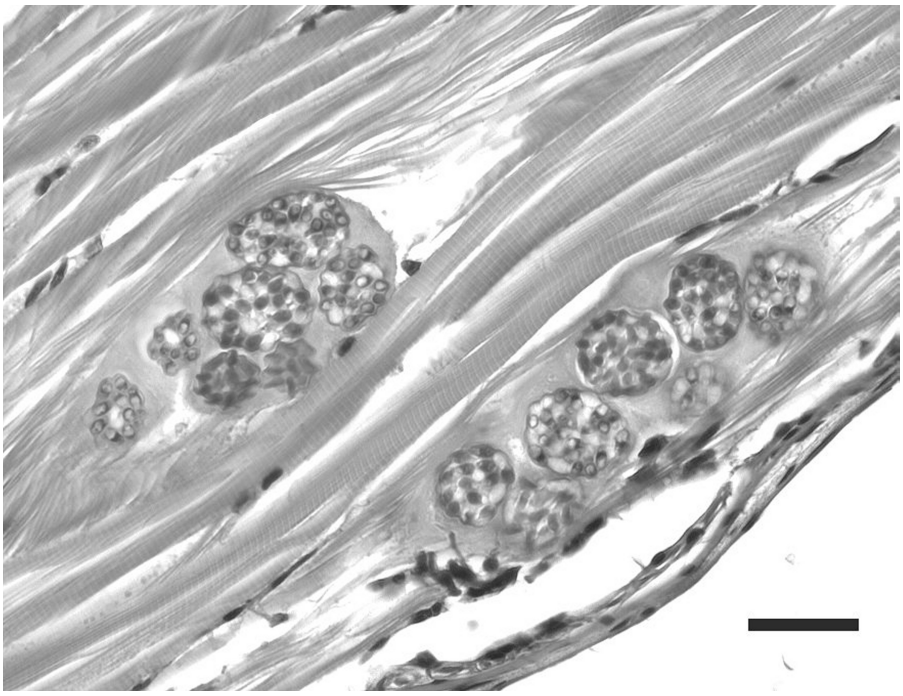

Figure 2. Microsporidia consistent with *Pleistophora hyphessobyrconis* in the ventral muscle of a zebrafish from India. Hematoxylin and eosin. Bar = 25 µm.

Concluding remarks and future directions

Misdiagnosis of parasite infections, including microsporidia, as toxicopathic lesions exemplifies the importance of including proper histopathology in wild fish surveys. The importance of microsporidian infections in fish will continue to increase with the continued expansion of food fish aquaculture and the use of fish in biomedical research.

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Wild and Cultured Fish as Potential Sources of Zoonotic Infections in Humans

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Abstract

Microsporidians are extremely common in aquatic habitats and are regularly found infecting numerous species of fish that are destined for human consumption. Fish protein is an essential part of a healthy diet and increasing the seafood content of our diet is considered to be important for our future well-being and for combating the global rise in non-communicable diseases. Increases in fish production are now required to fulfil this demand, and the shortfall is being met by a significant rise in aquaculture production, as wild capture fisheries are unable to sustain further increases. Such consistent increases in aquaculture production need to be balanced against human, animal and environmental well-being using a 'One Health' approach. It is not known whether some fish microsporidians can cause zoonotic infections in humans, but we are likely to be consuming significant numbers of microsporidian spores from fish in our diet. Some microsporidians infecting humans are morphologically and phylogenetically related to fish microsporidians, so the possibility of zoonoses remains a concern.

Importance of aquaculture and fish in the human diet

Overfishing, population growth, and marine pollution have led to a shift in the human diet, towards terrestrial livestock products and nutrient-poor highly processed foods (Gomez 2014). This, combined with other factors, has led to a dramatic increase in non-communicable diseases (NCDs) (Fig. 1A), known as the epidemiologic transition (Hanson et al. 2011). In affected countries, NCDs are the leading cause of mortality (36 million deaths/yr. globally), overtaking deaths due to infectious diseases, with 80% of NCD deaths occurring in low and

middle income countries (WHO 2011). Marine aquaculture species contain a better range of essential nutrients, such as the omega-3 fatty acids EPA and DHA, which are limited in other animal food products, and it is generally accepted that increasing such nutrients in our diet would help to stem the global pandemic of NCDs (Gomez 2014). Currently nearly half of all seafood consumed by humans comes from aquaculture; global aquaculture production is increasing at a rate of 6% every year (Bostock et al. 2010) and is one of the most rapidly growing global food sectors (FAO 2012). Such intensification of food production can lead to environmental problems and animal health concerns including epizootics. Therefore, disease outbreaks in aquaculture facilities are inevitable, and common fish-infecting parasites like microsporidians will have a far greater chance of entering the human food chain and may become emerging zoonotic diseases. The increases required from aquaculture to meet the rising global demands for fish protein should be achieved by using an integrative approach that follows the 'One Health' concept (Fig. 1B). This should lead to a sustainable expansion in aquaculture production that will minimize risks to public health, animal health and the environment (Gomez 2014).

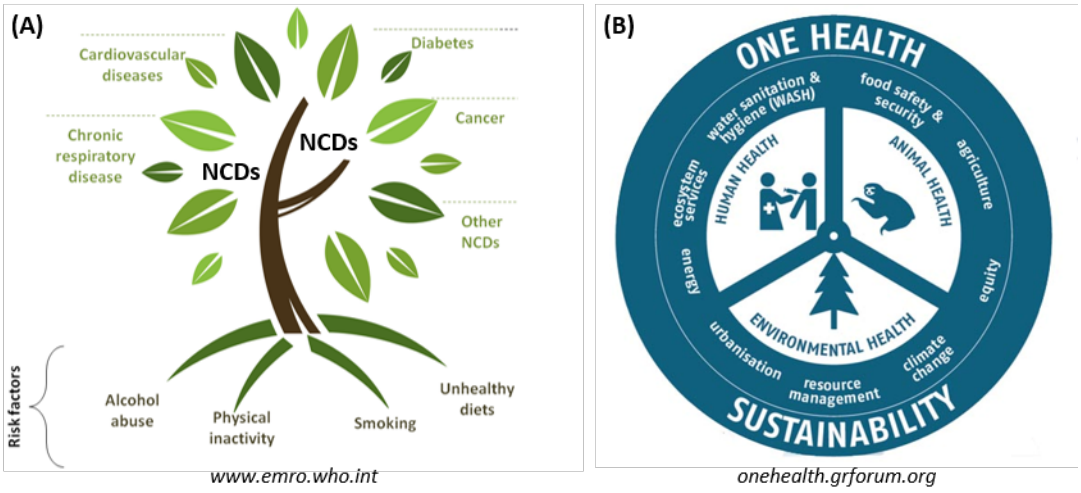

Figure 1. (A) Non-communicable diseases (NCDs), are non-infectious chronic diseases, like diabetes, cardiovascular diseases and various forms of cancer. They are often caused by, or exacerbated by, long-term poor lifestyle choices including having an unhealthy diet. (B) The 'One Health' concept is a global initiative to attain optimal health for people, animals, and the environment. It recognises the equal importance of all three facets, and that the health of each is inextricably connected to the others.

Fish as hosts for microsporidia

Fish are extremely common hosts for microsporidians. In a recent review, 187 genera of microsporidians were known (Vávra and Lukeš 2013), with almost half being from aquatic

organisms, of which 20 genera are described infecting fish (Stentiford et al. 2013). As aquatic organisms are generally not as well-studied as those from terrestrial systems, this suggests that microsporidians are very well-suited to life in aquatic organisms and that there are probably many more genera that have yet to be discovered in aquatic animals, including fish. The suitability of microsporidians to the aquatic environment may simply be a result of the effective means by which infective spores can be transmitted to naïve individuals in an aqueous medium, facilitating both host transmission and dispersal to new locations. If it is true that microsporidians are more common in aquatic systems, then it is possible that this increased exposure to these parasites over time has simply allowed more aquatic hosts to become infected. However, it is also possible that fish are more susceptible to infection by microsporidians than other vertebrate taxa, and this could be a function of their immune systems. It is widely recognised that microsporidians are opportunistic parasites, and that immunocompromised vertebrates are more vulnerable to opportunistic infections. Recent genomic research has revealed that certain fish, such as the Atlantic cod, have extremely unusual immune systems, and have undergone a secondary loss of certain adaptive immune functionality that is extant is all other vertebrates (Star et al. 2011). The loss of part of the adaptive immune system (MHC-II) in cod has been replaced with a unique system, rich in MHC-I, that we can assume functions well in its natural environmental. However, in an artificial environment such as net pen aquaculture, where stocking densities are high and pathogen presence is increased, their immune system does not function efficiently, and cases of chronic microsporidiosis in Atlantic cod, causing significant cumulative mortalities, occur (Kristmundsson et al. 2005; Kahn 2005). Currently it is not known how common such transformed immune systems are in fish, but this could be an explanation for the predominance of microsporidian infections in some teleost groups. Currently there are no effective treatments against microsporidia in fish. As the parasites are transmitted directly between fish, intensive rearing of susceptible fish like Atlantic cod is problematic.

Are fish microsporidians zoonotic or potentially zoonotic?

Currently, we are not able to confirm whether fish microsporidians are causing, or have ever caused, zoonotic infection in humans. However, as microsporidians are well-documented as being very opportunistic parasites, and are able to take advantage of an immunocompromised host that would otherwise not readily become infected, we need to carefully evaluate the eventuality that it is a possibility. Fish are considered to be lower vertebrates and are poikilothermic, meaning that their body temperature can vary significantly and is similar to that of its surroundings. In addition, the structure and function of adaptive immunity in lower

vertebrates, and its coevolution with innate defences, has evolved differently to that in higher vertebrates (Boehm et al. 2012). Therefore, the major, and most significant barriers for fish microsporidians to overcome in order to infect humans is higher body temperatures and a potentially more sophisticated adaptive immune system. Temperature differences between hosts are still regarded as one of the main obstacles that microsporidia must overcome in order to infect new hosts. Immunosuppression in the novel host can facilitate this transition, however, species that maintain similar body temperatures are more likely to successfully share microsporidian parasites.

Fish microsporidians are often found infecting the trunk muscles, which is the part of the fish we eat, therefore we are certainly likely to be consuming some fish microsporidian spores on a regular basis in our diet. Microsporidians from the genus *Pleistophora* are typically found infecting fish muscles and are exclusively found in lower vertebrate hosts. However, cases of myositis in immunodeficient patients have been identified as being caused by *Pleistophora* spp. (Grau et al. 1996), and *Pleistophora ronneafiei* has been described as the first documented case of a true *Pleistophora* infection to occur in a warm-blooded host, albeit an immuno-compromised human (Cali and Takvorian 2003). Temperature in certain parts of the human body is lower than the core temperature, and microsporidian infections are often found in such areas, such as ocular microsporidiosis (Microsporidial keratitis) and myositis (Curry et al. 2007), which may suggest they are zoonotic infections.

Enterocytozoon bieneusi is the most commonly identified microsporidian in humans and has also been reported worldwide in domestic and companion animals where it causes intestinal infection and diarrhoea (Fayer and Santin 2014). The discovery of *E. bieneusi* in numerous animals has raised the question of the importance of animal reservoirs in the epidemiology of this microsporidian and whether infections in humans are zoonotic. High genetic diversity has been found in isolates of *E. bieneusi* with over 200 genotypes identified (54 in humans) based on ribosomal DNA sequences (Fayer and Santin 2014). In phylogenetic analyses, *E. bieneusi* is robustly placed within a clade of microsporidians that is predominantly from aquatic animals, in particular fish and crustaceans (Stentiford et al. 2013), which raises the possibility of a fish reservoir for the parasite.

Inactivation of spores in microsporidian-infected fish products

If the complete removal of microsporidian spores from fish products is not possible, then other steps can be taken to inactivate the spores, and prevent any chance of zoonotic infection.

Microsporidian spores can remain viable for years at a temperature of 4 °C (Fenoy et al. 2009). However, studies of the effect of freezing and heating on the viability of several species of microsporidians, using different methods, have produced contradictory results. Some fish microsporidian spores such as *Loma salmonae* are inactivated by freezing, but others remain viable, such as *Glugea stephani* isolated from flatfish fillets (Amigó et al. 1996). Likewise heat inactivation has varied results depending on the species of microsporidian. *Nosema ceranae*, a parasite of the honey bee, remains viable after heat treatment at 60 °C for 6 h whereas treatment of a related bee-infecting microsporidian, *Nosema apis*, at the same temperature for 15 min completely inactivates the spores (Fenoy et al. 2009). Likewise, spores of the human pathogen *Encephalitozoon cuniculi* heated at 100 °C for 1 min failed to grow in cell culture, but spores of *E. intestinalis* and *E. hellem* had to be heated for 5 and 10 min, respectively for 100% inhibition of growth (Li and Fayer 2006). Heat inactivation of the isolated spores of the fish microsporidian, *Spraguea*, were achieved at 60 °C for 15 min. Studies have also shown that treatment of *Spraguea* spores in a microwave oven at 750W for 30–60 s inactivated the spores (Leiro et al. 2012). However, if spores are treated *in situ* in fish fillets its it debatable whether sufficient temperatures will be achieved, unless the fillet is very well-cooked, which is not always the desired way to prepare fish. Therefore, it would seem difficult to make generalised cooking recommendations for the inactivation of microsporidian spores in fish, which is a product that many cultures prefer to cook lightly or consume raw.

