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Chlamydial infections of fish: diverse pathogens and emerging causes of disease in aquaculture species

Running title: Chlamydial infections of fish

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Abstract

Chlamydial infections of fish are emerging as an important cause of disease in new and established aquaculture industries. To date, epitheliocystis, a skin and gill disease associated with infection by these obligate intracellular pathogens, has been described in over 90 fish species, including hosts from marine and fresh water environments. Aided by advances in molecular detection and typing, recent years have seen an explosion in the description of these epitheliocystis-related chlamydial pathogens of fish, significantly broadening our knowledge of the genetic diversity of the order *Chlamydiales*. Remarkably, in most cases, it seems that each new piscine host studied has revealed the presence of a phylogenetically unique and novel chlamydial pathogen, providing researchers with a fascinating opportunity to understand the origin, evolution and adaptation of their traditional terrestrial chlamydial relatives. Despite the advances in this area, much still needs to be learnt about the epidemiology of chlamydial infections in fish if these pathogens are to be controlled in farmed environments. The lack of *in vitro* methods for culturing of chlamydial pathogens of fish is a major hindrance to this field. This review provides an update on our current knowledge of the taxonomy and diversity of chlamydial pathogens of fish, discusses the impact of these infections on the health, and highlights further areas of research required to understand the biology and epidemiology of this important emerging group of fish pathogens of aquaculture species.

Chlamydia-related agents of epitheliocystis: an under-recognised threat to aquaculture industries?

First reported as 'Mucophilosis' in the common carp (*Cyprinus carpio*) in 1920 (Plehn, 1920), the term 'Epitheliocystis' was coined after cyst-like inclusions within the epithelial cells of the gills were observed in the freshwater species, bluegill (*Lepomis macrochirus*) (Hoffman et al., 1969). Since this initial diagnosis, epitheliocystis has been reported from over 90 species of fish globally (Appendix S1) (Nowak and LaPatra, 2006; Stride et al., 2013a), including fishes from marine and freshwater sources as well as from wild and farmed environments. Taxonomically, the host range for this disease is diverse, including but not limited to; [a] Acipenseridae: white sturgeon (*Acipenser trasmonanus*) (Groff et al., 1996), [b] Cyprinidae: carp (*Cyprinus carpio*) (Plehn, 1920), [c] Salmonidae: coho salmon (*Oncorhynchus kisutch*) (Carvajal et al., 1990), Atlantic salmon (*Salmo salar*) (Carvajal et al., 1990), Arctic charr (*Salvelinus aplinus*) (Draghi et al., 2007) and brown trout (*Salmo trutta*) (Karlsen et al., 2008), [d] Carangidae: amberjack (*Seriola dumerili*) (Crespo et al., 1990), yellowtail kingfish (*S. lalandi*) (Venizelos and Benetti, 1996) and yellowtail (*S. mazatlana*) (Venizelos and Benetti, 1996), [e] Latidae: barramundi (*Lates calcarifer*) (Meijer et al., 2006), [f] Muglidae: golden grey mullet (*Liza aurata*) (Paperna and Sabnai, 1980), thinlip grey mullet (*L. ramada*) (Paperna, 1977), greenback mullet (*L. subviridis*) (Paperna and Sabnai, 1980) and striped mullet (*Mugil cephalus*) (Paperna and Sabnai, 1980), [g] Terapontidae: silver perch (*Bidyanus bidyanus*) (Meijer et al., 2006), and [h] Centrachidae: bluegill (*Lepomis macrochirus*) (Hoffman et al., 1969) and largemouth bass (*Micropterus salmoides*) (Goodwin et al., 2005) (Appendix S1).

Only a limited number of the affected species are cultured for commercial purposes. In these fish species, epitheliocystis is usually considered insignificant. However, there have been

cases in aquaculture, such as in Atlantic salmon farmed in Norway, where epitheliocystis has been linked to high mortalities (Draghi et al., 2004; Mitchell and Rodger, 2011). While its exact effect on the fish is still not fully understood, aquaculture species are more at risk of being infected due to the higher stocking densities and greater stresses placed upon the fish (Grau and Crespo, 1991; Nowak and LaPatra, 2006). Consequently, there has been an increase in the general awareness of the impact and prevalence of this disease (Nowak and LaPatra, 2006).

