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Title: A naïve population of European oyster *Ostrea edulis* with reduced susceptibility to the pathogen *Bonamia ostreae*: are *S*-strategy life traits providing protection?

Running Title: *S*-strategy in *Ostrea edulis*

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Abstract

European populations of the native flat oyster, *Ostrea edulis,* have been heavily depleted by two protozoan parasites, *Marteila refringens* and *Bonamia ostreae,* with mortalities of up to 90% reported in naïve populations. However, in studies carried out over a ten-year period, researching the parasite-host relationship of *B. ostreae* and *O. edulis* in several age cohorts within a naïve *O. edulis* population from Loch Ryan (LR), Scotland, 1,364 specimens were challenged and only 64 (5%), across multiple testing protocols, screened positive for *B. ostreae*. This article presents a case for the development of *S*-strategy life traits in the LR population that coincide with enhanced immune

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function and survival. Oysters are considered typical *r*-strategists (small in size with fast development and high fecundity) while *S*-strategists, as outlined in Grime's (1977) C-S-R (competitor-stress tolerant-ruderal) triangle theory, are characterized by slow growth and investment in the durability of individuals. This study hypothesises that slower growth and reduced reproductive output in LR oysters has resulted in the investment of an enhanced immune function and reduced susceptibility to *B. ostreae* i.e. *r*-strategists with *S*-strategy life traits equates to protection from significant pathogens. The findings presented here within provide a strong case study for local adaptation of energy allocation and provides empirical support for the C-S-R triangle theory in a marine organism.

Keywords: *Bonamia ostreae*, C–S–R triangle, ecoimmunology, ecological strategies, European flat oyster, haplosporidia, immune function, pathogen

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A naïve population of European oyster *Ostrea edulis* with reduced susceptibility to the pathogen *Bonamia ostreae*: are *S*-strategy life traits providing protection?

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Introduction

The theory of *r*- and *K*- selection has underpinned life history theory since it was first proposed in 1967 (MacArthur & Wilson, 1967 *in* Pianka, 1972; Figure 1a). Subsequently, three-strategy life history frameworks, which separate disturbance and resource availability onto different axes, have been recognised (Figure 1b, c). The first of these, the C-S-R (competitor-stress tolerant-ruderal) triangle theory was developed for plants (Grime*,* 1977) but has since been adapted to fit with empirical studies of fish, echinoderms and insects, among others (Lawrence, 1990; Winemillar & Rose, 1992; Braby, 2002; Grime & Pierce, 2012). All these theories recognise that natural selection favours a range of survival strategies for species, which are defined by decisions or trade-offs of energy allocation. Growth, reproduction and self-maintenance are the three principle vertices involved in these trade-offs (Figure 2). At polar ends of the spectrum there are species that produce high numbers of offspring for which they provide little parental investment; *r*-strategists, and those that produce a reduced quantity of offspring with a corresponding increased parental investment; *K*-strategists (Figure 1a). These strategies are frequently associated with other traits including size, growth rate, age of sexual maturation and expected lifespan and, indeed; a third strategy, the *S*-strategist in the CSR theory, is characterized by slow growth and investment in the durability of individuals (Grime & Pierce, 2012; Figure 1b).

The adaptation of increased immunity is commonly observed as a consequence of parasitic stressors (Martin, 2009; Powell *et al.,* 2011; Guo *et al.,* 2015), and subsequent reduction in other life history traits has frequently been reported (Hoang, 2001; Colditz, 2008; Rauw, 2012). This phenomenon is

assumed to result, in some instances, from antagonistic pleiotropy: where a gene that has a positive effect on immune defence has a negative effect on another fitness component (Siva-Jothy *et al.,* 2005). However, using selection experiments in insects, changes have also been shown to happen in the other direction: increased energy allocation to certain non-immune-related traits has resulted in reductions in immune parameters. Dung flies, *Scatophaga stercoraria*, selected for larger reproductive organs were found to have a correlating reduction of prophenoloxidase activity, an indicator of immunocompetence in arthropods (Hosken, 2001). Even more interesting, genetic lines of mosquitos, *Aedes aegypti*, that were chosen for a later age of pupation had improved encapsulation abilities without any parasitic or immune-stimulatory influence (Koella & Boëte, 2002). This demonstrates how variations in immunity can develop without the influence of a direct stressor.

Immune function is an important but costly life history trait (Sheldon & Verhulst, 1996, Cotter *et al.,* 2011). Trade-offs between immune defences and other life history components have been reported in numerous species (Mauk *et al.*, 2005, Lee, 2006). In a study looking at 13 amphibian (11 anuran and two caudate) host species and a virulent parasite; species that developed quickly and metamorphosed at a smaller size (*r*-strategists) were particularly prone to infection and pathology (Johnson *et al.*, 2012). Similarly, across seventy neotropical bird species, the species which invested in longer developmental times had more developed immune systems (Lee *et al.*, 2008). Furthermore, when species are selectively bred for increased growth, a strong and significant decrease in immune function is frequently reported (van der Most *et al.,* 2011).

Vertebrates have two immune systems, innate and adaptive, which require different degrees of energy investment (McDade *et al.*, 2016). Although invertebrate immune response is defined by an innate system only, different elements of this system also have unique activation costs (Moret, 2003). In the marine environment, fast-growing branching species of corals appear to invest more in relatively low-cost pathways of the immune system (e.g. antioxidant activity) whereas slow-growing

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massive species, which devote more energy to more complex and selective immune defences (e.g. anti-microbial protein production; Pinzón *et al.*, 2014). Corals fit relatively well with the C-S-R triangle life history framework and when Darling *et al.* (2012) compared life history traits in scleractinian corals they found that the species divided into four different life history strategies (competitive (C), ruderal/weedy (R), stress-tolerant (S) and generalist). Their stress-tolerant group reproduced by broadcast spawning, were large, had high fecundity during episodic spawning events and slow growth.