Inactivation of spores, can, of course, prevent the possibility of zoonotic infections occurring, but the remnants of the parasites are still consumed. Allergic responses to parasitic remains in cooked fish have been reported for anisakid nematodes (Faeste 2014), and more recently for other spore-forming parasites that infect the muscles of fish (the myxosporeans), which have caused a wave of food poisonings in Japan and Korean (Kawai et al. 2012). Therefore, there is a concern that the remains of microsporidians in fish muscle could either cause some kind of allergic response in some humans or contain toxic substances that cause a similar reaction to other spore-forming parasites of fish muscle, such as *Kudoa septempunctata* (Iwashita et al. 2013), however, there is currently no evidence to support this possibility.

Concluding remarks and future directions

Due to the rapidly increasing human population on our planet, significant increases in sources of quality protein are going to be required to meet our future food security needs, in both poor and developed countries. One clear way to achieve this is to produce more fish protein for our diets.

This expectation is also extremely well-aligned to our 'One Health' strategy to combat the dramatic increase in non-communicable diseases that we have seen over recent decades in the developed world. As fish are known to be very common hosts to microsporidian parasites in both freshwater and marine environments globally, humans are highly likely, or even inevitably, going to consume more microsporidian spores in our diet in the future that have originated in fish.

Currently, there are no effective medicines to remove microsporidians from fish reared as food in aquaculture facilities, and some human pathogens, such as *Enterocytozoon* spp. potentially have an origin in fish or marine products. Therefore, future research should focus on the discovery of possible aquatic hosts for human microsporidian pathogens and develop effective chemotherapeutants for use in the aquaculture industry. It is also essential to fully investigate unusual cases of fish-like microsporidiosis in humans, such as *Pleistophora ronneafiei*, with molecular sequencing methodologies, to validate a fish origin.

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The *Enterocytoozoon* Clade of Microsporidians: Invertebrate, Fish and Human Pathogens

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The Microsporidia are a diverse phylum infecting host groups from all major taxa in all environments. Although most research has focused on terrestrial hosts (including pest and beneficial insects through to humans), almost half of the known microsporidian genera infect aquatic hosts (Stentiford et al. 2013). An increasing focus on the taxonomic status of the microsporidian parasites found infecting an incredible diversity of aquatic hosts (from protists to vertebrates) and further, their relation to pathogens of terrestrial animals and humans is highlighting some important linkages between those parasites infecting hosts from different ecological settings (Stentiford et al. 2016). Global change and system-level pressures which alter host susceptibility to microsporidian infection and disease is increasing the contact rate between hosts from different environments and provides the means for the zoonotic transfer of parasites (such as microsporidians). This has provided an urgency to the development of our understanding of the role of this parasite phylum in ecological communities and their potential to cause emergent diseases in aquatic animals, and in humans.

The type species of the genus *Enterocytozoon* and family Enterocytozoonidae (*Enterocytozoon bieneusi*) was described infecting the enterocytes of immunosuppressed human patients with developed AIDS (Desportes et al. 1985). It is now considered as the most prevalent human microsporidian (Mathis et al. 2005). Recent work has shown that *E. bieneusi* is a widely distributed pathogen in mammals and even in birds (e.g. Fayer and Santin-Duran, 2014). *E. salmonis*, described by Chilmonczyk et al. (1991) infecting Chinook salmon was later

synonymised to *Nucleospora salmonis*, a parasite described earlier by Hedrick et al. (1991). *N. salmonis* and *E. bieneusi* show 19.8 % genetic divergence in the SSU and LSU, genes, evidence which led to establishment of *Nucleospora* as a separate genus to *Enterocytozoon*. Further members of the genus infecting marine fish have further established this distinction and populated the *Nucleospora* genera (Lom & Dyková 2002; Mullins et al. 1994; Nilsen et al. 1995). Despite the distinction between these genera, at that time *Nucleospora* and *Enterocytozoon* remained more closely related to each other than to any other microsporidian genera described to that point, based on both morphological and molecular characteristics. Specifically, the exhibition of distinctive features of these genera (e.g. formation of polar tube precursors and electron dense discs in the multinucleate sporonts) emerged as a taxonomic feature of the family Enterocytozoonidae. A major distinguishing feature between the two however, was the propensity for *Nucleospora* to inhabit the nucleoplasm of host cells rather than the cytoplasm (Desportes-Livage et al. 1996; Hedrick et al. 1991).

In the following years, other studies revealed further examples of marine members of the Enterocytozoonidae. Phylogenetic studies of microsporidian parasites infecting parasitic copepods (genus *Desmozoon*) and free-living daphnids (undescribed '*Microsporidium* sp.') have shown relatively close affinity between these pathogens and existing members of the family (Freeman et al., 2003; Refardt et al., 2002, respectively), though divergence exceeded 10% in ssrDNA sequence data (Freeman and Somerville, 2009). Parallel investigations showed how the microsporidian *Paranucleospora theridion* (= *Desmozoon lepeophtherii* Freeman et al. 2003) infecting parasitic copepods can cycle between the copepod and the salmonid hosts on which they reside. This finding provided the first definitive evidence that members of the Enterocytozoonidae may cycle between hosts of different trophic levels and specifically between crustaceans and vertebrates (Nylund et al. 2009).

Simultaneously, an intranuclear microsporidian infection was described from the hepatopancreas of European edible crab (*Cancer pagurus*) and hermit crab (*Eupagurus bernhardus*) by Stentiford et al. (2007) and Stentiford & Bateman (2007); leading to description of a new genus *Enterospora* within the family *Enterocytozoonidae* (albeit on solely morphological criteria). A microsporidian was also described infecting the hepatopancreatocytes of tiger shrimp (*Penaeus monodon*) and assigned a new species, *Enterocytozoon hepatopenaeii* by Tourtip et al. (2009). However, the authors acknowledged that erection of a new species rather than a new genus (based upon only 85% similarity in the SSU rRNA gene to the sister species *E. bieneusi*) was likely conservative. The description of these two crustacean

pathogens within the family Enterocytozoonidae provided further evidence of the potential marine domain of this family.

Most recently, a study by Stentiford et al. (2011) demonstrated that the crustacean pathogens *Enterospora canceri* (from European edible crabs) and *Enterocytozoon hepatopenaeii* (from penaeid shrimp) were phylogenetically more similar to the human pathogen *E. bieneusi* than to another crustacean hepatopancreatic pathogen from a new genus, *Hepatospora*. This relatively higher phylogenetic similarity between the shrimp parasite *E. hepatopenaei* Tourtip et al. 2009, and the crab parasite *E. canceri* Stentiford et al. 2007 leading to proposition that the shrimp parasite should be reclassified as a member of the genus *Enterospora* (rather than Enterocytozoon), with *Hepatospora* branching as a sister group to the Enterocytozoonidae within clade IV of Vossbrinck and Debruner-Vossbrinck (2005). Finally, and most recently, the description of *Enterospora nucleophila*, infecting farmed sea bream from the Mediterranean has revealed not only the close phylogenetic relationship between this parasite and *E. canceri* infecting crabs but, further evidence that members of this family may well transmit between vertebrate and invertebrate hosts (Palenzuela et al. 2014). The intriguingly close relationship between marine decapod crustacean parasites and the mammalian pathogen *E. bieneusi* does however raise interesting questions on the ecological and evolutionary relationship between parasites from these distant host taxa and the potential role of invertebrates as a source of zoonotic infections. A phylogeny of the Enterocytozoonidae and related taxa, based upon ssrRNA partial gene sequence is given in Figure1.

The placement of pathogens comprising the family Enterocytozoonidae within Clade VI of the Terresporidia indeed poses some intriguing questions about relationships between members. Host taxa infected by parasites within the clade inhabit marine, freshwater, and aquatic environments and span several trophic levels (including hyperparasitic copepods, decapods, fish, and humans). In addition to similarity in their ssrRNA gene, parasites grouped in Clade VI share distinctive morphological traits (including the potential for intranuclear infection) and generally, infect gut epithelial cells.

In humans, *Enterocytozoon bieneusi* is a common pathogen in immunosupressed patients (such as those with AIDS), a feature also associated with infection and disease of sea bream caused by *E. nucleophila* (Palenzuela et al. 2014). Previously, immune-suppression has been associated with increased severity of microsporidiosis in model fish hosts (e.g. zebrafish infected with *Pseudoloma neurophilia*, Ramsay et al. 2009) while in other scenarios, infection by microsporidian parasites have directly impaired immunity, presumably making their hosts more

susceptible to infection by other pathogens (e.g. *Nucleospora salmonis* infection of salmonids, Wongtavatchai et al., 1995). It appears likely that an association between sub-optimal environmental conditions, relative immune-suppression and host proximity in aquaculture settings can encourage microsporidiosis and will lead to further emergence of yield-limiting diseases in farmed animals.

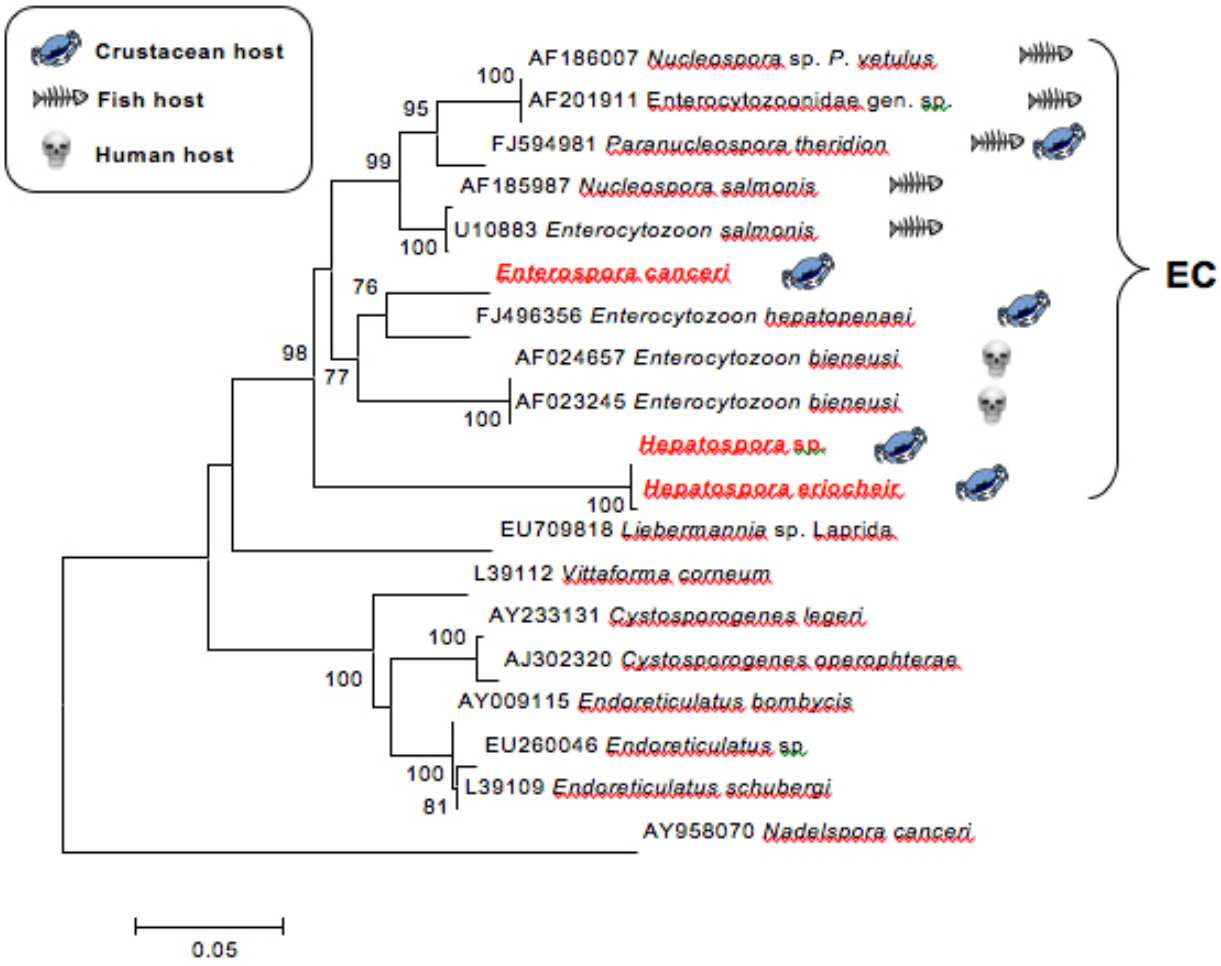

Figure 1. Neighbor-joining tree based on partial SSU-rRNA gene sequences of representatives of the Enterocytozoonidae clade (EC). The clade consists of gut-infecting fish, crustacean and human pathogens united not only by molecular phylogenetics but also by distinct morphology of the sporogonal plasmodium. To date, the human pathogen *E. bieneusi* is the most closely related to a pathogen infecting the hepatopancreatic epithelia of shrimp (*E. hepatopenaeii,* Tourtip et al. 2009) and crab (*E. canceri* Stentiford et al. 2007). Other representatives within the clade (e.g. *P. theridion*) are known to cycle through fish and crustacean hosts. The phylogenetic analysis was performed using MEGA version 2.1. Scale bar represents substitutions per nucleotide site. Adapted form Stentiford and Dunn (2014).