Initially, it was believed that the same aetiological agent caused epitheliocystis in all fish species. However, as early as 1977 it was recognised that the epitheliocystis forms were a distinct taxonomic entity that demonstrated a high degree of host specificity (Paperna, 1977). In addition, it could not be determined whether epitheliocystis was due to *Rickettsia*-like organisms (RLO) or *Chlamydia*-like organisms (CLO), due to both bacterial groups being defined as 'gram-negative, obligate and intracellular'(Zachary and Paperna, 1977; Bradley et al., 1988; Venizelos and Benetti, 1996). The identification of the actual causative agent(s) of epitheliocystis as belonging to the *Chlamydiales* was only a recent advance, primarily aided through the use of molecular techniques (Everett et al., 1999; Draghi et al., 2004).

Chlamydiales pathogens exhibit a two-stage developmental cycle of replication (Everett et al., 1999; Corsaro and Greub, 2006). This developmental cycle rotates between (i) an extracellular infectious elementary body (EB), which is endocytosed by eukaryotic cells and resides within a cytoplasmic inclusion and; (ii) an intracellular vegetative reticulate body (RB), which replicates by binary fission (Everett et al., 1999; Corsaro and Greub, 2006). The unique developmental cycle has hampered efforts to culture the bacteria *in vitro*. Many attempts have been made to culture the aetiological agent(s) *in vitro* as a means of proving

causation and fulfilling Koch's postulates, however all have been unsuccessful to date (Hoffman et al., 1969; Bradley et al., 1988; Valdron et al., 1994; Venizelos and Benetti, 1996; Kumar et al., 2012).

In the absence of an *in vitro* culture system for this group of CLOs, researchers have focussed on fulfilling Fredricks and Relman's molecular postulates to establish a role for these organisms in epitheliocystis (Fredricks and Relman, 1996). Using these methods, the first CLO agent characterised in fish was *Candidatus* Piscichlamydia salmonis, which was described in Atlantic salmon in 2004 (Draghi et al., 2004). Since the description of this new chlamydial species, seven new *Candidatus* species have been described with many more partially sequenced from epitheliocystis cases in different host species. Although other agents have been associated with epitheliocystis (Toenshoff et al., 2012; Mitchell et al., 2013), the majority of evidence points to a role for *Chlamydia*-related bacteria, the obligate intracellular parasites of animals and humans. Much more, however, still needs to be known about the impact of these infections on fish, their epidemiology and methods to control the disease. In this review we provide an update on the current taxonomy and diversity of *Chlamydia*-like epitheliocystis agents, discuss the impact of chlamydial infections on fish health and review the potential source and dissemination of these infections in aquaculture.

An update on the taxonomy, genetic diversity and geographical distribution of *Chlamydia*-like agents of epitheliocystis

Bacteria belonging to the order *Chlamydiales* are an extremely important and diverse group of pathogens of vertebrates, which include respiratory diseases of fishes (Corsaro and Greub, 2006). Since the epitheliocystis review of 2006 (Nowak and LaPatra, 2006), there have been numerous reports of epitheliocystis (Table 1). Many of these studies have focussed on

molecular characterisation of the aetiological agent(s) involved and this has resulted in several new *Candidatus* species being described. It is important to note that all members of the order *Chlamydiales*, not just those associated with epitheliocystis in fish underwent a significant review in 1999, which substantially altered the taxonomy of the order (Everett et al., 1999). The taxonomic guidelines were updated to reflect the increasing use and reliance of molecular techniques and DNA sequences. Consequently, for any future bacterial isolate or sequence to be included within the order *Chlamydiales* it required a ≥ 80% sequence similarity of the 16S rRNA gene to the known and accepted members already within the order. The review went further and proposed that ≥ 90% sequence similarity conferred the same family and ≥ 95% sequence similarity conferred the same genus (Everett et al., 1999). The 95% sequence similarity criterion for genus assignment however, has been challenged by members of the *Chlamydiaceae* research community (Stephens et al., 2009; Greub, 2010), and researchers should use these criteria with common sense and flexibility. These criteria are also to be used for full-length 16S (or 23S) rRNA, or near full-length segments that cover all the variable domains of those genes to ensure the accuracy of the results. All new *Candidatus* sequences that have since been proposed have followed these updated taxonomic guidelines for inclusion within the order *Chlamydiales*. Currently 49 sequences associated with epitheliocystis, differentiated by PCR of the 16S rRNA regions, have been reported in the literature and/or registered with the National Centre for Biotechnology Information (NCBI) GenBank database (Figure 1). As the taxonomy stands today, there is no single genus of *Chlamydia*-like bacteria associated with all epitheliocystis cases (Stride et al., 2013a) (Figure 1).