Oysters are typically considered *r*-strategists (Rushton, 1995; Figure 1a) mostly because of their high fecundity (between 500,000 – 1 million eggs per spawning for *Ostrea edulis*) and low parental investment (Foighil & Taylor, 2000; FAO, 2019). The European flat oyster, *Ostrea edulis*, (Figure 3) typically matures at three years of age (Cole, 1942), is oviparous, brooding its fertilised eggs for 6-8 days (Helm & Bourne, 2004) and typically grows 20 g $year^{-1}$ in the UK (www.marlin.ac.uk) or 30 g $year^{-1}$ in warmer Mediterranean regions (Acarli *et al.,* 2011; Celik *et al.*, 2015). Oysters, as bivalve molluscs, have an innate immune system and depend on circulating haemocytes to encapsulate and phagocytose foreign particles and humoral molecules to incapacitate and eliminate invaders (Bachère *et al.*, 1995; Wang *et al.*, 2018). They have two major types of haemocytes; granulocytes and hyalinocytes (Auffret, 1989; Renault *et al.*, 2001; Cochennec-Laureau *et al.,* 2003). Granulocytes are the primary cell type that is used in defence by oysters and the most efficient at fighting disease (Cochennec-Laureau *et al.,* 2003). Other defence mechanisms include circulating enzymes and production of reactive oxygen species (Tiscar & Mosca, 2004). Haemocyte counts, lysozyme activity, reactive oxygen species and phagocytic capacity are principle immune components used to assess immune activity levels in oysters. At a transcriptomic level, gene expression studies of immune-related genes complement functional assays. Recently, studies have shown that oysters also display innate immune-memory, allowing for within- and multi-generational priming towards viral and bacterial pathogens (Zhang *et al.*, 2014; Green & Speck, 2018).

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As filter-feeders, adult oysters filter up to 50 gallons of water per day (Oesterling & Petrone, 2012) which results in a continuous interaction with large volumes of marine particulate, including numerous microscopic pathogens. Diseases, primarily oyster herpesvirus (OsHV-1) and the protozoan parasites *Marteila refringens* and *Bonamia* spp., particularly *Bonamia ostreae* and *Bonamia exitiosa,* are a significant problem for oyster populations world-wide. In fact, *B. ostreae* (Figure 4) has decimated populations of the European flat oyster. This parasite was first imported unintentionally to Europe in oysters originating from California, U.S.A. After arriving in France in 1979 (Pichot *et al.*, 1980), it quickly spread with oyster consignments to other European populations in France, Spain, Denmark, the Netherlands, England and Ireland (Culloty *et al.*, 2004). When initially introduced to a naïve population, *B. ostreae* can cause over 90% mortality (Culloty and Mulcahy, 2007). Although eradication of such parasitic infections is unlikely, especially in populations grown in the wild; nearly 40 years of research has resulted in a better understanding of this parasite-host relationship (Grizel *et al.,*1988, Montes *et al.,* 1994, Engelesma *et al.,* 2010, Morga *et al.,* 2012, Arzul & Carnegie, 2015) and selective breeding programmes for disease tolerance and resistance to *B. ostreae* have shown that reduced proliferation of this parasite within oysters, along with reduced prevalence of infection and susceptibility, can occur (Lynch *et al.,* 2014). Such progress has led, in recent years, to a movement to restore *O. edulis* production. With increased interest in restoration of this species, there is a greater importance, now more than ever, to understand the survival strategies of *O. edulis.*

The theories proposed to explain the evolution of varying life history traits, by the likes of McArthur & Wilson (1967), Grime (1977) and Winemillar & Rose (1992), provide strong theoretical bases from which to study population dynamics. However, when using such frameworks, it is important to remember that they also include lines between the proposed apical strategies on which species, with suites of varying traits, may be placed (Darling *et al.,* 2012). Empirical examples of *S*-strategy appear, thus far, to be rare in the marine environment. However, it has been directly reported in cnidarians (Darling *et al.,* 2012; Pinzon *et al.,* 2014). Indeed, *S*-strategy with energy allocation

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towards enhanced physical defences has also been observed in crustacea (e.g. *Callinectes sapidus*; Davis *et al.,* 2005) and echinoderms (e.g. *Colobocentrotus atratus* and *Heterocentrotus mammillatus*; Lawrence, 1990). Previously, the oyster has been used as a classic example of an *r*-strategist (Rushton, 1995). However, in this article we have presented data to suggest that at least one population of *O. edulis* is better placed towards the *S*-strategy vertex. Below, studies carried out over a ten-year period, researching the parasite-host relationship of *B. ostreae* and *O. edulis* within a naïve *O. edulis* population are outlined. They provide support to the hypothesis that the development of *S*-strategy life traits (reduced energy allocation towards growth and reproduction in favour of self-defence) in a naïve population of *O. edulis* has resulted in enhanced immune function and protection from *B. ostreae* infection; providing a strong case study for local adaptation of energy allocation and empirical support for the C-S-R triangle theory in a marine organism.