One recent high profile example exists in aquaculture. Penaeid shrimp represent one of the highest value traded seafood commodities (Stentiford, *et al.*, 2012). Historically low prevalence microsporidian infections such as *E. hepatopenaeii* have been associated with 'slow growth' syndromes in *Penaeus monodon* (Tourtip *et al.*, 2009). However, increasingly intensive farming of the congeneric penaeid *Penaeus vannamei* in Asia, which now dominates the global market with first sale values exceeding $10bn per annum, has led to host-switching of *E. hepatopenaeii* to *P. vannamei*, with accompanying high prevalence and high intensity infections being observed in both hosts in association with the recently emergent and devastating syndromic condition Early Mortality Syndrome (EMS) (Tangprasittipap *et al.*, 2013). As described above, phylogenetic analysis place this parasite within the *Enterocytozoon* clade, closest to the human gut pathogen *E. bieneusi* and another intranuclear pathogen, *E. canceri*, infecting the hepatopancreas of European edible crab (Stentiford et al., 2007). The rapid emergence of this microsporidian has prompted high profile warnings to industry from regional bodies such as the Network of Aquaculture Centres in the Asia Pacific (NACA) (http://www.enaca.org) advising that *E. hepatopenaei* should be added to list of pathogens screened for during production of post-larvae for eventual stocking to commercial farms. Once again, the link between microsporidiosis and either sub-optimal environmental conditions experienced within the farm or population immune-suppression associated with inbreeding may have played a role in recent and rapid emergence across major shrimp farming regions (Doyle, 2014).

The close relationship between microsporidian taxa infecting aquatic crustaceans and fish, and the human pathogen *E. bieneusi* raises interesting questions regarding the ecological and evolutionary linkage between these parasite taxa and to the potential role of invertebrates, fish (and water) as a source of zoonotic infections in humans.

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Clues for Multiple-Taxa Lifecycles from Invertebrate Research

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Introduction

Microsporidia are ubiquitous parasites within the Animal Kingdom. They have been recorded from nearly every major animal phylum. However, distribution of microsporidia among host taxa is far from even. Of about 1400 described species (200 genera), nearly 70% parasitize invertebrates, predominantly arthropods of the classes Insecta and Crustacea; 10% parasitize fish. Only about 1% of known species have been found in endothermic ("warm-blooded") vertebrates, birds and mammals, including hominids (Becnel and Andreadis, 2014, Kent et al., 2014, Stentiford and Dunn, 2014, Vávra and Lukeš, 2013). This host range strongly suggests that the Phylum Microsporidia evolved as parasites of invertebrates and, to a lesser extent, fish. However, a few species managed to establish themselves in the cells of birds and mammals in spite of the temperature barrier and advanced immune defenses. In *Homo sapiens*, the best studied representative of warm-blooded animals**,** as many as 14 species belonging to 8 genera have been reported (Table 1)[1]. What is special about these species? How high are the risks of acquisition of new microsporidia parasites by humans given the abundance of microsporidia in invertebrates, many of which may traverse food chains leading to humans and other mammals?

Table 1. Microsporidia discovered in humans, their relatives and host groups[1]

Genus and Species	Tissue tropism	Group of animal host	Closest relatives and their host groups
Encephalitozoon			*Mockfordia xanthocaecilliae* (79-81%)[2], **Insects,** Psocoptera *E.romaleae* (93-96%), **Insects,** Orthoptera; *E. lacertae, E. cuniculi_Pogona* (96-98%[3]), **Reptiles**
E.cuniculi	Brain, IGT, disseminated.	Mammals, birds, reptiles	
E.hellem	Disseminated		
E.intestinalis	IGT, gall-bladder, kidney, eye		

[1] Table 1 lists 16 species, the records on *Nosema ocularum and Microsporidium africanum* does not contain molecular data or electron microscopy, and does not allow proper identification.

Enterocytozoon			*Paranucleospora theridion* (82%), **Crustaceans,** Copepoda**, Fish** *En.hepatopenaei* (84%), **Crustaceans**: Decapoda *Nucleospora salmonis* (80%), **Fish**
E. bieneusi	IGT, gall-bladder, kidney, eye	Mammals	
Anncaliia			*Anncaliia* spp. (97-99%), **Insects:** Diptera, Coleoptera**; Crustaceans:** Amphipoda[4];
A.vesicularum	Surface, eye, skin, muscle, dissemi-nated (*A.connori*)	Insects (*A.a*); Primates (*A.v,, A.c.)*	
A. algerae			
A.connori			
Tubulinosema			*Tubulinosema* spp (99%), **Insects:** Diptera**,** Lepidoptera, Coleoptera, Hymenoptera, Orthoptera
T.acridophagus	Muscle, disseminated	Insects, Primates	
Trachipleistaphora			*T.extenrec* (98%)*,* **Mammals***,* exp. infection in insects; *Vavraia culicis* (97%), *V. oncoperae* (96%), **Insects:** Diptera, Lepidoptera
T. hominis	Eye, sinus, muscle	Unknown; Exp. infection in insects	
T. anthropopthera	Eye, brain, disseminated		
Vittaforma			*Endoreticulatus spp.* (89%), *Cystosporogenus sp.*(98%), **Insects:** Lepidoptera, Coleoptera, Orthoptera
V.corneae	Eye, bladder	Unknown; Exp.infection in mammals	
Endoreticulatus *group*			*Endoreticulatus* spp. (83-91%) **Insects**: Lepidoptera
Microsporidium sp.	Muscle	Unknown	
Pleistophora			*Pleistophora* spp, **Fish**
*P.ronneafiei**	Muscle	Unknown	
Pleistophora sp.*		Unknown	
Nosema		Identification under question No EM or molecular data available	
*N.ocularum**	Eye		
Microsporidium			
*M.africanus**	Eye		
*M.ceilonenesis**			

[1]Sources of information: Cali and Takvorian, 2003, Cali et al., 1998, Cali et al., 2005, Cheney et al., 2000, Docker et al., 1997, Franzen et al., 2006a, Franzen et al., 2006b, Koudela et al., 1998, Lange et al., 2009, Nylund et al., 2010, Pilarska et al., 2015, Plischuk et al., 2015, Richter et al., 2013, Sokolova et al., 2007, Sokolova et al., 2010, Suankratay et al., 2012, Tourtip et al., 2009, Vávra et al., 2006, Vávra et al., 2011, Weiss, 2014. [2]Percent of identity ("relatedness") inferred from SSUrDNA-based pairwise distance analysis (in brackets); [3]YS, unpublished data; [4]Tokarev et al., unpublished data;
*No molecular data available

Origin of Microsporidia

The evolutionary origin of microsporidia has been significantly elucidated during the last 2-3 years. The consensus tree, based on phylogenies inferred from several genes with high statistical support places Microsporidia within the Aphelidea-Rozellamycota-Microsporidia (ARM) clade, a basal Fungi or sister-to-Fungi lineage (Karpov et al. 2013, Letcher et al. 2013). Aphelids are parasites of algae, and the Rozellamycota lineage comprises species parasitizing fresh-water chitrids and amoebas, as well as numerous "cryptic" species known only by their sequences. Within the ARM Clade, microsporidia cluster with rozellids. Association with rozellids has been proven recently by genomic and proteomic studies on *Pararmicrosporidium* spp. and *Mitosporidium daphnia*, the "missing links" between Rozelids and microsporidia

(Corsaro et al. 2014; Haag et al. 2014). *Paramicrosporidium* spp., intranuclear parasites of free-living amoebae, bear striking morphological similarity with hyperparasitic metchnikovellids (subphylum Rudimicrosporidia), presumably a basal lineage of Microsporidia[2] (Corsaro et al. 2014; Sokolova et al. 2013; Sokolova et al. 2014). Hence, the common ancestor of *Paramicrosporidium* and Microsporidia may have been an intranuclear parasite of a protist. This inference is supported by (i) obligatory intranuclear development in three genera (*Nucleospora, Desmozoon (Paranucleospora) and Enterospora*)[3], (ii) occasional development of some species within host nucleoplasm (YS, unpublished observations); and (iii) existence of unusual metabolic relationships of cytoplasmic microsporidia with the host nucleus, i.e. targeting microsporidian hexokinase to the host nucleus during intracellular development (Senderskiy et al. 2014). *Paramirosporidium*-like hyperparasites of Archigregarina[4] infecting gut lumens of the common ancestor of annelids and arthropods, probably gave rise to some lineages of contemporary microsporidia (metchnikovellids and *Chytridiopsis*-like "primitive" (Larsson 2014) microsporidia). Insects and annelids are the major host groups for both Gregarina (Perkins et al. 2000) and Microsporidia (Becnel and Andreadis 2014), and a hypothesis that cannot be excluded posits that gregarines might have functioned as a "Trojan Horse," enabling dispersal of microsporidia from marine and brackish water annelids to terrestrial arthropods and insects (Sokolova et al., 2013).

Distribution among invertebrates and fish

Estimates based on the analysis of distribution of microsporidia among hosts suggest that ancestors of Microsporidia switched to parasitism in oligochaetes and polychaetes during their colonization of land, migrating from marine through brackish waters of river estuaries to fresh water basins during the Cambrian and Silurian Periods. The radiation and flowering of Microsporidia likely took place during the Carboniferous and Triassic and was associated with diversification of Arthropods (Issi 1986). Currently, 70% of microsporidia species parasitize aquatic hosts, mostly crustaceans and insects connected with aquatic habitats (Stentiford and Dunn 2014). The distribution of microsporidia among groups of terrestrial and freshwater

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[2] So far no rDNA sequences for metchnikovellids are available through public database, opening three possible positions of Metchnikovellids: as a basal taxon of Microsporidia, as a close sister group to Mictrosporidia, and as a sister to *Paramicrosporidium.*

[3] All intranuclear microsporidia are parasites of enterocytes, and ability to develop in the nucleus could be considered a rudimental trait, a "pre-adaptation" employed by nucleus-dwelling microsporidia to avoid degradation by the lysosome system of enterocytes.

[4] Archigregarines that parasitize annelids and occasionally harbor metchnikovellids, is the earliest diverging lineages within Apicomplexa, a "polyphyletic stem" from which all other gregarines evolved (Leander, B.S. (2008) Marine gregarines: evolutionary prelude to the apicomplexan radiation? *Trends in Parasitology* 24, 60-67).

arthropods included numerous host switches via polyxenous life cycles, common parasites, and food chains. The result is the contemporary abundance of species, with evolutionary bonds that have been increasingly elucidated by SSUr-DNA-inferred phylogenies (Vossbrinck and Debrunner-Vossbrinck 2005; Vossbrinck et al. 2014), though the whole puzzle is far from assembled.

Examples of adaptation of microsporidia to parasitism in invertebrates are numerous and exquisite, from bizarre ectospore appendages, multiple spore morphotypes, and polyxenous life cycles, to effects on host behavior, population dynamics and sex ratio (Becnel and Andreadis 2014; Stentiford and Dunn 2014; Vávra and Larsson 2014). Microsporidia demonstrate an arsenal of adaptations to evade the innate immunity of invertebrate hosts including modification of the phenol-oxidase cascade, accumulation in specialized haemocytes and adipocytes, stimulating host cells to grow into gigantic cells with prolonged cell cycles (e.g., “cysts” in insects, and “xenomas” in fish), that form protected and nutrient-supplied niches for developing parasites. Biochemical and molecular studies have revealed that microsporidia are able to modulate host cell cycles by inhibition of apoptosis, and influence host gene expression and metabolism by secreting diverse regulatory factors into the host cell (Senderskiy et al. 2014; Williams et al. 2014).