As already mentioned, the first epitheliocystis agent molecularly characterised was *Ca.* Piscichlamydia salmonis (AY462243-4) which was found in Atlantic salmon farmed in

Ireland and Norway (Draghi et al., 2004). These near full-length sequences were found to have ≥ 80% nucleotide sequence homology to members of the order *Chlamydiales*. Following the molecular taxonomic guidelines (Everett et al., 1999), this result firmly and definitively placed the epitheliocystis causative agent of Atlantic salmon within this order, and the first molecular proof that these bacteria were CLOs and not RLOs as previously speculated.

In 2008 a second aetiological agent of epitheliocystis was characterised, *Ca.* Clavochlamydia salmonicola, sequenced from both farmed Atlantic salmon (DQ011662) and wild brown trout (EF577392) from Norway (Karlsen et al., 2008). This bacterium was genetically distinct from the previously described *Ca.* Piscichlamydia salmonis with only 80.7% sequence similarity (from a comparison of near full-length sequences). It was only 90.5% similar to its nearest relative, *Chlamydia abortus*, placing it within the same family but creating a new genus. As more information on this bacterium has been reported, this classification has been updated and along with a name change to *Clavichlamydia*, now stands within its own family, *Ca.* Clavichlamydiaceae. All reports of *Ca.* Clavichlamydia salmonicola have come from freshwater sourced fishes within Europe; Atlantic salmon from Ireland (FN545849-52 (Mitchell et al., 2010)) and Norway (EF577391 (Karlsen and Nylund, unpublished) and, DQ011662 (Karlsen et al., 2008)), and brown trout from Norway (EF577392 (Karlsen et al., 2008) and JN123362 (Nylund, unpublished)) and Switzerland (HQ416712 (Schmidt-Posthaus et al., 2012)).

Three taxonomically distinct CLOs have been reported from farmed salmonids across the Northern hemisphere (Atlantic salmon, Arctic charr and brown trout), the third being an uncultured *Neochlamydia* sp. described from Arctic charr in West Virginia, USA (Draghi et

al., 2007). The specific agent has never been submitted to GenBank, however it was described as being 100% identical to an uncultured clinical sample from a cat with ocular disease (AY225593 (von Bomhard et al., 2003)).

Candidatus Parilichlamydia carangidicola (JQ673516), isolated from farmed Australian yellowtail kingfish (*Seriola lalandi*), was the first epitheliocystis agent to be characterised from the Southern hemisphere (Stride et al., 2013a). This bacterium was only distantly related (87% sequence similarity) to its nearest described relative *Ca.* Piscichlamydia salmonis and formed another new family within the order, *Ca.* Parilichlamydiaceae. Not long after this case was reported, *Ca.* Actinochlamydia clariae (JQ480299-301) was characterised from farmed African catfish (*Clarias gariepinus*) in Uganda (Steigen et al., 2013). Interestingly, this bacterium was 92% similar to the agent from yellowtail kingfish placing it within the same family. These reports however, were released only three months apart and they both proposed a new family (Figure 1). For the purposes of this review we will use the first reported family, *Ca.* Parilichlamydiaceae. The final decision on the taxonomic nomenclature of this clade however, is yet to be decided by the '*International Committee on Systematics of Prokaryotes Subcommittee on the taxonomy of the Chlamydiae*'.

There have been three more additions to *Ca.* Parilichlamydiaceae; *Ca.* Similichlamydia latridicola from farmed and wild Australian striped trumpeter (*Latris lineata*) (JQ687061 and KC686678-9 (Stride et al., 2013b)), an uncultured *Chlamydiales* bacteria from wild Nile tilapia (*Oreochromis nilotica*) in Uganda (JQ480302-3 (Steigen *et al*. unpublished)) and *Ca.* Similichlamydia laticola from farmed Australian barramundi (KF219613 (Stride et al., 2013c)). All these epitheliocystis agents are from fish hosts exclusively sourced within the Southern hemisphere. With the evidence currently available, it is hypothesised that all the

epitheliocystis agents of the *Ca.* Parilichlamydiaceae form a Southern hemisphere clade (Figure 1).

In 2003, a case of an 'epitheliocystis-like organism' (ELO) was reported in wild blue-striped snapper (*Lutjanus kasmira*) from Hawaii. Membrane-bound inclusions were found in internal organs and not in the gills or skin of the fish. Although the infection was found to be Gram-negative and the morphology of the ELO being somewhat comparable to epitheliocystis, the infection was seen exclusively within the kidney and spleen (Work et al., 2003). In 2012, the agent responsible for these ELO inclusions in the wild blue-striped snapper was molecularly characterised and classified as *Ca.* Renichlamydia lutjani (JN167597 (Corsaro and Work, 2012)). While this bacterium belongs to the order *Chlamydiales*, it is not considered to be a true epitheliocystis agent according to previously established definitions (Hoffman et al., 1969; Groff et al., 1996).