Case Studies (2004 to 2013) with the naïve population of oysters from Loch Ryan (Scotland)

Numerous studies have been carried out in Ireland on *O. edulis* and *B. ostreae* since the parasite was first detected in 1987 (Rogan *et al.,* 1991; Culloty *et al.,* 1996; Culloty *et al.,* 1999; Culloty *et al.,* 2001; Lynch *et al.,* 2005; Lynch *et al.,* 2008; Lynch *et al.,* 2010; Flannery *et al.,* 2014a; Prado-Alvarez *et al.,* 2015). In many of these trials the use of naïve (i.e. no prior exposure to *B. ostreae*) oysters was required to test hypotheses. A naïve population of slow growing (11 g year^{-1} compared to the UK average 20 g year^{-1}; Hugh Jones, 2003; Laing *et al.*, 2005) Scottish oysters from Loch Ryan, Scotland (Figure 5), were frequently used for such studies. The oysters were sourced from Loch Ryan because *B. ostreae* has never been detected at that site and the private fishery is classified as *Bonamia*-free (FHI, 2019). Outlined below are a succession of independent studies (two in the field and three in the laboratory) investigating *B. ostreae* in multiple *O. edulis* stocks, including Loch Ryan

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oysters that were used as a negative control. Together they show how this naïve population exhibit an atypical immune tolerance or reduced susceptibility towards *B. ostreae.*

(i) Field trials (2004 and 2005)

In spring/summer 2004, naïve Loch Ryan (LR) *O. edulis* (n=540) of three different ages (spat (<1 year), half-grown (2-3 years) and adults (5-6 years)) were held at a *B. ostreae*-endemic site (Rossmore, Cork, Ireland; Figure 5) for four months to investigate latent (early) stages of infection using OIE recommended diagnostic techniques (heart smear imprints to visualise the pathogen and polymerase chain reaction (PCR) to detect the pathogen's DNA; OIE, 2012). Rossmore has been a *B. ostreae*-endemic site since 1987 (Lynch *et al.,* 2014) and other studies carried out in this area with naïve and disease-resistant, selectively-bred Rossmore oysters showed that *B. ostreae* mean prevalence was ~20% in 2003 (Lynch *et al.,* 2005); with a mean prevalence of 4% to 15% (heart smears) and 9% to 24% (PCR) in all Rossmore age groups being recorded from 2003 to 2007. However, all LR *O. edulis* (n=540) in the 2004 trial were observed to be free of infection when screened using heart imprints, while *B. ostreae* DNA was detected in two out of the 540 oysters using PCR screening (0.4% prevalence).

In spring/summer 2005, LR oysters (n=240, 1-2 years) were again deployed to Rossmore with 60 oysters sampled on Day 1, Week 1, Week 4 and Week 7 of the trial. *B. ostreae* was not detected in any of the 240 oysters screened using heart imprints and PCR. In contrast, a previous study (July 2003) monitoring infection susceptibility of naïve oysters from Tralee, Ireland and Seasalter hatchery, England, at Rossmore, recorded 10% and 29% infection prevalence respectively after being held at the Rossmore site for a similar duration (2 months; Lynch *et al.,* 2005).

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(ii) Laboratory transmission trial (2012)

In 2012, a transmission trial study was set up, using naïve LR *O. edulis* as control specimens. The aim was to compare *B. ostreae* prevalence and mortality rates of long and short-term infected *O. edulis* populations (Clew Bay (CB) and Lough Foyle (LF) Ireland, respectively). The initial health screening (heart imprints and PCR analysis) showed 33% infection of CB and LF samples and no infection in LR samples. *B. ostreae* was not detected in the LR *O. edulis* throughout the 6-month trial. This study held 50 oysters from each of the four populations together in four tanks (20L volume) and supplemented the exposure of the oysters to *B. ostreae* with the addition of homogenised *O. edulis* tissue containing *B. ostreae* (approx. $1x10^6$ viable cells). Heart imprints, PCR analysis of gill and heart tissue and ISH was carried out on all LR *O. edulis* to screen for the presence of *B. ostreae.* Fifty percent prevalence of infection was recorded in CB and LF oysters at two and eight weeks into the trial, respectively. Whereas, *B. ostreae* was not detected in the initial LR sample and no subsequent infection was found by heart imprint or PCR analysis of the experimental samples (n=104) over the course of the trial. Interestingly, when the LR *O. edulis* were screened using ISH, positive signals for *B. ostreae* DNA were observed in 51% of these oysters (53/104). These positive signals were observed in several tissues 24 hours after introduction of *B. ostreae* to the tank system and continued up until the end of the study. In some samples the cytoplasm of the *O. edulis* blood cells stained positive, indicating the presence of *B. ostreae*. ISH was used alongside heart imprints and PCR analysis as *B. ostreae* can be visualised in the tissue using this technique, thus the progress of the parasite can be traced in the animal. Using ISH, *B. ostreae* DNA was observed in all tissues (gills, mantle, digestive tract and connective tissue), except for the haemocytes of the heart, in the LR stock. The fact that it was observed so extensively in tissues yet not in the heart imprints is a common occurrence during the latent stage of infection.

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(iii) Laboratory immune trials (2009 and 2013)

Immunological parameters in LR *O. edulis* were compared in four laboratory-based studies with those of *O. edulis* from a long-term (25+ years) and a short-term (5 years) *Bonamia*-infected population (Rossmore (R) and Loch Foyle (LF), respectively). In winter 2009, natural infection levels and immune parameters of LR, LF and R *O. edulis* were initially compared in a 21-day study. For this study the animals initially endured an excessive handling pre-treatment and then prolonged (stress) exposure to a warm dry environment immediately before each sampling time point. Following this, further immunological parameters of LR and LF *O. edulis* were subsequently monitored in three additional 21-day studies completed in spring, summer and autumn 2010. The oysters from each site were kept in separate 500L tanks at ambient temperature, acclimatising for 7-10 days before trials began. The animals were checked daily and dead and dying animals were removed. During the studies, the animals were sampled at five time points, Day 1, 3, 7, 14 and 21.