Tight ecological bonds within the aquatic habitats via numerous intersecting and overlapping food chains could have played a leading role in microsporidian host switches from invertebrates to fish (and in reciprocal host transfers), as suggested by phylogenetic analyses (Stentiford et al. 2013). Circumstantial evidence indicates that the most common parasite of the White Atlantic shrimp, *Agmasoma penaee,* cycles between shrimp and perciform fish feeding on juvenile penaeids (Johnson 1995; Overstreet 1973; Pasharawipas and Flegel 1994; Sokolova et al. 2015). The microsporidian parasites could have been spread among aquatic inhabitants also by parasites similar to sea fleas, *Lepeophterius* sp. (Copepoda), which are common fish ectoparasites related to free-living cyclopids. These crustaceans, like freshwater copepods, can be parasitized by several microsporidian species, at least two of which display close evolutionary distances with a fish microsporidium, *Nucleospora salmoni* (Freeman and Sommerville 2009; Jones et al. 2012). This suggests the presence of polyxenous fish-crustaceans life cycles now or in the past. Existence of such a cycle has been recently demonstrated for *Desmozoon* (*Paranucleospora) theredion*, a species that parasitizes simultaneously an Atlantic salmon and its copepod parasite (Nylund et al. 2010). The much broader distribution of microsporidia among fish versus birds and mammals can be explained by

the fact that switching to parasitism in fish did not demand special adaptations to the elevated body temperatures, a major factor together with humoral immunity that has limited the spread of microsporidia among warm-blooded animals.

"Human microsporidia" and related species

The importance of the "clues from invertebrate research" for understanding the origin and accessing the threat of microsporidia to the human population are evident. Excluding two *Pleistophora* species, likely related to fish congeners, 12 species and 6 genera of microsporidia recorded as infectious to humans are either insect parasites themselves, like *Tubulinosema acridophagus* and *Anncaliia algerae*, or have close relatives among insect parasites (Table). The only exception, *Enterocytozoon bieneusi*, is also likely derived from a microsporidium infecting an arthropod given the broad distribution of enterocytozoonids among marine crustaceans (Stentiford et al., 2013).

Mammals are the very recent hosts for microsporidia parasites from the evolutionary perspective. Microsporidia were adapted to intracellular parasitism in invertebrates well before switching to mammals, and a few lineages were apparently more successful in expanding their host range to vertebrates than others. Hence, addressing phylogenetic bonds between the species infecting humans and those parasitizing invertebrates might help to answer questions posed in the first paragraph of this short review.

***Cystosporogenes/Endoreticulatus/Vittaforma* clade.** *Vittaforma corneae* was once isolated from corneal stroma of immunocompetent HIV-negative patient, and was the first human microsporidium placed in culture. However, some authors maintain that this species cannot be considered a true human pathogen (Van Frankenhuyzen et al., 2004). It shares 98.2% identity of its rDNA sequence with the lepidopteran microsporidium *Cystosporogenes legeri* and most likely is an unknown isolate of a closely related *Cystosporogenes* species accidentally developing in the immune-privileged site. Infection with *V.corneae* in immunocompetent patients is associated with self-limited short-term conjunctivitis caused presumably by traumatic inoculation of environmental spores of the insect pathogen (Weiss, 2014). Recently another species clustering within the same *Endoreticulatus-Cystosporogenes-Vittaforma* clade was found to cause myositis in the immunocompetent patient (Suankratay et al., 2012). Comparatively low identity (83-91%) of this *Microsporidium* sp. precluded the authors from assigning it to *Endoreticulatus*. The *Endoreticulatus-Cystosporogenes* clade is composed predominantly of parasites of Lepidoptera. *Endoreticulatus* spp. though also has been isolated from two other orders of insects, Coleoptera and Orthoptera (Pilarska et al., 2015).

Cystosporogenus legeri, a common parasite of insect rearing facilities, infects as a many as 5 families of Lepidoptera, demonstrating an unusually broad host range (Van Frankenhuyzen et al., 2004). So, among representatives of this clade there are generalist parasites with broad host ranges among natural hosts. Another feature that might facilitate the transition to a new group of hosts is resistance of *Cystosporogenes* spp. spores to high (up to 42^{o}C) temperatures (Van Frankenhuyzen et al., 2004). Butterflies and moths, some of which are known as facultative blood and tear feeders (Plotkin and Goddard, 2013, Zaspel et al., 2014), could be vectors for transmission of microsporidia belonging to this clade.

***Anncaliia/Tubulinosema* clade** is composed of two genera with extraordinarily broad host ranges for insect microsporidia. The host range of *Anncaliia* spp. includes representatives of at least two insect orders, Coleoptera, and Diptera (Franzen et al., 2006b), and also amphipod crustaceans (Y.Tokarev and YS, unpublished data). *Tubulinasema* spp. parasitize as many as 5 insect orders (Lepidoptera, Orthopteran, Coleoptera, Diptera and Hymenoptera) (Franzen et al., 2006a). *Tubulinosema acridophagus* from a grasshopper was found to cause myositis and disseminated infection in a patient with a bone marrow transplant (Weiss, 2014). This is a typical opportunistic infection, but as in the case involving the *Endoreticulatus*-related *Microsporidium* sp., it clearly demonstrates insignificance of temperature as a limiting factor for the parasite development. *Anncallia* spp. probably diversified further as parasites of mammals. *Anncallia (Brachiola) algerae*, a common mosquito parasite of several genera of mosquitoes (Andreadis, 2007), occasionally infects brain and eye tissues, and causes disseminated disease in immunocompromised individuals. It also may induce skin and muscle infections presumably transmitted by a mosquito vector in immunologically healthy humans (Weiss, 2014). *Anncaliia algerae* is known to develop infections in SCID mice (Koudela et al., 2001), and tolerate elevated temperatures; it can be cultivated in cell lines at >36^{o} C (Trammer et al., 1999). Resistance of *A.algerae* to high temperatures could be an ecological adaptation, since this parasite in nature infects mosquito larvae inhabiting small pools heated during the summer period. The two other representatives of the genus, *A.connori* and *A.vesicularum*, have been recorded from humans with immunodeficiency, and their environmental source is unknown (Weiss, 2014).

***Trachipleistophora/Vavraia* clade.** Reperesentatives of this clade*, Trachipleistaphora hominis* and *T. anthropophtera*, the most widespread causative agents of human myositis due to microsporidia, have been recorded from several immunodefficient individuals (Suankratay et al., 2012). Insect origin of these infections was suggested by successful experimental infection of

insect larvae with human-isolated *T. hominis* (Weidner et al., 1999). One representative of this genus was described from a Madagascar insectivore, *Hemicentatis semispinosus* (Vávra et al., 2006). Interestingly, this mammal belongs to the peculiar family Tenrecidae (order Afrosoricida), which members are characterized by lower body temperatures. The spores isolated from the animal were also infectious to *Spodoptora littoralis* (Lepidoptera) larvae. It is unclear whether *T. extenrec* is a native parasite of tenrecs, or an insect pathogen. It may develop in both types of hosts, suggesting a potential transmission route from insects to insectivorous mammals. *Trachipleistaphora hominis* is a close relative of the mosquito microsporidium *Vavraia culicis*, sharing with the latter 98% of SSUrDNA sequence similarity. *Vavraia culicis* parasitizes mosquitoes belonging to 6 genera (Andreadis, 2007). Microsporidia from mosquitoes, as a rule, are species- or genus-specific "specialists." Among >30 mosquito-infecting microsporidia a similarly broad range of hosts is known only for the above mentioned human-pathogenic species, *Anncaliia algerae* (Andreadis, 2007). One additional *Vavraia* (*V.oncoperae)* was described from a lepidopteran host (Malone and McIvor, 1995).

Interestingly, all three lineages of insect microsporidia that contain forms known to infect humans include taxa of generalist pathogens as well as species tolerating high temperatures. Such consistency may suggest specific biochemical pre-adaptations required for transmission to a foreign warm-blooded host for these groups. Genes and regulatory factors responsible for these adaptations are yet to be identified by genomic and proteomic analyses. Factors analogous to LRR proteins or products of the *InterB* multigene family (Williams et al., 2014) might potentially play a role in regulating limits of host specificity within certain lineages.

Enterocytozoon bieneusi and ***Encephalitozoon*** spp.

Enterocytozoon bieneusi, a specialized parasite of enterocytes, is the most common microsporidium known to cause diseases in humans, particularly in patients with AIDS. *E. bieneusi* is widely distributed among several orders of mammals, and also has been recorded in birds (Fayer and Santin-Duran, 2014). The evolutionary history of *E. bienusi* parasitism in vertebrates is probably relatively short, since the taxon has not diversified into separate species, but is represented by numerous genotypes with different levels of host specificity (Fayer and Santin-Duran, 2014). The closest relatives of this microsporidium infect fish and crustaceans (Stentiford et al., 2013), so *E.bieneusi* ancestors probably transferred to parasitism in vertebrates from these hosts via food chains. The previous presentation by Grant Stentiford covered the aspects of phylogeny and biology of enterocytozoonids.

Four of five species of the genus *Encephalitozoon*, *E.cuniculi, E. intestinalis, E. hellem,* and *E. lacertae,* parasitize vertebrates, mammals, birds and reptiles, and one species, *E.romaleae,* has been found in an insect, the lubber grasshopper *Romalea microptera* (Lange et al., 2009). *Encephalitozoon cuniculi* is the best known and most ubiquitous microsporidium of mammals. It has diverged into at least three mammalian host-specific genotypes (Didier et al., 1995). In reptiles, *E.cuniculi*, or morphologically identical species, cause multisystemic granulomatous disease (Koudela et al., 1998, Richter et al., 2013). Infection discovered recently in a bearded dragon *Pogona vitticeps* (Richter et al., 2013; YS, unpublished data), were caused by a yet unknown genotype that may represent a new species.

E. hellem is a natural pathogen of birds, and *E. intestinalis* is more restricted to humans (Snowden, 2014). *Encephalitozoon* spp, unlike *E. bieneusi*, are not confined to infecting gastrointestinal tracts, but often cause disseminated microsporidiosis (Weiss, 2014). In phylogenetic reconstructions, the *Encephalitozoon* lineage clusters within the Clade 4 of "Terresporidia" (Vossbrinck and Debrunner-Vossbrinck, 2005) composed of predominantly insect microsporidia.

The *Encephalitozoon* branch forms a dichotomy with *Mockfordia xanthocaeciliae,* a parasite of *Xanthocaecilia sommermanae,* Order Psocoptera. Psocoptera is considered to be the most basal order of hemipteroids, originating during the Permian Period 295-248 million years ago. Psocoptera are closely related to Phthiraptera, sucking lice, which parasitize warm-blooded animals including humans. These two orders are placed in the infraorder Psocodea and share a common ancestor, based on robust morphological and molecular evidence (Johnson and Mockford, 2003); for other references, see Sokolova et al., 2010). Though the majority of barklice are free-living species, various species of Psocoptera inhabit plumage of birds and the pelage of mammals, as well as their nests. This short-term commensal-type relationship presumably gave rise to obligate parasitism characteristic to Phthiraptera (Johnson et al., 2004). Evidence of a close relationship of *M. xanthocaecilliae* to *Encephalitozoon* spp. (Sokolova et al., 2010), ubiquitous parasites of birds and mammals, supports the idea that the association of ancestral Psocodea with mammals and birds could be one of the avenues of transfer of Microsporidia from arthropods to warm-blooded hosts.

Within the *Encephalitozoon* clade the position of *E. romaleae* (Lange et al., 2009), which shares 96% of SSUrDNA similarity with *E. hellem*, certainly creates a problem. As an explanation of striking genetic relatedness of *E. romaleae* to *E. hellem*, perhaps this species evolved as a result of reciprocal transfer of the *E. hellem*-related bird-infecting microsporidium back to insects

(Sokolova et al., 2010). The genomic survey revealed that the genomes of *E. hellem* and *E. romaleae* contained the gene for purine nucleotide phosphatase (PNP), a component of the purines salvage pathway of insect origin (Pombert et al., 2012, Selman et al., 2011). This gene is absent in the genomes of *E. intestinalis*, *E. cuniculi* and other microsporidia with sequenced genomes, and likely was acquired from an insect host by a common ancestor of *E. romalea* and *E. hellem*. The narrow distribution of this gene is most consistent with its recent gain (Selman et al., 2011) and conforms to the idea of reciprocal transfer that might have occurred relatively recently. Of note, the *Encepahlitozoon* spp.-derived PNP genes cluster with the orthologue from *Pediculus humanus* (Phthiraptera) (Fig.1, Salmon et al., 2009). This suggests that a lice-related ectoparasite of birds harboring an ancestral encephalitozoonid could have been a source for the PNP gene transfer from insects to the *E.hellem-E.romalea* lineage. Further molecular studies based on broader sampling and robust analyses could test this hypothesis.