Epitheliocystis does not exclusively affect teleost fishes, with two cases of CLOs being reported from cartilaginous fishes. In 2010, a novel *Chlamydiales* bacterium (FJ001668) from a leopard shark (*Triakis semifasciata*) was reported from Switzerland (Polkinghorne et al., 2010). More recently, a novel *Chlamydiales* bacterium (KC454358) from a spotted eagle ray (*Aetobatus narinari*) was reported from Florida, USA (Camus et al., 2013). The signature sequences isolated from both specimens were distantly related and shared only 80% sequence similarity (Camus et al., 2013). Both specimens were held in captivity and neither case has been named with only the short 298 bp signature sequence confirmed.

Our knowledge of the genetic diversity of CLOs associated with epitheliocystis in fish has also been expanded by the partial amplification of 16S rRNA sequences of CLOs from

numerous other fish species. Molecular characterisation of archival fixed cases of epitheliocystis from Australian fish revealed the presence of three unique partial 16S rRNA sequences, CRG18 (AY013394), CRG20 (AY013396) and CRG98 (AY013474), from Australian silver perch, leafy seadragon (*Phycodurus eques*) and barramundi, respectively (Meijer et al., 2006). These sequences were found to be quite distinct from each other and from those previously described (Figure 1), however they have not been subsequently described elsewhere.

Until recently, all epitheliocystis agents, although taxonomically diverse, fell within the order *Chlamydiales*. This changed in 2012 with the report of a novel betaproteobacterium in the gill cysts of Atlantic salmon farmed in Norway and Ireland (Toenshoff et al., 2012). There was strong evidence that this bacterial agent could be found within the epitheliocystis cysts within the gills as shown by fluorescence *in situ* hybridisation (FISH). This was the first report of a molecularly characterised epitheliocystis bacterium that was not a CLO. The novel bacterium was named '*Ca.* Branchiomonas cysticola', and sequences from Atlantic salmon in Norway (JN968376 (Toenshoff et al., 2012)) and Ireland (JQ723599 (Mitchell et al., 2013)) have now been reported (Figure 1).

With the ongoing descriptions of new bacterial species with each fish host investigated, questions are raised over whether this phenomenon is a reflection of the amazing diversity and host specificity of organisms within this taxonomic group, or whether these observations reflect geographic differences in the distribution of these pathogens. Knowledge over the geographical distribution of these agent(s) is limited. However, since its first description, *Ca.* Piscichlamydia salmonis has been detected multiple times from several species of farmed salmonids across the northern hemisphere. It has been found in epitheliocystis infected brown

trout (*Salmo trutta*) from Norway (EF153480, Karlsen and Nylund, unpublished) and Switzerland (HQ416711 (Schmidt-Posthaus et al., 2012)), Arctic charr from Canada (GQ302987-8 (Draghi et al., 2010)), and further specimens of Atlantic salmon from Norway (DQ310810 (Watanabe et al., 2006), EU326495 (Nylund et al., 2008) and JQ065095-6 (Toenshoff et al., 2012)). There is only one report of epitheliocystis in Atlantic salmon cultured in Tasmania, Australia, and this was of very low prevalence and intensity by histology only (Nowak and Clark, 1999). While it has been reported that *Ca*. Piscichlamydia salmonis occurs in Tasmania (Mitchell and Rodger, 2011), there is no molecular evidence to support this supposition. A recent survey of Tasmanian Atlantic salmon for epitheliocystis by histology and PCR resulted in all samples being negative (Stride, Polkinghorne and Nowak, unpublished data). Although it is currently unknown what the status of epitheliocystis in farmed Atlantic salmon in Chile is, it would be worth considering. With a recent addition of *Ca.* Piscichlamydia cyprinis (JX470313 (Kumar et al., 2012)), an epitheliocystis agent from grass carp (*Ctenopharyngodon idella*) farmed in Austria, there is mounting evidence that the genus *Ca*. Piscichlamydia is an agent exclusive to the northern hemisphere.

The impact of chlamydial infection on fish health

Mortality of some farmed fish have been attributed to epitheliocystis, with early life stages more affected by the condition, however mortalities do occur in grow-out. In hatchery and juvenile production fish mortalities have been reported up to 10% in largemouth bass (Goodwin et al., 2005) and 100% in sharpsnout sea bream (Katharios et al., 2008), while adult fish in grow-out have seen rates of up to 44% in Arctic charr (Draghi et al., 2007) and 100% in yellowtail kingfish (Marty Deveney, Pers. Comm.). However, under what circumstances mortalities occur has yet to be determined.