Immunological parameters: lysozyme activity, nitric oxide production, haemocyte cell counts and phagocytic capacity, as well as prevalence and intensity of *B. ostreae* infection and oyster mortalities were recorded. Lysozyme activity was analysed using a standard assay kit comparing samples with hen egg white lysozyme standard while nitric oxide production was measured using an assay based on the Griess reaction. Comparing LF and LR *O. edulis,* no difference was found in phagocytic capacity; however, LR *O. edulis* had a significantly greater proportion of granulocytes (LF: 32.87 ± 5.54%, LR: 38.67 ± 4.97%; t-test: t = 3.02, df = 28, $p < 0.01$) and large hyalinocytes (LF: 22.67 ± 6.38%, LR: 41.0 ± 8.21%; t-test: t = 6.83, df = 28, $p < 0.001$). LR *O. edulis* also had significantly higher lysozyme activity than LF *O. edulis* (absorbance at 450nm; LF: 0.53 ± 0.10 AU, LR: 1.25 ± 0.22 AU; t-test: t = 11.79, df = 28, $p < 0.001$). Interestingly, in the stress exposure trial, LR *O. edulis* had significantly higher nitric oxide production compared to infected and uninfected LF *O. edulis*, and infected R *O. edulis* (LR: 67.74 ± 2.42 µM; LF infected: 44.04 ± 7.39 µM, LF uninfected: 58.92 ± 3.60

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µM, R infected: 57.12 ± 3.78 µM; one-way ANOVA: F = 25.26, df = 4, $p < 0.01$). However, uninfected R *O. edulis* (69.57 ± 2.87 µM) were found to have similar levels (Figure 6).

Following this, in 2013, an experiment involving 450 LR (naïve) oysters and 450 LF (*B. ostreae* infected) oysters was carried out over 4 weeks to evaluate the effect of oral administration of immunostimulants on oyster immune capacity and progression of the *B. ostreae* infection (Prado-Alvarez *et al.*, 2015). Three recirculation systems consisting of three 100 L tanks were set up for each stock of oysters (LR and LF separately). Two systems for each stock of oysters were selected for the experimental exposure to encapsulated stimulants and the third acted as a control. Immuno-stimulants (zymosan A: 13 beta (β)-glucan from yeast *Saccharomyces cerevisiae* (Sigma-Aldrich) and curdlan: 1,3-β-glucan from bacteria *Alcaligenes faecalis* (Sigma-Aldrich)) were administered in one dose combined with phytoplankton to boost filtering and incorporation. Samples were collected at 3, 24, and 48 hours and 7, 10, 14, 21 and 28 days post-administration. LF (*B. ostreae* infected) oysters experienced mortality from Day 1 with a gradual increase over the experiment, reaching 14% of cumulative mortality at Day 21 (504 hours). LR (naïve) oysters, on the contrary, showed a minimal increase in cumulative mortality. Survival analysis using Cox proportional hazards regression showed that immune-stimulant treatment did not significantly affect survival ($p > 0.05$) but infection status (LF vs LR) was a significant factor of cumulative survival (HR = 4.67, df = 1, $p < 0.05$; Figure 7). Results indicated that the general status of *Bonamia*-infected oysters (LF oysters) had deteriorated compared to naïve LR oysters and LR oysters were able to resist the less favourable conditions under confinement. Prevalence of *B. ostreae*, determined by heart imprints, varied between 5% and 40% in LF oysters but was not detected in LR oysters at any of the sampling points or the initial sample. The activation of the immune system was evaluated by estimation of cell and humoral immune parameters. To evaluate the transcriptomic response of haemocytes after immune stimulation, a battery of genes related to *Bonamia* infection (galectin, lysozyme, superoxide dismutase and filamin genes; Stossel *et al.,* 2001; Di Lella *et al.,* 2011; Morga *et al.*, 2011) were assayed by real-time SYBR Green RT-PCR (Prado-Alvarez *et al.*, 2015). Oral administration of immuno-stimulants enhanced the

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immune response of both stock of oysters. However, testing significant differences between the two oyster groups, using the t-test and the Mann-Whitney Rank Sum test when normality tests failed, overall immune activation was higher in LR oysters, with zymosan treatment inducing significantly higher lysozyme activity (U=172.5, $p < 0.05$) and gene expression of galectin (t=9.742, $p < 0.001$) and lysozyme (t=61.635, $p < 0.001$), while curdlan triggered significantly greater transcription of galectin (t=5.540, $p = 0.01$), lysozyme (t=9.797, $p < 0.001$) and superoxide dismutase (t=4.809, $p < 0.01$). These results showed an enhanced immune activation in LR oysters; especially the immune-related genes which showed an increase of 5- to 200-fold gene induction compared to LF oysters and are indicative of a more robust defence to boost an efficient immune response after encountering any aggressor.