Concluding remarks and further directions

In fact, only four species belonging to two genera can be considered true mammalian parasites: *Enterocytozoon bieneusi*, *Encephalitozoon cuniculi*, *Enc. intestinalis*, and *Enc. hellem.* They have evolved as parasites of warm-blooded vertebrates and might represent a serious threat to human populations as zoonotic infections (Fayer and Santin-Duran, 2014). Records of other microsporidia in mammals, including humans, are more or less accidental. However, it is hard to argue the opinion expressed by my major professor Dr. Irma Issi, that “now microsporidia represent a numerous and aggressive group of parasites expanding the range of their hosts” (Issi, 1986). Recorded cases of microsporidiosis demonstrate the consecutive stages of microspordida transforming into parasites of humans: from transient arthropod-related microsporidia known by sequences in stools of AIDS patients (Genebank accessions CQ408913, CQ408914, (Sokolova et al., 2011), through accidental surface infections in immunocompromised patients (*Endoreticulatus*-like *Microsporidium* sp., *Tubulinosema*) and development in immune privileged tissues of eyes (*Vittaforma*), skin, and muscles due to accidental exposure to spores of a “generalist” microsporidium (*Trachipleistaphora, Anncaliia)*, to specialized infections of gut epithelium (*Enterocytozoon)*, and systemic microsporidiosis disseminated by macrophages *(Encephalitozoon*). Potential sources of human infection with invertebrate microsporidia are likely associated with “generalist” parasites, and, particularly, with human (mammalian) hyperparasites. Further surveys of Microsporidia in Psocoptera, Phthiraptera and related orders, as well as in other

ectoparasitic or bloodsucking insects (fleas, bed-bugs, dipterans and hematophagous lepidopterans) and acarines (ticks, mites and chiggers) will shed light on evolutionary routes of host transfers as well as on possible risks of infection.

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Is Microsporidian Infection/Disease Becoming More Common in Bumble Bees?

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Abstract

Microsporidia, specifically *Nosema*, have been suggested to be emerging infectious diseases (EIDs) in bumble bees. Two potential drivers – commercial bumble bees and managed honey bees – have been identified as possible sources of pathogen spillover. In addition, declines in bumble bee populations may lead to lower genetic diversity and subsequent higher susceptibility to infection. However, in the absence of long-term data on infection or disease prevalence in wild or managed populations, and a lack of definitive data on spillover, it is impossible to say whether microsporidian infection or disease is becoming more common. In addition, much of the published literature on microsporidians in bumble bees is hard to interpret, due to methodological issues. Future studies should combine microscopy and molecular analyses at the tissue-level, in order to produce definitive data on infections. Studies of museum collections using next-generation approaches, or identification of population genetic signals in current infections, may enable an assessment of temporal trends in the presence/absence of microsporidia in bumble bees.

Introduction

Microsporidia were first recorded in bumble bees in 1913 (Fantham and Porter 1913, 1914). Nearly a century later, they are at the heart of a controversy about the role of emerging infectious diseases (EIDs) in driving bumble bee declines (Brown 2011). Bumble bees are

important pollinators for a range of crops, and so understanding why they are declining is an important question for agricultural sustainability. Determining whether microsporidian infections and disease have become more common in bumble bee populations, as the emerging infectious disease hypothesis requires, is complicated by underlying uncertainties about the identity, diversity, and impact of these pathogens. Here, I assess what can be definitively stated about microsporidia in bumble bees, and identify issues that are holding back our understanding of this host-pathogen interaction.

The diversity of Microsporidia in bumble bees

An array of microsporidia, including *Nosema apis*, *N. bombi*, *N. ceranae*, *N. thomsoni*, and *Tubulinosema pampeana*, have been shown or suggested to infect bumble bees (Fantham and Porter 1913, 1914; Plischuk et al. 2009; Li et al. 2012; Plischuk et al. 2015). Consequently, assessing prevalence, and possible changes in prevalence, requires accurate identification of the infectious agent. Prior to the development of molecular tools (Fries et al. 2001), nearly all microsporidian infections in bumble bees were identified as *N. bombi* (Table 1). Best practice at the time meant that identifications should have been based on the presence of spores (and other life-stages) in the Malpighian tubules, the main site of infection identified in the original description of the species (Fantham and Porter 1914). However, many studies fail to detail their screening method for *Nosema* in sufficient detail, and combined with the controversy about whether *N. apis* can infect bumble bees (Fantham and Porter 1913; Uspenskii 1949, as cited in Showers et al. 1967; Showers et al. 1967; van den Eijnde and Vette 1993), and the recent discovery of *N. ceranae* and *T. pampeana* infections (Plischuk et al. 2009; Li et al. 2012; Plischuk et al. 2015), this makes the species identification in many earlier studies uncertain (Table 1, Table 2). Since the development of molecular tools it has been possible to combine dissection and microscopy with molecular screening, to produce definitive accounts of prevalence (e.g., Cameron et al. 2011; Cordes et al. 2012). However, at the same time the use of molecular screening on its own has resulted in studies that measure the presence and absence of pathogen DNA, without determining if this represents a true infection (Szentgyörgyieta et al. 2011; Li et al. 2012; Fürst et al. 2014; Schmid-Hempel et al. 2014; Table 1, Table 2). As false positives can be generated by pathogen spores that are being vectored, or that have been ingested, or by mis-priming during PCR reactions, interpretation of prevalence based on molecular screening alone is problematic.

In terms of assessing prevalence in the field, *N. bombi* (as originally described by Fantham and Porter 1914, redescribed by McIvor and Malone 1995, and molecularly described by Fries et al. 2001), *N. ceranae* (Fries et al. 1996; Plischuk et al. 2009), and *T. pampeana* (Plischuk et al. 2015) are the only microsporidia that have been definitively shown to infect wild bumble bees (Figure 1). Whether *N. bombi* as known today is the same as the original microsporidian described under this name by Fantham and Porter (1914), and reported in subsequent microscopy studies, is unlikely to be resolved, although descriptions of tissue specificity make this likely. For the rest of this paper, I will assume that the species identification given by authors for microsporidian infections is accurate, whilst bearing in mind the caveats detailed above.

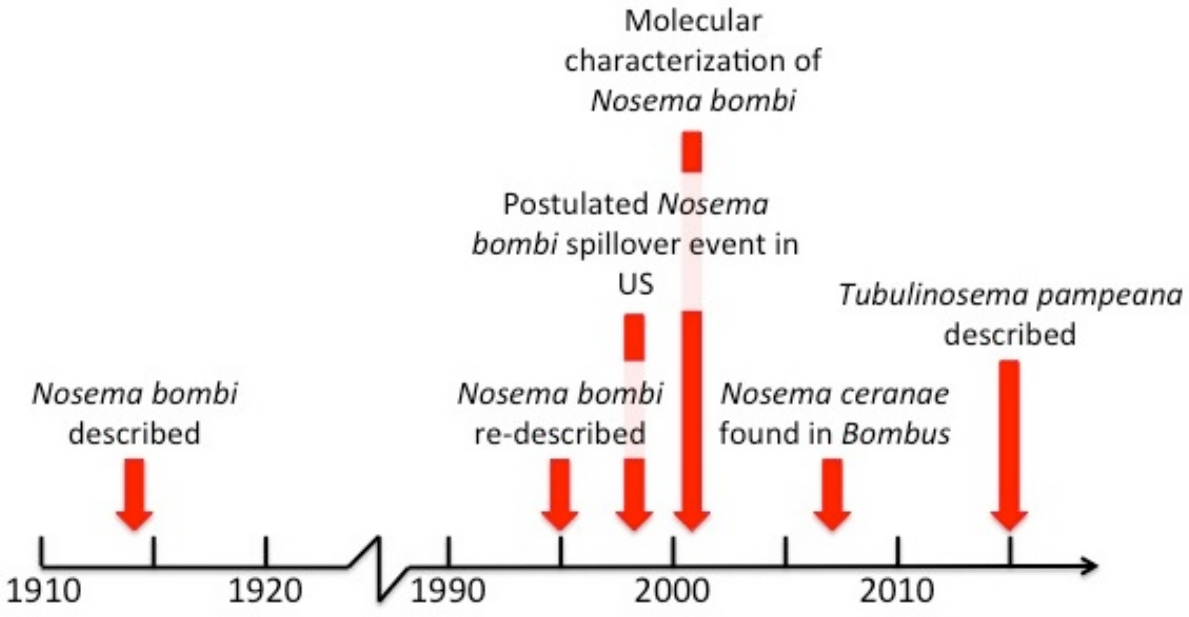

Figure 1. Important events in our understanding of microsporidia in bumble bees.

Impact of microsporidians in bumble bees

One reason that *N. bombi* and *N. ceranae* cause concern as potential EIDs is their apparently high virulence. Obviously, an EID with low impact is unlikely to be a driver of host population declines. Whittington and Winston (2003) report the suggestion by bumble bee suppliers that *N. bombi* may have been behind the collapse of commercial *B. occidentalis* breeding in the late 1990s. Recent experimental studies have indeed demonstrated significant negative impacts on individual health and colony-level reproductive fitness by *N. bombi* (Otti and Schmid-Hempel 2007, 2008; Van der Steen 2008; Rutrecht and Brown 2009), and on individuals by *N. ceranae*

(Graystock et al. 2013; but see Fürst et al. 2014). All of these studies have been conducted on either *B. lucorum* or *B. terrestris*, two common Palearctic species, with the latter being one of the main species produced commercially for pollination services (Velthius and van Doorn 2006). Whether impacts vary across other bumble bee species remains to be determined. The impact of *T. pampeana* has yet to be investigated.

Patterns of prevalence of microsporidians in bumble bees

Bumble bees, like other eusocial insects, have three castes – males, queens and workers – and studies have suggested caste-specific prevalence in *N. bombi* (reviewed by MacFarlane et al. 1995). Queens are available for sampling for a relatively short period after hibernation, and thus spot samples are likely to produce a relatively good measure of prevalence. In contrast, workers and males are produced over a period of months. The seasonal progression of the annual *Nosema* epidemic, both within (Rutrecht and Brown 2008a) and among (Imhoof and Schmid-Hempel 1999) colonies, poses a challenge to generating a meaningful assessment of prevalence in workers or males and making comparisons across species or years. Nevertheless, most studies have focused on workers as they are more abundant, and collecting them puts less pressure on declining bumble bee populations (Figure 2).

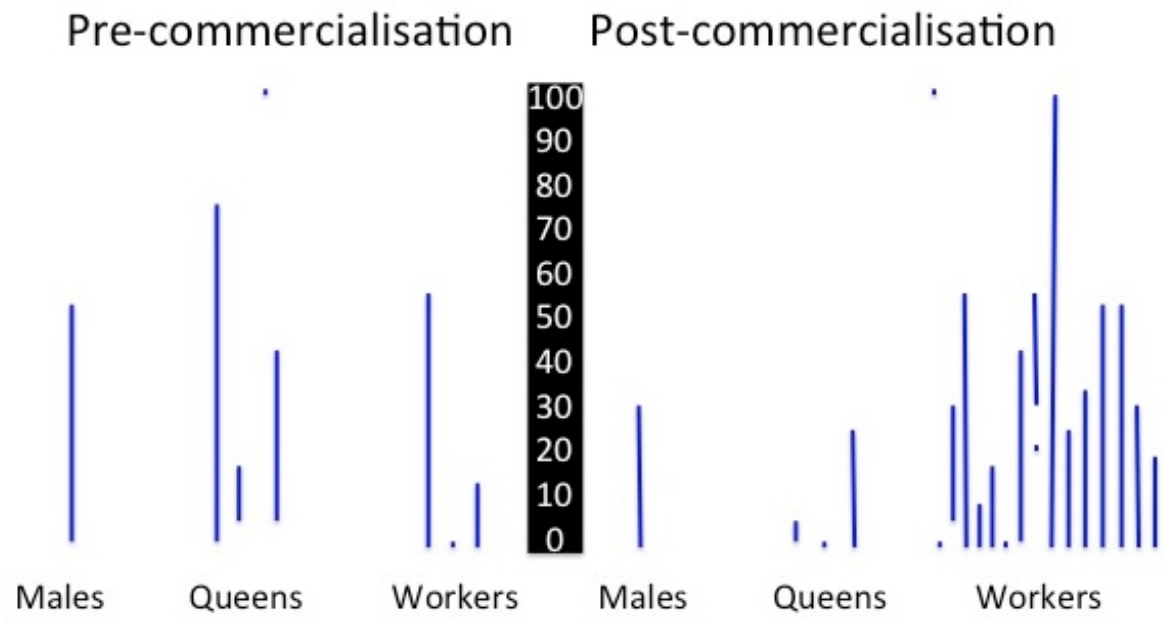

Figure 2. Reports of microsporidian prevalence pre- and post-commercial use of bumble bees. Each bar shows the prevalence range found in a specific study for a specific caste of bumble bee.