Gross pathological signs are sometimes but not always observed with epitheliocystis infected fish and may include lethargy and a weak swimming behaviour, gasping at the water surface, decreased feed consumption, excessive mucous production and white nodular lesions on the gills or skin (Nylund et al., 1998; Draghi et al., 2007; Mitchell and Rodger, 2011). The large surface area of the gills allows the physiological mechanisms of ammonia excretion, gas exchange, acid base balance and salt reduction to function efficiently. While fish can afford to lose approximately 50% of their respiratory function before serious issues arises (Speare and Ferguson, 2006), gill diseases, such as epitheliocystis, can cause gill pathology and impact on the efficiency and efficacy of these functions. However, there have been very few studies into the effects of epitheliocystis on fish physiology. In one of the few studies to investigate these effects further, Lai et al. (2013) recently found that serum osmolality, which is a measure of soluble salts within the serum or plasma, was significantly increased in farmed striped trumpeter when an increase in the severity of epitheliocystis infections occurred. Infected fish had a 10% increase in their serum osmolality. In addition, chloride cells were affected by epitheliocystis infections and were absent from areas around the epitheliocystis cysts within the gill lamellae (Lai et al., 2013).

The host response to epitheliocystis presents itself in two forms, either proliferative or non-proliferative (Nowak and LaPatra, 2006; Mitchell and Rodger, 2011). While epitheliocystis infections are often benign and without any proliferative host response (Nowak and LaPatra, 2006; Katharios et al., 2008; Mitchell and Rodger, 2011; Stride et al., 2013a), when a host response does occur there is no consistency as to the reaction. This varied response includes, focal to multi-focal epithelial hyperplasia with interlamellar filling (Draghi et al., 2004; Draghi et al., 2007; Draghi et al., 2010; Polkinghorne et al., 2010; Kumar et al., 2012; Camus et al., 2013; Mitchell et al., 2013; Steigen et al., 2013), lamellar fusion (Draghi et al., 2004;

Kumar et al., 2012; Mitchell et al., 2013; Steigen et al., 2013), epithelial bridging (Draghi et al., 2010) and cellular hypertrophy of epithelial cells, lymphocytes or histiocytes (Draghi et al., 2007; Karlsen et al., 2008; Schmidt-Posthaus et al., 2012; Camus et al., 2013; Steigen et al., 2013). All these morphological changes occur with varying degrees of severity, ranging from mild and patchy, to diffuse and extremely severe.

Little is still known about the factors that influence the outcome of an epitheliocystis infection. One of the few studies to investigate this examined the role of the innate immune response of farmed striped trumpeter to severe epitheliocystis infections (Lai et al., 2013). A significant positive correlation between the severity of epitheliocystis and lysozyme activity was found. In fact, lysozyme activity in infected fish was 40% higher than in uninfected fish (Lai et al., 2013). Although this was the first report testing fish lysozyme levels in response to epitheliocystis infections, it has been previously tested for other bacterial infections in farmed fish, such as *Aeromonas salmonicida* in Atlantic salmon (Moyner et al., 1993).

The epidemiology of *Chlamydia*-like epitheliocystis in fish: more questions than answers?

While *Chlamydia*-like organisms can affect fish at their larval, juvenile and adult phases (Nowak and LaPatra, 2006), little is still known about how each developmental phase of fish acquires their CLO and what factors influence the rate of infection and disease. Unlike RLOs, where the route of infection is known to be horizontally via fish-to-fish (Gollas-Galvan et al., 2013), the route of infection for CLOs is less clear. Several hypotheses exist on the potential reservoirs or vectors for CLO infection associated with epitheliocystis, including (i) vertical or horizontal transmission; and/or (ii) the utilisation of a reservoir such as environmental amoebae (Corsaro and Venditti, 2004; Horn and Wagner, 2004; Stride et al., 2013c). A recent

study into epitheliocystis infections in farmed barramundi found that vertical transmission was a plausible route of infection following the molecular detection of the CLO from both fertilised pre-hatch eggs and several older cohorts of fish (Stride et al., 2013c). This is the first documented evidence that vertical transmission via the broodstock may play a part in transmitting the bacteria to its progeny. In terms of alternative methods of transmission, there is anecdotal evidence of horizontal transmission through co-infection studies using both goldfish (*Carassius auratus*) and bluegill as infection models (Mitchell and Rodger, 2011).