Discussion

Over a period of 10 years, a series of studies have shown that naïve LR oysters consistently prevent or suppress the development of *B. ostreae* infection when challenged in the field and laboratory. When 1,364 specimens were challenged in transmission trials only 64 (5%) screened positive for *B. ostreae* at a very low level of infection and were not commonly associated with mortalities (Table 1). Furthermore, nine of these were found positive only by PCR screening which may have detected trace DNA rather than a true infection. These findings are at odds with numerous other studies that have shown that naïve populations of *O. edulis* are highly susceptible to *B. ostreae* infection (Polanco *et al.,* 1984; Van Banning 1991; Cigarría & Elston, 1997; Culloty *et al.,* 2001), acquiring the pathogen and subsequent disease rapidly and with significant mortalities (Culloty *et al*., 2001). The results from the immune studies suggest that when LR *O. edulis* take in *B. ostreae* they are able to keep the parasite at a low/sublethal level of infection and eventually eliminate the disease without succumbing to the pathogen. These naïve oysters have an enhanced cellular (increased proportions of granulocytes ($p < 0.01$) and large hyalinocytes ($p < 0.001$)) and humoral (higher nitric oxide

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production ($p < 0.001$) and lysozyme activity ($p < 0.05$)) immune response, similar to a selectively bred, resistant stock. Previous observations of LR oysters have demonstrated that they are characteristically slow growing (Hugh Jones, 2003, Laing *et al.*, 2005) with low reproductive outputs (Eagling *et al.,* 2017). These previous observations, combined with the statistically supported findings of the field and laboratory trials outlined above, present a strong case for the development of *S*-strategy life traits in the LR population that coincide with enhanced immune function and survival.

Abiotic and biotic factors ("local effects") have been shown to actively shape specific genomic regions through natural selection in marine organisms (Vera *et al.,* 2019). We propose that slower growth may be a key driver of *B. ostreae* tolerance in LR *O. edulis*, which is supported by predictive models and real-life examples that have illustrated how increased investment in the immune system leads to decreased investment in other competing life history functions and *vice versa* (Mauk *et al.,* 2005; Lee *et al.*, 2008; Ardia *et al.,* 2011; Lozano-Durán *et al.*, 2013). The slow growing phenotype of LR oysters could be a consequence of genetic and/or environmental factors. Low environmental temperatures have been reported to result in slower growth and associated, improved immunity in a range of different species (Yang *et al.,* 1992; Mauk *et al.,* 2005). In line with this, it has been noted in the UK that disease-free oyster sites tend to be in more northern areas where seawater temperatures are lower, growth rates are slower and spawning is less frequent and reliable (Laing *et al.*, 2005). This is surprising in one sense because *B. ostreae* has higher survival rates and infectivity at low temperatures (Arzul *et al.*, 2009; Murray *et al.*, 2012).

Temperature is a strong influencer of growth rate in *O. edulis* (Wilson, 1987). It also effects reproduction (Joyce *et al.,* 2013). The LR population of *O. edulis* exist in an environment where nutritional resources are plentiful (Giménez *et al.,* 2017) but water temperatures are low, fluctuating around the minimum required for reproductive ripeness (Eagling *et al.,* 2017). The temperatures at Loch Ryan are at the cusp of what is required for reproduction (max. temperatures ranging 16 – 18

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°C, occasionally reaching 20 °C; pers. obs.). However, *O. edulis* populations have a wide tolerance to environmental temperatures and exist at higher latitudes with lower minimum but similar maximum temperatures (2 – 18 °C; https://www.seatemperature.org/europe/) than Loch Ryan (in Scotland as well as Europe and Canada). *B. ostreae* was first detected in Canada in 2004 (Marty *et al.*, 2006), in Denmark in 2014 (Madsen and Thomassen, 2015), Norway in 2006 (Mortensen *et al.,* 2019) and at Loch Sunart, and West Loch Tarbert in Scotland (www.oie.int; Munro & Wallace, 2016). While there is very little published literature relating to these northern infected populations, reports of prevalence (www.oie.int) suggest that the incidents are frequently isolated and there has been no associated mass mortalities (Nielsen & Petersen, 2019).

Although temperature may influence growth rates, and hence immune parameters, we suggest that this is a secondary driver and it is the growth rate that is the principle driver of reduced susceptibility to *B. ostreae*. Supporting this theory, a naïve *O. edulis* population with a 'slow growth-enhanced immune function' phenotype was presented in another study investigating growth, mortality and disease susceptibility of *O. edulis* populations obtained from different geographical origins (da Silva *et al.,* 2005). For this study, naïve oysters were obtained from four regions, including a *Bonamia*-free site in Greece. Although the site details were not provided in the study, it is worth noting that sea temperatures commonly reach a maximum >25°C around Greece (https://www.seatemperature.org/europe/greece.htm). Adult oysters, transported to Galicia, were mated and the resulting spat were kept in family groups and on-grown on oyster rafts located in the Ría de Arousa, an oyster-culture area heavily affected by bonamiosis since the 1980s. Growth, pathological condition, infection prevalence and survival were monitored monthly for two years. Interestingly, one of the five Greek families displayed significantly lower mortality and prevalence of *B. ostreae*. Bonamiosis has not been reported from Greece (Anonymous 2002; Virvilis *et al.*, 2003) and therefore, the parents of these specimens would not have been exposed to or built up a tolerance to the disease. Furthermore, da Silva *et al.* (2005) report that this family (GR5) "showed much lower mortality but slow growth". Slow growth is a common attribute of this Greek naïve

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population and the naïve Loch Ryan population of *O. edulis,* which both have shown reduced susceptibility to *B. ostreae*. Greece is at the eastern extreme of the range of *O. edulis.* Therefore, it may be that extreme local environmental conditions in this area have also resulted in a slow growth-enhanced immune function phenotype in some individuals.

It may be that this 'slow growth – enhanced immune function' phenotype was lost in many populations due to indirect (Bromley *et al.,* 2016) or direct (Newkirk & Haley, 1982; Mahon, 1983) selection for a faster growing phenotype. It has also been found that mitochondrial genetic diversity is severely reduced in many European populations because of unbalanced sex ratios and/or sex-biased differential reproductive success between males and females (Diaz-Almela *et al.,* 2004). The LR population is now considered unusual in that it maintains a 1:1 sex ratio, a characteristic deemed a consequence of the low temperatures (Eagling *et al.,* 2017). It is possible that the unique immune features reported here are related to a retention of a resistant mitochondrial DNA (mtDNA) genotype or increased mtDNA diversity. Such a theory is worthy of further investigation.