The concept of microsporidians as EIDs lies behind the question of whether microsporidian infection and disease is increasing in bumble bees. However, as yet, there is no definitive evidence that either *N. bombi* or *N. ceranae* are emergent in bumble bees. While high prevalence of *N. bombi* is associated with declining bumble bee species in North America (Cameron et al. 2011), the absence of historical data and the presence of species-specific prevalence by this parasite in other geographical areas (e.g., Shykoff and Schmid-Hempel 1991) makes interpreting this pattern difficult (Brown 2011). Similarly, the basis for interpreting *N. ceranae* as an EID relies on an association of its presence in UK bumble bees with its presence in European honey bees (Fürst et al. 2014), and the idea that *N. ceranae* is an EID in the European honey bee, *Apis mellifera*, with a host-switch occurring in the mid-1990s (reviewed in Fries 2010). However, recent data suggest that *N. ceranae* was present in *A. mellifera* in the US in 1975 (Traver and Fell 2015) and in Brazil in 1979 (Teixeira et al. 2013). Furthermore, phylogenetic studies show that *N. ceranae* is more closely related to *N. bombi* than to *N. apis* (Shafer et al. 2009), suggesting the possibility that it has actually switched species from *Bombus* to *Apis*, rather than the other way round.

Irrespective of these complications, the obvious way to address whether microsporidian infection and disease is increasing in bumble bees is to look across time at prevalence measures taken in the same geographical area. Unfortunately, no such dataset exists. In the absence of such data, a crude approach might be to to look at data collected pre- and post-commercialisation. *N. bombi*, as identified by microscopy, was present in Europe (Denmark, Switzerland, UK), New Zealand, and North America, with prevalences varying from 0-100% in spring queens, 0-55% in workers, and 0-50% in males, prior to commercialisation (reviewed by MacFarlane et al. 1995)(Figure 2). Studies post-commercialisation in Europe (Jones and Brown 2014) and North America (Cameron et al. 2011; Kissinger et al. 2011; Koch and Strange 2011; Cordes et al. 2012; Blaker et al. 2014; Tripodi et al. 2014) show similar prevalence ranges in queens and workers (Figure 2). Given that *N. bombi* is only suggested to be an EID in North America (Thorp and Shepherd 2005), a fairer comparison might be between studies in North America pre- and post-commercial production of bumble bees. Unfortunately, only 2 studies of *N. bombi* from North America prior to commercialisation exist (Fantham et al. 1941; Liu 1973). Interestingly, both report low levels of infection (<5% in Liu 1973).

Can space be a substitute for time? If microsporidians are EIDs, then they should exibit higher prevalence in areas nearer to the proposed source population (commercial bumble bees for *N.*

bombi, managed honey bees for *N. ceranae*). Colla et al. (2006) found *N. bombi* in 14% of bumble bees next to a Canadian greenhouse using commercial *B. impatiens*, as opposed to <4% of bees at non-greenhouse sites. However, a 2nd greenhouse site had no infected bees, making this result hard to interpret. In a larger-scale study, Murray et al. (2013) showed a gradual decline of *N. bombi* prevalence in male *B. terrestris* as distance from Irish strawberry farms using commercial bumble bees increased (prevalence in workers showed no trend in either direction). This could be interpreted as increased transmission, and thus prevalence of the microsporidian near commercial operations (that is, pathogen spillover), or alternatively as commercial males exhibiting philopatry (although current evidence of male dispersal argues against this; Kraus et al. 2009). Whitehorn et al. (2013) found generally low prevalence of *N. bombi* around Scottish fruit farms, irrespective of whether they were using commercial bumble bees or not, a result reflected in a study of fruit farms in England (Graystock et al. 2014). Overall, there is no definitive evidence that *N. bombi* infections are higher in areas where managed bumble bees are present. In contrast, in areas with relatively low *N. ceranae* prevalence, patterns in bumble bees in the UK match those of honey bees (Fürst et al. 2014). This pattern disappears at higher levels of infection. Graystock et al. (2014) found that *N. ceranae* prevalence increased away from greenhouse sites that were not using commercial bumble bee. No obvious explanation for this pattern exists. Further work is needed to show whether *N. ceranae* actively passes from honey bees to bumble bees in the field, and whether this in turns leads to higher prevalence in bumble bees.

A third potential way to ask whether microsporidian infections are increasing in prevalence comes from patterns across species. Cameron et al. (2011) found higher prevalences in declining bumble bee species in North America, as might be predicted by the spillover theory. However, interpretation of prevalence patterns is not straight-forward, for at least four reasons. First, there are no prior data on patterns of microsporidian prevalence in the US, and so whether prevalence has increased in declining bumble bee species cannot be determined (Brown 2011). Second, a key declining species, *Bombus occidentalis*, also hosts a high prevalence of microsporidian infections in regions where the bee remains abundant (Koch and Strange 2012). Third, patterns of species specificity are common in both pathogens and parasites in bumble bees (Schmid-Hempel 1998 and references therein). Fourth, bumble bee decline goes hand-in-hand with reduced genetic variability (Cameron et al. 2011), and a previous study showed that bumble bees with lower genetic diversity have higher parasite prevalence, perhaps due to increased susceptibility (Whitehorn et al. 2011).

Overall, while there appears to be no solid evidence for or against the contention that microsporidian infections and disease are increasing in wild bumble bee populations, this reflects a lack of historical data, and a paucity of well-designed studies to examine possible spillover.

Concluding remarks and future directions

The question posed by the title of this paper remains unanswered. Future studies of museum specimens (specifically, time-series), or the use of genetic approaches to identify recent rapid expansion in microsporidia populations, appear to be the only way to resolve the issue. Nevertheless, given the widespread use of commercial bumble bees, well-designed field studies that incorporate molecular techniques may still provide insight into whether local prevalence levels are raised when commercial bumble bees are deployed. At the same time, future studies must incorporate both dissection/microscopy techniques, to identify the presence of real infections, and molecular approaches that combine species-specific primers and sequencing, to confirm the identity of the microsporidian species involved. As scientists increasingly turn to molecular tools, data quantity cannot be allowed to compromise the essential data quality needed if we are to monitor disease in our wild pollinators.

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Table 1. Records of *Nosema* spp. infection in wild bumble bees. Papers were assessed for methodology, pathogen species identified, and whether this identification could be viewed as definitive (based on the methods given).

Year	Country	Micro-scopy	Mole-cular	*N. apis*	*N. bombi*	*N. ceranae*	*T. pampeana*	Other	Definitive identify-cation*	Reference
2013	Colombia	No	Yes	No	No	Yes	-	-	No	Gamboa et al. 2015
2013	Korea	Yes	Yes	No	Yes	No	-	-	Yes	Kwak et al. 2013
2009-13	Argentina	Yes	Yes	-	-	-	Yes	-	Yes	Plischuk et al. 2015
2011-13	USA	No	Yes	-	Yes	-	-	-	No	Tripodi et al. 2014
2011	USA	Yes	Yes	-	Yes	-	-	-	Yes	Blaker et al. 2014
2011	UK	Yes	No	-	Yes	-	-	-	Yes	Jones & Brown 2014
2011	UK	Yes	Yes	-	-	Yes	-	-	Yes	Graystock et al. 2013a, 2014
2011	UK	No	Yes	Yes	Yes	-	-	-	No	Graystock et al. 2013a, 2014
2004, 10-12	Chile	No	Yes	No	Yes	No	-	Yes	No	Schmid-Hempel et al. 2014
2011	UK	No	Yes	-	-	Yes	-	-	No	Fürst et al. 2014
2010	UK	Yes	No	-	Yes	-	-	-	No	Whitehorn et al. 2013
2010	USA	Yes	Yes	-	Yes	-	-	-	No	Koch & Strange 2012
2010	UK	Yes	No	-	Yes	-	-	-	No	Goulson et al. 2012
2009	Sweden	Yes	Yes	-	Yes	-	-	-	No	Huth-Schwarz et al. 2012
2008	Ireland	Yes	No	-	Yes	-	-	-	No	Murray et al. 2013
2008	China	No	Yes	-	Yes	Yes	-	Yes	No	Li et al. 2012
2007-8	USA	Yes	Yes	No identification to species given					No	Gillespie & Adler 2013
2007-9	USA	Yes	Yes	-	Yes	-	-	-	Yes	Cameron et al. 2011; Cordes et al. 2012
2006-7	USA	Yes	Yes	-	Yes	-	-	-	Yes	Kissinger et al. 2011
2006-7	USA	Yes	No	No identification to species level					No	Gillespie 2010
2005	UK	Yes	No	-	Yes	-	-	-	Yes	Henson et al.

										2009
2004-8	Poland, Russia	No	Yes	-	Yes	-	-	-	No	Szentgyörgyi et al. 2011
2004-5	Canada	Yes	No	-	Yes	-	-	-	No	Colla et al. 2006
2003-5	Denmark, Sweden	Yes	No	-	Yes	-	-	Possibly	Yes	Larsson et al. 2007
2003	Ireland	Yes	No	-	Yes	-	-	-	Yes	Rutrecht & Brown 2008b
2002-3, 8	USA	Yes	Yes	-	Yes	-	-	-	Yes	Sokolova et al. 2010
2001	Switzerland	Yes	No	-	Yes	-	-	-	-	Korner & Schmid-Hempel 2005
200?	Denmark, Ireland, Netherlands, Sweden, Switzerland, UK	Yes	Yes	-	Yes	-	-	-	Yes	Tay et al. 2005
1998-9	Switzerland	Yes	No	-	Yes	-	-	-	No	Shykoff & Schmid-Hempel 1991
1996-8	Turkey	Yes	No	-	Yes	-	-	-	No	Aytekin et al. 2002
199?	New Zealand	Yes	No	-	Yes	-	-	-	Yes	McIvor & Malone 1995
1987, 2005-8	Argentina	Yes	Yes	-	-	Yes	-	-	Yes	Plischuk et al. 2009
1986-7	New Zealand	Yes	No	-	Yes	-	-	-	No	Fisher & Pomeroy 1989
197?	Canada	?	No	-	Yes	-	-	-	?	Liu et al. 1973
1962	Denmark	Yes	No	-	Yes	-	-	-	No	Skou et al. 1963
194?	Canada	Yes	No	-	Yes	-	-	-	Yes	Fantham et al. 1941
191?	UK	?	No	-	Yes	-	-	-	?	Betts 1920
191?	UK	Yes	No	-	Yes	-	-	-	Yes	Fantham & Porter 1914

*=either correct tissue screened microscopically, or infection shown microscopically and species confirmed molecularly; -=species not screened for; ?=lack of data through inability to access report.

Table 2. Records of *Nosema* spp. infection in commercial bumble bees. Papers were assessed for methodology, pathogen species identified, and whether this identification could be viewed as definitive (based on the methods given).

Year	Country	Micro-scopy	Mole-cular	*N. apis*	*N. bombi*	*N. ceranae*	*T. pampeana*	Other	Definitive identification*	Reference
201?	Mexico	No	Yes	-	*Yes*	-	-	-	No	Sachman-Ruiz et al. 2015
2011-12	UK	Yes	Yes	No	Yes	Yes	-	-	No	Graystock et al. 2013b
2011-12	UK	Yes	Yes	No	No	Yes	-	-	Yes	Graystock et al. 2013a,

										2014
2011-12	UK	No	Yes	Yes	Yes	No	-	-	No	Graystock et al. 2013a, 2014
2008	Ireland	Yes	No	-	Yes	-	-	-	No	Murray et al. 2013
2002	Canada	Yes	No	-	Yes	-	-	-	No	Whittington & Winston 2003
200?	Japan	Yes	No	-	Yes	-	-	-	Yes	Niwa et al. 2004

**=either correct tissue screened microscopically, or infection shown microscopically and species confirmed molecularly; -=species not screened

Interactions of Microsporidia with the Global Honey Bee Population

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Abstract

The microsporidium *Nosema ceranae* is an emerging pathogen of European honey bees, *Apis mellifera,* although it is unknown when this species first invaded *A. mellifera* populations. Prevalence of nosemosis has increased globally and the pathogen appears to have a competitive advantage over *Nosema apis,* once thought to be the only microsporidium infecting honey bees. *N. apis* has been nearly completely displaced in warmer temporate areas and *N. ceranae* is competitive in most areas where *A. mellifera* occurs. Because of high prevalence and deleterious chronic effects on the host, the presence of *N. ceranae* in colonies potentially increases susceptibility to other pathogens and pesticides. It is a concern that the only pesticide registered for treatment of nosemosis in honey bees has lower efficacy against *N. ceranae* than *N. apis.* Resistance management may be possible but should address the tendency of *Nosema*-tolerant bees to carry higher spore loads.