Several new species of the order *Chlamydiales* have been reported as living in association with free-living or environmental amoebae (Horn, 2008). It has been hypothesised that the CLOs utilise these amoebae as either symbionts or intermediary hosts (Corsaro and Venditti, 2004; Horn and Wagner, 2004; Corsaro and Greub, 2006). Despite this hypothesis, and the many attempts to co-culture the CLOs with different species of amoebae (e.g. *Acanthamoeba* spp.), there have been no successful isolations of any of the fish CLOs. Most of the epitheliocystis aetiological agent(s) are host specific (Draghi et al., 2004; Corsaro and Greub, 2006; Meijer et al., 2006; Draghi et al., 2007; Karlsen et al., 2008; Draghi et al., 2010; Mitchell et al., 2010; Polkinghorne et al., 2010; Kumar et al., 2012; Toenshoff et al., 2012; Camus et al., 2013; Mitchell et al., 2013; Steigen et al., 2013; Stride et al., 2013a; Stride et al., 2013b; Stride et al., 2013c) and since no epitheliocystis agent has been found in association with amoebae, it seems unlikely that amoebae are the environmental reservoir of CLOs associated with epitheliocystis.

Increased stocking densities are a known risk factor for many important bacterial, viral and parasitic diseases of aquaculture species. However, the risk factors that increase the incidence of epitheliocystis infections are still speculative (Nowak and LaPatra, 2006; Rigos and

Katharios, 2010). While farmed fish appear to have a greater prevalence and intensity of epitheliocystis infections over the levels experienced by wild fish (Nowak and LaPatra, 2006), stocking densities alone do not appear to be the definitive risk factor for epitheliocystis infections. Even when lower stocking densities are used, for example within mesocosm-reared fish, hyperinfection of epitheliocystis still occurred (Katharios et al., 2008). This suggests that another mechanism for inducing infection is occurring.

Poor water quality in the form of increased levels of pollution (Tricklebank, 2001; Agamy, 2013) or excessive nutrients available in the water column (Katharios et al., 2008), have both been attributed to increasing levels of epitheliocystis infections. More specifically, sub chronic exposure to light crude oil induced a proliferative epithelial response with lamellae fusion and a high prevalence of epitheliocystis inclusions in juvenile rabbit fish (*Siganus canaliculatus*) after only nine days of exposure (Agamy, 2013). Wild fish sampled around sewage outfalls, while not statistically different, also had a higher prevalence of epitheliocystis infections when compared to wild fish from 'clean' sites (Nowak, 1996). There is also anecdotal evidence that specific nutritional deficiency within aquaculture feeds, such as a taurine deficiency in yellowtail kingfish, can induce epitheliocystis infections (Marty Deveney, Pers. Comm.).

Seasonal differences in water temperature or changes in salinity may also affect the levels of epitheliocystis infections in both farmed and wild fish. Lower water temperatures were previously linked to higher epitheliocystis infection rates in farmed amberjack (Crespo et al., 1990) and striped bass (*Morone saxatilis*) (Zachary and Paperna, 1977), and wild jack mackerel (*Trachurus declivis*), sand flathead and tiger flathead (Stride and Nowak, 2014), while higher water temperatures were associated with increased epitheliocystis infections in

Australian Atlantic salmon (Nowak and Clark, 1999). Although not yet investigated directly, salinity is thought to be a risk factor in epitheliocystis infections. Currently, there are several CLOs affecting salmonids, however only *Ca*. Clavichlamydia salmonicola has been observed in brown trout and Atlantic salmon sampled from freshwater origins. This bacterium has not been observed in any marine sourced fish and it has therefore been hypothesised that this infection ceases upon seawater transfer (Mitchell et al., 2010).

Narrowing down what risk factors induce and/or increase the incidence of epitheliocystis will go a long way to helping aquaculture farmers reduce morbidity and mortality of their fish. While epitheliocystis is treatable with antibiotics, this is not ideal for any fish product destined for human consumption. Epitheliocystis can be effectively treated with 25 ppm of oxytetracycline and this treatment regime was first used for infected largemouth bass (Goodwin et al., 2005). Interestingly, this finding was discovered by accident as the farmer believed he was treating an infection of columnaris and not epitheliocystis. Within three days, mortalities had ceased entirely with the farmer losing about 10% of his stock. Even though an effective treatment is available for epitheliocystis infections, unless a rapid, direct and non-destructive means of diagnosing epitheliocystis becomes available, diagnosis of disease outbreaks will continue to be confirmed by indirect, destructive means. Without the means to culture the bacterium *in vitro*, future research on this area will continue to be limited.

Future directions

Recent studies have greatly increased our knowledge of the taxonomic diversity, host range, natural source and distribution of epitheliocystis and the CLOs associated with these infections, revealing at least 30 new host species and six more characterised *Candidatus* bacterial species. While some researchers and diagnosticians believe it to be negligible when

associated with fish morbidity and mortality, there is mounting evidence that it is more important to fish health than currently recognised.