Conclusions

There has been significant interest in restoring *O. edulis* populations across Europe (Laing *et al.,* 2006; Lallias *et al.,* 2010; Gercken & Schmidt, 2014; Sawusdee *et al.,* 2015). Regarding this, major research has been completed to build detailed knowledge of the genetic diversity within the European population (Beaumont *et al.,* 2006; Lallias *et al.,* 2010; Horvath *et al.*, 2013; Vera *et al.,* 2019) and to understand what genes are important for *Bonamia*-tolerance or resistance (Pardo *et al.,* 2016; Ronza *et al.,* 2018; Vera *et al.,* 2019). The findings that we have presented here could contribute significantly to discovering additional genetic markers linked to *Bonamia*-tolerance or resistance. However, consideration for the adaptability of this unique population to new environments will also be important. Of significance, the LR population of *O. edulis* present an

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interesting case study of local adaptation through resource allocation and energy trade-offs that have resulted in a *S*-life history strategy, differing from the general species survival strategy. This case study highlights the variation of life history strategies that may exist, even within a single species and provides empirical support for the C-S-R triangle theory in a marine organism.

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Table Caption

Table 1. Overview of results from the *Bonamia ostreae* transmission studies carried out with Loch Ryan (LR) *Ostrea edulis* by University College Cork, Ireland. PCR = polymerase chain reaction; ISH = *in-situ* hybridization. N.A. = Not applicable.

Figure Captions

Figure 1. Life history strategy theories: a) McArthur & Wilson's (1967) *r-K* scale of reproductive strategy, considering fecundity and parental investment (Rushton, 1995). b) Grime's (1977) CSR triangle model describing the various equilibria between competitive (c), stress-tolerant (s), and disturbance or ruderal (r) strategies in vegetation. c) Winemillar & Rose's (1992) model based on fundamental demographic trade-offs and selection in response to different kinds of environmental variation.

Figure 2. Representative diagram of the principle life history traits in the oyster that require energy and between which trade-offs exist.

Figure 3. The European flat oyster, *Ostrea edulis.*

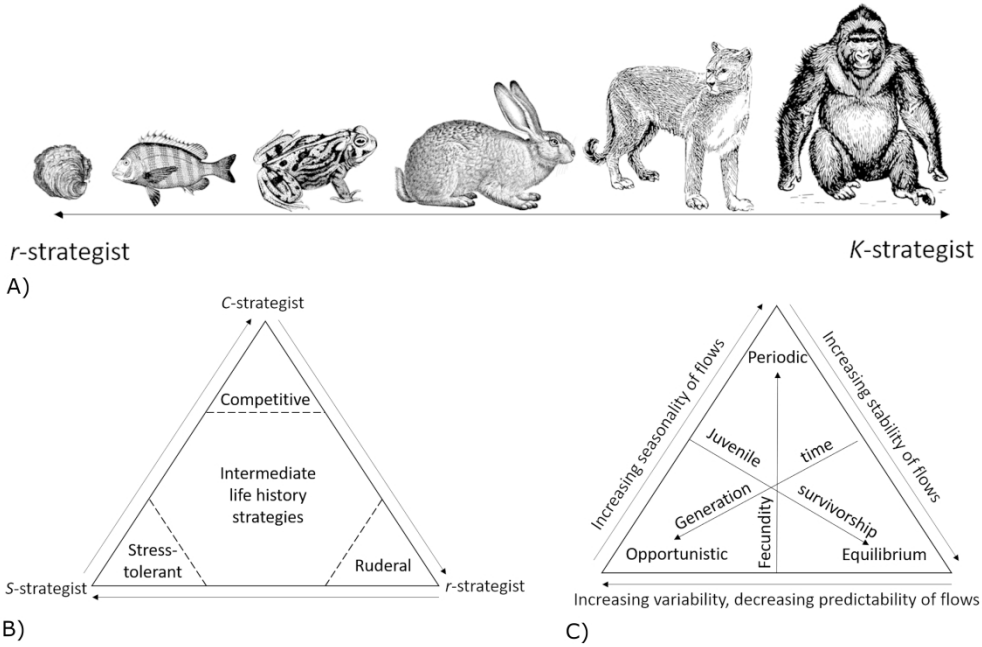
Figure 4. Extracellular and intracellular *Bonamia ostreae* cells in oyster haemolymph.

Figure 5. Map of Loch Ryan, Scotland a *Bonamia*-free site where the naïve population of *Ostrea edulis* were sourced from for transmission and immune trials and, Rossmore, Cork, Ireland a *Bonamia*-infected site where the field transmission trials were conducted.

Figure 6. Haemolymph nitric oxide levels measured in *Ostea edulis* from three sites (Loch Ryan (LR), Rossmore (R) and Loch Foyle (LF)) after having undergone excessive handling pre-treatment and prolonged exposure to a warm dry environment immediately before sampling (n = 10).

Figure 7. Cox regression analysis of overall survival according to oyster group: Loch Foyle (*Bonamia ostreae* infected oysters) vs Loch Ryan (naïve osyters), with immunostimulant treatment as covariate.