Introduction

Honey bees, particularly the European species *Apis mellifera,* are globally vital pollinators and are also managed domestic animals. In addition to honey production, the more important pollination services are variously estimated to contribute to productivity of crops worth $100-800 billion (US) dollars globally on an annual basis (Hein 2009). (Estimates have been extremely difficult to produce due to the varied and complex cropping systems.) Recent global declines of honey bee colonies have alarmed producers, scientists and the public, and determination of

cause has proven elusive due to numerous putatively interacting factors. Because they are both eusocial and mass-reared by apiculturists, honey bees are “ideal” hosts for density dependent pathogens. Several species of pathogenic fungi and bacteria and nearly 30 species of viruses have been identified from honey bees and, as well, macroscopic parasites (mites, beetles, moth larvae) further weaken colonies and may aid in pathogen transmission. Two microsporidian pathogens, *Nosema apis* and *Nosema ceranae*, infect honey bees and although the infections they produce are typically chronic, both species deleteriously impact colony health. The ubiquitous presence and often high prevalence in colonies raise questions about synergies with parasites, pesticides and other pathogens, and have implicated these microsporidia, particularly *N. ceranae,* as one of several primary factors in global honey bee decline.

Microsporidia in the *Nosema-Vairimorpha* clade are most commonly reported from Lepidoptera (moths and butterflies) but have been recovered from several other insect orders, including Hymenoptera, as well as occasionally from other arthropod taxa. The two honey bee pathogens, *N. apis* and *N. ceranae,* share an evolutionary history but have distinct genetic differences (Chen et al. 2013), and are different species within the taxon. *N. apis* was the only microsporidium recorded in honey bees until *N. ceranae* was described from the Asian honey bee, *Apis cerana,* by Fries et al. in 1996. In 2007, Huang et al. reported *N. ceranae* infecting *A. mellifera* in Taiwan and shortly afterward the pathogen was reported in *A. mellifera* colonies in Europe, North America and South America (Fries et al. 2006; Higes et al. 2006; Klee et al. 2007; Cox-Foster et al. 2007; Chen et al. 2008, and others). Genetic studies of stored honey bee samples have placed the pathogen in North American honey bees as early as 1975 when most sampled bees were co-infected with *N. apis* (Travor and Fell 2014) and other studies show presence in South America in 1979 (Teixeira et al. 2013).

Transmission and pathogenesis

The “true” *Nosema* species, those infecting Lepidoptera and closely related to the type species *Nosema bombycis,* are systemic pathogens. Although some level of tissue tropism is exhibited by other species in the *Nosema-Vairimorpha* clade (Vavra et al. 2010), the restriction of both *N. ceranae* and *N. apis* to the midgut tissues (and possibly the proximal Malpighian tubules) is unusual in the taxon, particularly because the known species most closely related to *N. ceranae* and *N. apis* are not midgut-specific pathogens. In general, entomopathogenic microsporidia that are restricted to the midgut tissues are chronic, horizontally transmitted pathogens, a pattern also observed for *N. apis* and *N. ceranae* infections.

Both *N. apis* and *N. ceranae* infect and are transmitted among adult honey bees, possibly by trophallaxis, the transfer of nectar and pollen from foraging adults to housekeeping adults (Smith 2012) and cleaning of fecal matter in the hive (Huang and Solter 2013). Although *N. ceranae* was recently reported to infect late instar larvae inoculated in laboratory studies (Eiri et al. 2015), it is not known if larvae are typically included in the cycle of transmission in field colonies. In other studies, newly eclosed adult bees that were isolated on emergence, including those from heavily infected field colonies, were never infected at up to 30 days post eclosion (Huang et al. 2013; Huang and Solter 2013).

The pathogenic effects of *N. apis* have been well-documented (Bailey 1955; Bailey and Ball 1991) and similar pathology was reported for *N. ceranae* (Fries et al. 2006 and others). Both pathogens reproduce in the cytoplasm of midgut epithelial cells, resulting in disruption of cells and energetic costs; field studies of *N. ceranae* show significantly reduced survival time; changes in flight behavior that include reduced number of days foraging; and reduced number of total flights, but a longer time in the field (Alaux et al. 2014).

N. apis- N. ceranae interactions

Current data support an Asian origin for *N. ceranae* and global spread (Botías et al. 2012; Gómez-Moracho et al. 2015). *N. ceranae* has been reported in five species of honey bees and fourteen species of bumble bees (*Bombus* spp.) globally (Figure 1; also *A. cerana* and *A. mellifera*), while *N. apis* has only been reported from *A. mellifera*.

Bumble bees

China (Li et al. 2012)
- *B. sibericus*
- *B. waltoni*
- *B. impetuosus*
- *B. remotus*

Argentina (Plischuk et al. 2009)
- *B. atratus*
- *B. bellicosus*
- *B. morio*

UK (Furst et al. 2014)
- *B. terrestris*
- *B. lapidarius*
- *B. hortorum*

7 *Bombus* spp. (Graystock et al. 2013)

Honey bees

Borneo (Botías et al. 2012)
- *Apis koschevnikovi*

Thailand (Chaimanee et al. 2010)
- *Apis dorsata*
- *Apis florea*

Figure 1. Host range of *Nosema ceranae*

It is intriguing that *N. ceranae,* along with a more diverse group of *Nosema* isolates, was isolated from bumble bees at the presumed nexus of bumble bee origins in China (Li et al. 2012). Global movement of *A. mellifera* for pollination services presumably served to spread the pathogen. It is not known if *N. ceranae* infects non-apid Hymenoptera, but even in bumble bees the host range may not include all species.

Why is N. ceranae dominant (and where)?

In most laboratory studies, *N. ceranae* produced more infective spores than *N. apis* in the honey bee host. Virulence studies have produced highly varied results; some suggest that *N. ceranae* is a more virulent pathogen (Higes 2006; Paxton et al. 2007; Williams et al. 2014) while others show no significant differences (Chaimanee et al. 2012; Pettis et al. 2013; Huang and Solter, 2013;). Co-infection studies suggest that the "first invader wins", but simultaneous inoculation tends to favor *N. apis* (Milbrath et al. 2015; Natsopoulou et al. 2015), a result incongruous with dominance of *N. ceranae*. Climate may be a factor. Warmer climates seem to favor *N. ceranae* while *N. apis* appears to have remained competitive in colder climes such as northern Germany and Sweden (Gisder et al. 2010; Forsgren and Fries 2013). With a few isolated exceptions, *N. apis* is no longer found in US honey bees, even in the northernmost states.

What is the role of Nosema disease in loss of colonies?

N. apis has been described for more than 60 years as having an overall relatively minor impact on honey bee health, but the situation has apparently changed with the advent and dominance of *N. ceranae* in most temperate areas. Whether *N. ceranae* is actually a more virulent pathogen with more deleterious impacts than *N. apis* is debatable, but the environment has also changed in other ways due to the global spread of other honey bee parasites including Varroa mite, hive beetles and probably several viruses, as well as the impacts of pesticides. Various combinations of pathogens, parasites and pesticides have been tested and additive effects and synergies have been suggested. Some studies suggest synergy of *N. ceranae* and the (also ubiquitous) deformed wing virus (Zheng et al. 2015), as well as synergies with pesticides, particularly neonicotinoids (Alaux et al. 2010; Pettis et al. 2013).

What is not yet understood are the overall effects on colony health of various combinations of *N. ceranae,* Varroa mites, hive beetles and other macro-pests, multiple viruses, pathogenic fungi and bacteria, agricultural chemicals and chemical hive treatments, several of which are usually present in a single system at one time.

Treatment options?

The antibiotic fumigillan, the only drug registered for treatment of *Nosema* disease in honey bees, has been used for over 60 years to suppress *N. apis* infections. The drug also inhibits reproduction of *N. ceranae* at the manufacturer's recommended dosages (Williams et al. 2008), but laboratory studies by Huang et al. (2013) showed that *N. ceranae* was released from suppression at higher fumagillin levels than *N. apis.* In addition, at lower dosages representing degradation of the drug in summer hives (fumagillin is not used during foraging season due to toxicity to vertebrates- it is sequestered in honey), twice as many mature *Nosema* spores developed in exposed bees than in bees that had not been exposed to fumagillin. However, this effect has not been tested in the field.

Studies looking at the effect of increasing overall nutrition on *N. ceranae* infection found that addition of bee bread increased bee survival time but also increased spore production (Basualdo et al. 2014), as did pollen (Porrini et al. 2009; Fleming et al. 2015). Some evidence of tolerance to *N. ceranae* has been shown in breeding experiments in Denmark (Huang et al. 2012) with genes related to resistance being identified in bees with lower spore loads (Huang et al. 2013). However, tolerant bees, like those receiving high levels of nutrition, may actually produce more spores (Huang et al. 2012), potentially leading to higher levels of transmission.

Conclusions and future directions

Honey bee biology and management practices team up to provide a "hotbed" for increased pathogen prevalence and transmission. *Nosema ceranae* is a ubiquitous pathogen that frequently occurs at high prevalence levels, and there is evidence that it interacts with other pathogens, parasites and pesticides to reduce vitality of colonies leading to colony loss. Some studies and anecdotal evidence suggest that honey bees may develop tolerance to this pathogen, but additional research is needed to determine if tolerance to *Nosema* ultimately increases prevalence and, thus, susceptibility to other pathogens and to pesticides.

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Current and Future Models for Microsporidian Research

(Insights from the Nematode *C. elegans* as a Host for Studying Microsporidian Infection)

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Abstract

Microsporidia comprise a phylum of obligate intracellular pathogens that infect virtually all animals. We have developed a convenient model for studying microsporidia infection in a whole-animal host through the identification and characterization of a natural microsporidian pathogen of the nematode *C. elegans*, which is an organism commonly studied in the lab. The *C. elegans* natural microsporidian pathogen is named *Nematocida parisii*, and it causes a lethal intestinal infection in *C. elegans*. We have characterized the *Nematocida* genome, how *N. parisii* restructures host cells, and in particular how it hijacks host exocytosis for non-lytic exit in order to facilitate transmission. We have also described how the host responds to infection with ubiquitin-mediated responses, and how a natural variant of *C. elegans* is able to clear *N. parisii* infection, but only during early life.

N. parisii is a natural microsporidian pathogen of *C. elegans*

The nematode *C. elegans* is a highly tractable organism for study in the lab that has provided novel insights into questions of development, neurobiology and cell biology, among other basic biological processes (Corsi et al. 2015). Until recently however, little was known about its natural ecology. Now, due to greatly increased sampling of *C. elegans* from the wild, we are learning more about its life outside the lab (Frezal and Felix 2015). In particular it has become clear that *C. elegans* and other *Caenorhabditis* nematodes are commonly infected by

microsporidia in the wild (Bakowski et al. 2014b; Felix and Duveau 2012; Troemel et al. 2008). Microsporidia comprise a phylum of over 1400 species of obligate intracellular parasites that can infect nearly all animal hosts (Texier et al. 2010; Vavra and Lukes 2013; Williams 2009), so it is perhaps no surprise that it is a common pathogen of nematodes like *C. elegans*.

The first and best-described microsporidian pathogen of *C. elegans* was obtained from a wild-caught *C. elegans* isolated from a compost pit near Paris, and we named it *Nematocida parisii* (nematode-killer from Paris) strain ERTm1 (Troemel et al. 2008). *N. parisii* causes a lethal intestinal infection in *C. elegans* (Troemel et al. 2008), and we recently have shown how it can also cause developmental arrest of *C. elegans* larvae (Luallen et al. 2015). In our original study we also described two other *N. parisii* strains from wild-caught *C. elegans* in France (strains ERTm3 and ERTm4), as well as a related *Nematocida* species called *Nematocida* sp1 (strain ERTm2), which was found in a sister species of *C. elegans* called *Caenorhabditis briggsae* isolated from a park in India (Troemel et al. 2008). Genome sequencing of *N. parisii* strain ERTm1 demonstrated a genome size of 4.1 Mb, with 2661 predicted genes, expression of which was confirmed for most of them by transcriptome analysis of several stages of the *N. parisii* life cycle (Cuomo et al. 2012). The ERTm3 genome genome had a similar size and predicted gene set. *Nematocida* sp1 sequencing revealed a slightly larger genome of 4.7 Mb and 2770 predicted genes.