Beyond the importance of these pathogens as causative agents of disease in aquaculture species, research into CLOs of epitheliocystis may be profitable for an understanding of the evolution and adaptation of pathogens of the genus *Chlamydia*, the "traditional" pathogens of humans and terrestrial animals. Inspection of the specific branching of the fish CLOs in the phylogenetic and taxonomic trees of the *Chlamydiae* indicate that fish CLOs are the oldest and deepest branched organisms within the order *Chlamydiales* (Draghi et al., 2004; Draghi et al., 2007; Horn, 2008; Karlsen et al., 2008; Corsaro and Work, 2012; Steigen et al., 2013; Stride et al., 2013a; Stride et al., 2013b). As such, further research into fish CLOs may provide important insights into the origin of pathogenicity in this group of obligate intracellular pathogens, observations that have been limited thus far by study of the genomes of members of the genus *Chlamydia*, alone.

While a comparison of complete CLO genome(s) would be an important achievement in understanding the biology of fish CLOs, alongside other *Chlamydiae*, these tasks remain difficult in the absence of an *in vitro* protocol for pure culture and isolation of the causative agent(s) involved in epitheliocystis. The main problem is that CLOs do not replicate on traditional bacteriological media, and cell cultures or amoebal co-cultures are the most likely means of CLO cultivation. Despite the known protocols available for the 'traditional' *Chlamydiales* bacteria (i.e. *Chlamydia trachomatis*), these protocols have been unsuccessful in culturing CLOs from epitheliocystis infected fish. The inability to culture these CLOs *in vitro* from fish significantly hamper the development of an experimental challenge model and further studies related to the complete characterisation of these bacteria. Until this is possible,

all new CLOs characterised will remain as *Candidatus* status. The *in vitro* culture of the causative agent(s), whether they are CLOs or non-CLOs, will most likely take one of two pathways; amoebal co-culture, or host-specific cell line development. To date, two non-fish CLOs (*Waddlia chondrophila* and *Estrella lausannensis*) have been demonstrated to enter and multiply in the permanent fish cell lines EPC-175 (epithelioma papulosum cyprini cells) and RTG-2 (rainbow trout gonad cells), as well as multiple mammalian cell lines and various species of amoebae (Kebbi-Beghdadi et al., 2011).

Whether the recently described CLOs of several fish species prove to be species- or region-specific requires further investigation. We know that several epitheliocystis susceptible *Seriola* species are found in multiple locations around the world including, Australia, New Zealand, Japan and Ecuador. We also know that Atlantic salmon is currently affected by three genetically distinct CLOs, all of whom occur in the northern hemisphere. It would be extremely beneficial to conduct a global survey, especially throughout Norway, Ireland, Switzerland, Canada, USA, Chile and Australia, to establish the true status (absent or present) of epitheliocystis infections, calculate its prevalence and to confirm which causative agent(s) affect farmed and wild salmonids. This work is required to map the geographical distribution of each epitheliocystis agent and see what patterns may emerge.

These intriguing pathogens should continue to be targets of aquaculture disease research and continued studies, such as those proposed, will further highlight the potential impact of these microorganisms on growing aquaculture industries while providing us with a fascinating insight into the evolution and adaptation of these obligate intracellular bacteria.

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Tables and Figures

Table 1: Cases of epitheliocystis reported since Nowak and LaPatra (2006) review. New information on aetiological agents (gene sequences) or new host species is reported.