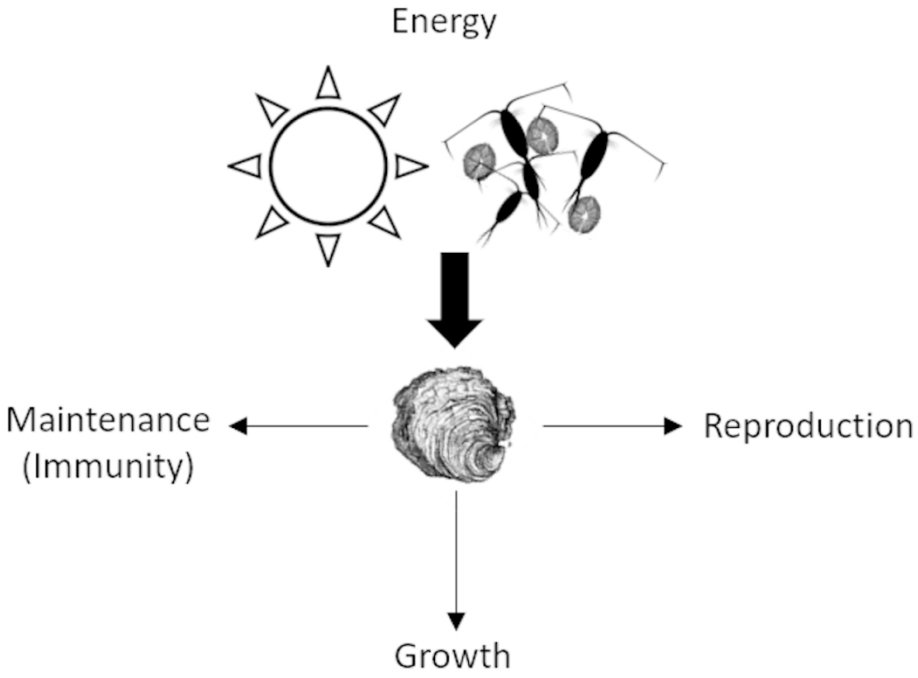
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Life history strategy theories: A) McArthur & Wilson's (1967) *r-K* scale of reproductive strategy, considering fecundity and parental investment (Rushton, 1995). B) Grime's (1977) CSR triangle model describing the various equilibria between competitive (*c*), stress-tolerant (*s*), and disturbance or ruderal (*r*) strategies in vegetation. C) Winemillar & Rose's (1992) model based on fundamental demographic trade-offs and selection in response to different kinds of environmental variation.

184x122mm (600 x 600 DPI)

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Representative diagram of the principle life history traits in the oyster that require energy and between which trade-offs exist.

85x60mm (400 x 400 DPI)

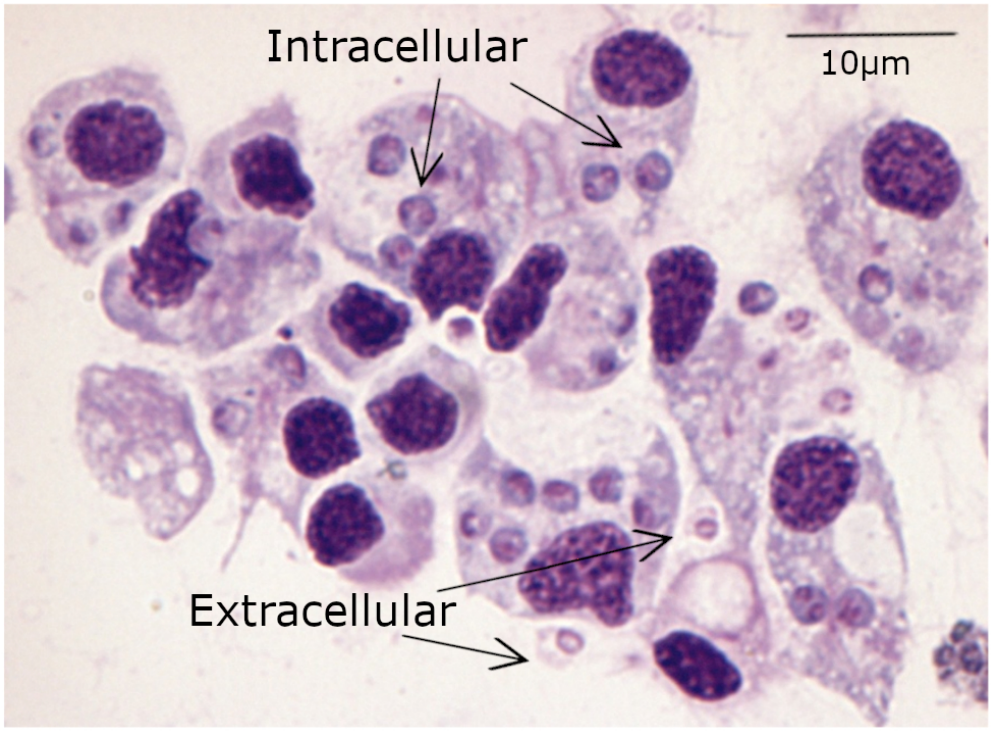
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The European flat oyster, *Ostrea edulis*.