Of note, genome sequencing indicated that the *Nematocida* sp1 genome was diploid, with most regions of the genome highly heterozygous with 1 SNP every 82 bp (Cuomo et al. 2012). These findings were the first to demonstrate that microsporidia can be diploid. *N. parisii* was similarly found likely to be diploid, although with much less heterozygosity than *Nematocida* sp1 (strain ERTm2). Genome sequencing of a second *Nematocida* sp1 strain (ERTm6) isolated from a *C. briggsae* in Cape Verde indicated that it also is heterozygous, although not as heterozygous as ERTm2 (Bakowski et al. 2014c). Intriguingly, several chromosomal arms in *Nematocida* strains have a loss of heterozygosity (LOH), indicating there has been a rare or recent recombination event, consistent with a sexual cycle for microsporidia (Cuomo et al. 2012). Passaging of *Nematocida* sp1 for 3 months in lab did not result in a change in the pattern of LOH, suggesting that a sexual cycle did not occur under these laboratory conditions (Cuomo et al. 2012). It would be interesting to examine the patterns of LOH under more natural conditions to determine if or when a sexual cycle may occur for *Nematocida* species.

Together with the microsporidian genome data described above, *C. elegans* provides a convenient whole-animal host to study microsporidia infection because of its transparent body plan and the ease of culturing it in the lab. In particular it provides an attractive model for intestinal infection, because its cells are very similar in structure to human intestinal epithelial cells, with apically polarized microvilli anchored into a cytoskeletal structure called the terminal web (Balla and Troemel 2013; Bossinger et al. 2004; McGhee, 2007). Because of the convenient culturing of C. elegans, together with the powerful genetic and molecular tools, it has been possible to molecularly characterize different aspects of its life cycle in the intestine of live, intact animals to determine how it molecularly interacts with the host, which we describe below.

N. parisii restructures *C. elegans* intestinal cells and exits via apical exocytosis

N. parisii infection causes extensive cytoskeletal restructuring in the *C. elegans* intestine as part of its exit process (Szumowski et al. 2012). Like most microsporidia, *N. parisii* survives outside of host cells in the transmissible spore form, and replicates exclusively inside of host cells in a form called a meront (Troemel et al. 2008). Early during infection, when *N. parisii* is replicating inside the cell as a meront, we found that there is a partial loss of cytoskeletal polarity of *C. elegans* intestinal cells (Figure 1).

Normally, the intestinal-specific isoform of actin called ACT-5 is restricted to the apical side of the cell. However, *N. parisii* infection at the meront stage causes ectopic expression of ACT-5 at the basolateral side of the cell (Estes et al. 2011). Soon after this redistribution of actin, there are gaps that appear in the terminal web, which is a conserved cytoskeletal structure composed of actin and intermediate filaments (Estes et al., 2011, Troemel et al., 2008). This redistribution of actin may in fact trigger the formation of terminal web gaps, because reducing actin expression with RNA interference caused the formation of terminal web gaps in the absence of infection (Estes et al. 2011). Our characterization of terminal web gaps indicated that they appear precisely when spores begin to form and exit from the cell. Once terminal web gaps form, there do not appear to be additional gaps that form later, although the existing gaps become larger likely due to distension of the lumen. These gaps appear to be specific to *N. parisii* infection, in that they do not appear in response to infection with other pathogens that cause lethal intestinal infections in *C. elegans*. Although the exact function of these gaps has not been established, we proposed that they serve to remove a barrier to exit for *N. parisii*, such that the spores are able to traverse this cytoskeletal structure in order to reach the apical surface of the intestinal cell for exit into the lumen.

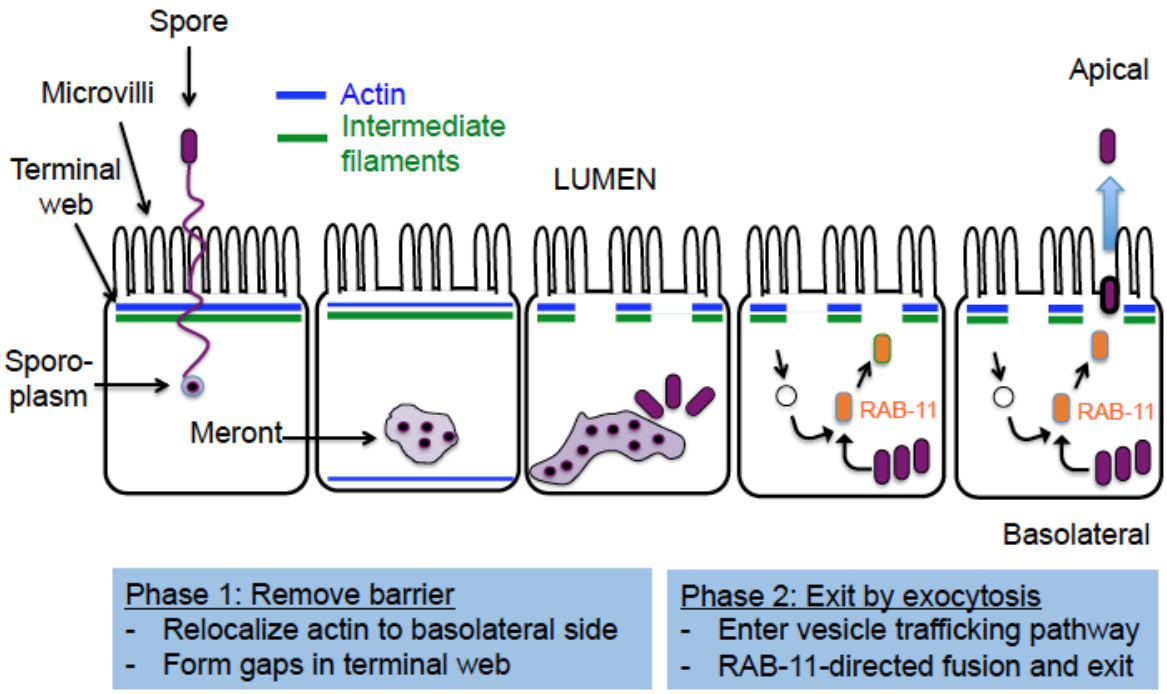

Figure 1. *N. parisii* restructures *C. elegans* intestinal cells and exits via RAB-11-directed apical exocytosis. *N. parisii* exit from *C. elegans* intestinal cells is a two-phase process. Phase 1) When *N. parisii* replicates as a meront inside *C. elegans* intestinal cells, the actin isoform ACT-5 is no longer restricted to just the apical side and instead appears to be ectopically expressed on the basolateral side of the cell. This relocalization may trigger gaps in the terminal web, which occur just as spores begin to form in the intestinal cell. Phase 2) N. parisii spores are found in separate membrane-bound compartments, become coated in the host small GTPase RAB-11, which is required for spore-containing compartments to fuse with the apical membrane and exit into the lumen.

Restructuring of the terminal web may remove a barrier to exit for *N. parisii* so it can reach the apical surface, but how does this pathogen actually traverse this membrane to exit from host cells? Very little was known about this question for any species of microsporidia. To learn more about the spore exit process for *N. parisii*, we tested when infected worms were contagious to other worms. Here, we found that animals infected with just a few spores were contagious to their neighbors (Troemel et al., 2008). These animals were alive and seemingly healthy, suggesting that intestinal cells were not lysed to release pathogen. This non-lytic exit was confirmed through the use of cell integrity assays, and analysis of GFP-labeled intestinal cells suggested that the exit was not due to budding off of membranes (Estes et al., 2011). Because

C. elegans only has 20 non-renewable intestinal cells, it is likely advantageous for *N. parisii* to exit from these cells non-lytically.

Further analysis with electron microscopy and molecular markers indicated that after *N. parisii* meronts differentiate into spores they are found in a separate membrane-bound compartment, suggesting a fusion mode of exit (Szumowski et al., 2014). Indeed, using a marker for the apical side of intestinal cells we found that *N. parisii* spores fuse with the apical membrane and are released into the intestinal lumen to be defecated out and spread to new hosts for disease transmission (Szumowski et al., 2014). This exit appears to be highly directional, with exit only out of the apical side into the lumen with no evidence of exit basolaterally to spread into other tissues of the animal (Estes et al., 2011). Furthermore, this exit is prodigious, with one to two thousand spores shed per worm per hour for several hours (Estes et al., 2011). Using a spore shedding assay, we screened through predicted small GTPases in *C. elegans* and found the small GTPase RAB-11 as a critical host factor required for spore fusion, spore exit and host contagiousness (Szumowski et al., 2014). RAB-11 localizes extensively to spores poised to exit into the intestinal lumen. Because RAB-11 directs recycling endocytosis to the apical side of the cell, by hijacking this pathway *N. parisii* can accomplish directional exit from the cell without causing a lysis event that would be likely be very detrimental to its host.

We have recently also described the formation of actin coats around exocytosing spores and performed a genetic screen to identify small GTPases required for formation of these coats (Szumowski et al., 2015). Analysis of animals that lack these actin coats indicate that actin coats are not required for spores to exit. It is possible that they facilitate exit, perhaps acting to stabilize the large spore cargo contained in an exocytic vesicle. Later during infection we observed large vesicles coated with actin that contain many *N. parisii* spores, and our analysis indicates that these vesicles are due to compensatory endocytosis to maintain the membrane balance after the extensive exocytosis of *N. parisii* spores (Szumowski et al., 2015).

C. elegans responds to *N. parisii* infection by upregulation ubiquitin-mediated defense

In order to describe the *C. elegans* host response to *N. parisii* infection we performed RNAseq analysis at five timepoints during *N. parisii* infection (Bakowski et al., 2014a). These studies demonstrated that the *C. elegans* transcriptional response to this natural intracellular pathogen

is distinct from responses to other previously described extracellular pathogens. Strikingly however, there was a very strong similarity in the gene expression response to a natural viral pathogen, indicating that some similar feature of the infection caused by these two very distinct pathogens is eliciting a transcriptional response in the host (Bakowski et al., 2014a, Sarkies et al., 2013). This response was characterized by an upregulation of ubiquitylation components, and we found that these had a functional role in protecting the host from infection, together with components of the autophagy pathway (Bakowski et al., 2014a). For example, if components of the autophagy pathway were reduced in expression with RNAi, then animals were more susceptible to infection, whereas if the autophagy pathway was upregulated, then animals were more resistant to infection. Furthermore, we observed localization of ubiquitin and autophagy components to meronts, suggesting that they are being targeted for destruction by ubiquitin-mediated autophagy, which is a common host defense strategy against intracellular pathogens. To learn more about the transcriptional response we developed GFP reporters and determined that many of the microsporidia and virus response genes are also induced by inhibition of the ubiquitin-proteasome system (Bakowski et al., 2014a). Thus, a trigger for transcriptional response to intracellular infection caused by microsporidia and virus may be perturbation of the ubiquitin proteasome system.

Natural variation in host resistance

Although the studies described above indicate that ubiquitin-mediated signalling in the N2 laboratory strain of *C. elegans* can provide some resistance against *N. parisii*, ultimately this host strain always succumbs to *N. parisii* infection. Interestingly, we have found natural variation in *C. elegans* resistance against *N. parisii*, showing that a *C. elegans* strain from Hawaii has the ability to clear infection, demonstrating striking immune capabilities for epithelial cells (Balla et al., 2015). Intriguingly, this capability is restricted to very young animals, and is lost rapidly as animals develop, even before attaining reproductive stage. We used quantitative genetics to demonstrate that this enhanced, early-life resistance of Hawaiian animals is a complex trait comprised of at least four distinct genetic loci. Using near isogenic lines we confirmed that two of these loci can confer resistance, and act additively (Balla et al., 2015). This enhanced resistance of Hawaiian worms enables them to outcompete the susceptible laboratory strain in just a few generations with selective pressure from pathogens. Altogether these findings indicate impressive abilities of epithelial cells in this wild *C. elegans* strain to clear intracellular infection by microsporidian pathogens.

Concluding remarks and future directions

The use of *C. elegans* as a model host for microsporidian infection has provided insight into the exit strategies of microsporidia and how they exploit host cells cytoskeletal and trafficking pathways to facilitate their life cycle. These studies have also provided insight into defense pathways and natural variation in host resistance. However, studies of microsporidia infection in *C. elegans* and any other host are hampered by the lack of ability to transform and genetically manipulate these parasites. With sequencing of microsporidian genomes and an increasing array of genome editing tools available, it is a propitious time to focus effort on developing these tools, in order to facilitate our understanding of the pathogenesis and life cycle of these ubiquitous parasites.

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