Host Species	Geographic Origin (Environment)	Histo	ISH/FISH IHC/ICC	TEM	Gene (sequence)	Name	Length (bp)	Reference
Arctic charr (*Salvelinus alpinus*)	Canada (F)	+ve	ISH +ve	+ve	16S rRNA not submitted	nd	nd	(Draghi et al., 2007)
Brown trout (*Salmo trutta*)	Norway (W)	nd	nd	nd	16S rRNA EF153480	uncultured *Chlamydiaceae* bacterium	1204	Karlsen & Nylund (GenBank)
Atlantic salmon (*Salmo salar*)	Norway (F)	+ve	ISH +ve	+ve	16S rRNA DQ011662	*Ca.* Clavichlamydia salmonicola	1294	(Karlsen et al., 2008)
Brown trout (*Salmo trutta*)	Norway (W)	+ve	ISH +ve	+ve	EF577392	*Ca.* Clavichlamydia salmonicola	1294	(Karlsen et al., 2008)
Sharpsnout sea bream (*Diplodus puntazzo*)	Greece (F)	+ve	nd	nd	nd	nd	nd	(Katharios et al., 2008)
Atlantic salmon (*Salmo salar*)	Ireland (F)	+ve	nd	nd	16S cDNA FN545849-52	*Ca.* Clavichlamydia salmonicola	1244	(Mitchell et al., 2010)
Leopard shark (*Triakis semifasciata*)	Switzerland (C)	+ve	IHC -ve	nd	16S rRNA FJ001668	UFC1	294	(Polkinghorne et al., 2010)
Arctic charr (*Salvelinus alpinus*)	North America (F)	+ve	ISH +ve	+ve	16S rRNA GQ302987-88	*Ca.* Piscichlamydia salmonis	263	(Draghi et al., 2010)
Brown trout (*Salmo trutta*)	Switzerland (W)	+ve	IHC +ve	+ve	16S rRNA HQ416711	*Ca.* Piscichlamydia salmonis	297	(Schmidt-Posthaus et al., 2012)
Brown trout (*Salmo trutta*)	Switzerland (W)	+ve	IHC +ve	+ve	16S rRNA HQ416712	*Ca.* Clavichlamydia salmonicola	294	(Schmidt-Posthaus et al., 2012)
Blue-striped snapper (*Lutjanus kasmira*)	Hawaii (W)	+ve*	nd	nd	16S rRNA JN167597	*Ca.* Renichlamydia lutjani	1435	(Corsaro and Work, 2012)
Atlantic salmon (*Salmo salar*)	Norway (F)	+ve	FISH +ve	+ve	16S rRNA JN968376	*Ca.* Branchiomonas cysticola	1464	(Toenshoff et al., 2012)
Yellowtail kingfish (*Seriola lalandi*)	Australia (F)	+ve	nd	+ve	16S rRNA JQ673516	*Ca.* Parilichlamydia carangidicola	1393	(Stride et al., 2013a)
Atlantic salmon (*Salmo salar*)	Norway (F) Ireland (F)	+ve	FISH +ve	nd	16S rRNA JQ723599	*Ca.* Branchiomonas cysticola	1464	(Mitchell et al., 2013)
Grass carp (*Ctenopharyngodon idella*)	Austria (F)	+ve	nd	nd	16S rRNA JX470313	*Ca.* Piscichlamydia cyprinis	296	(Kumar et al., 2012)
Striped trumpeter (*Latris lineata*)	Australia (F)	+ve	+ve	nd	16S rRNA JQ687061 KC686678-9	*Ca.* Similichlamydia latricola	1396	(Lai et al., 2013; Stride et al., 2013b)
African catfish (*Clarias gariepinus*)	Uganda(F)	+ve	ISH +ve	+ve	16S rRNA JQ480299-301	*Ca.* Actinochlamydia clariae	1280	(Steigen et al., 2013)
Nile tilapia (*Oreochromis nilotica*)	Uganda (nd)	nd	nd	nd	16S rRNA JQ480302-3	ON3/ON26	1410	Steigen et al. (GenBank)
Spotted eagle ray (*Aetobatus narinari*)	Florida (W)	+ve	ICC +ve	+ve	16S rRNA KC454358	UGA1	296	(Camus et al., 2013)
Rabbit fish (*Siganus canaliculatus*)	United Arab Emirates (F)	+ve	nd	nd	nd	nd	nd	(Agamy, 2013)
Jack mackerel (*Trachurus declivis*)	Australia (W)	+ve	nd	nd	nd	nd	nd	(Stride and Nowak, 2014)
Sand flathead (*Platycephalus bassensis*)	Australia (W)	+ve	nd	nd	nd	nd	nd	(Stride and Nowak, 2014)
Tiger flathead (*Neoplatycephalus richardsoni*)	Australia (W)	+ve	nd	nd	nd	nd	nd	(Stride and Nowak, 2014)
Barramundi (*Lates calcarifer*)	Australia (F)	+ve	ISH +ve	nd	16S rRNA KF219613-14	*Ca.* Similichlamydia laticola	1396	Stride submitted

nd – no data; F – farmed; C – captive; W – wild

Figure 1: Relationships between all known *Chlamydia*-like and non-*Chlamydia*-like sequences submitted to GenBank from fishes with epitheliocystis. Origins and GenBank accession no. are given in brackets. Maximum-likelihood analysis was conducted on the 49 sequences available and four traditional *Chlamydiaceae* sequences, which were aligned and trimmed to the shortest available sequence. There were a total of 216 nucleotides in the final dataset. Nodal support was inferred by 1,000 bootstraps using MEGA5 (Tamura et al., 2011), with values of less than 70% not shown.