89x47mm (400 x 400 DPI)

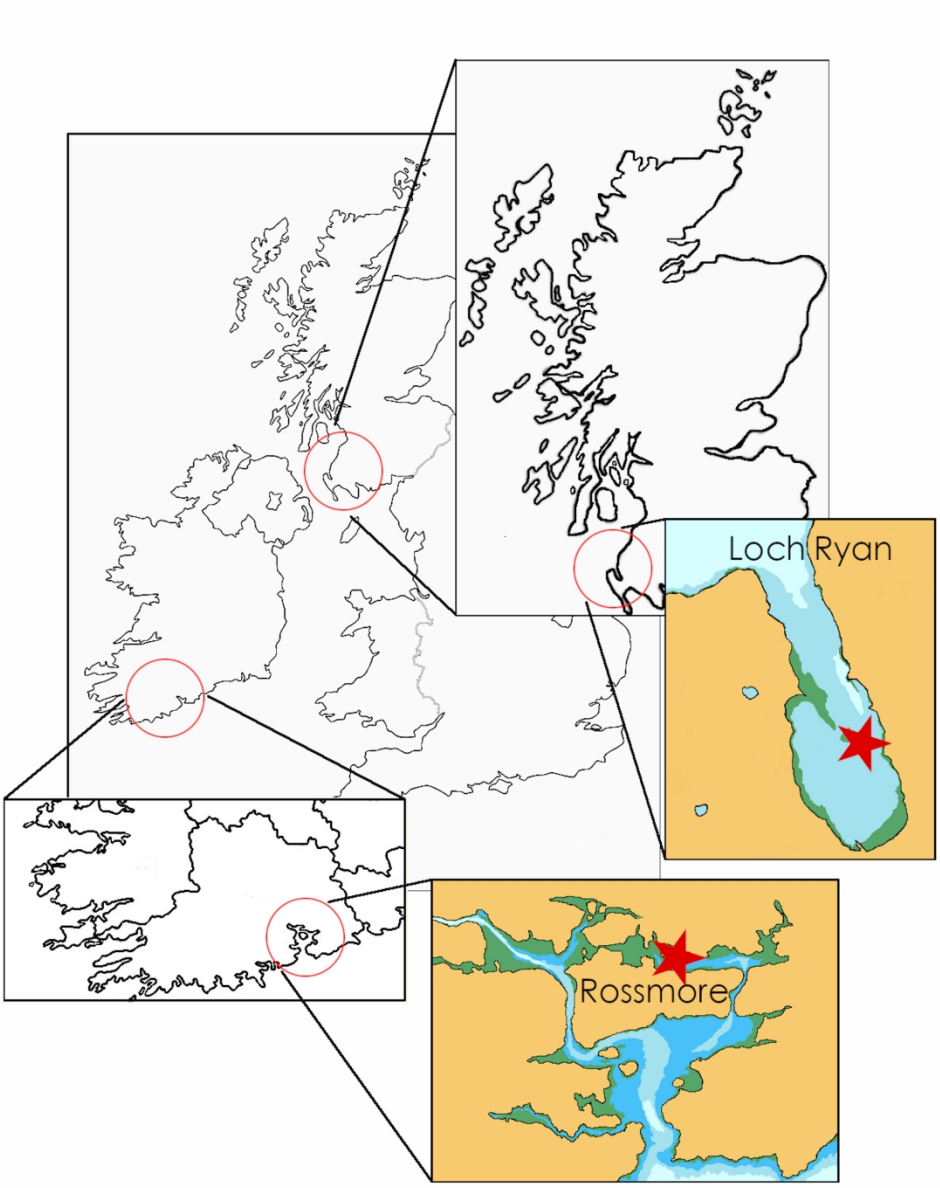
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Extracellular and intracellular *Bonamia ostreae* cells in oyster haemolymph.

83x61mm (300 x 300 DPI)

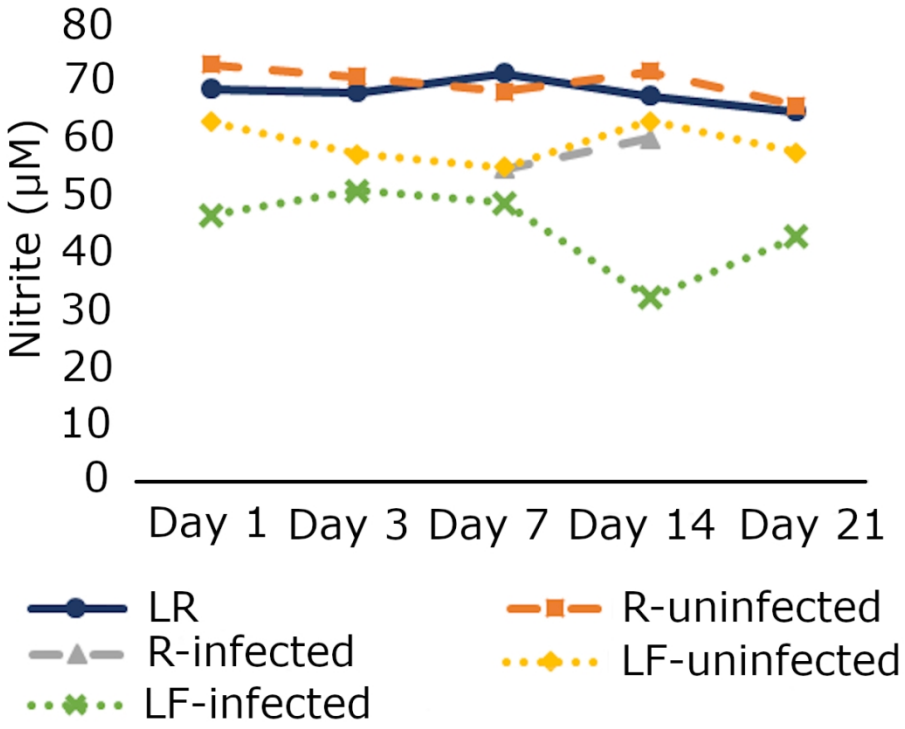
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Map of Loch Ryan, Scotland a *Bonamia*-free site where the naïve population of *Ostrea edulis* were sourced from for transmission and immune trials and, Rossmore, Cork, Ireland a *Bonamia*-infected site where the field transmission trials were conducted.

90x113mm (600 x 600 DPI)

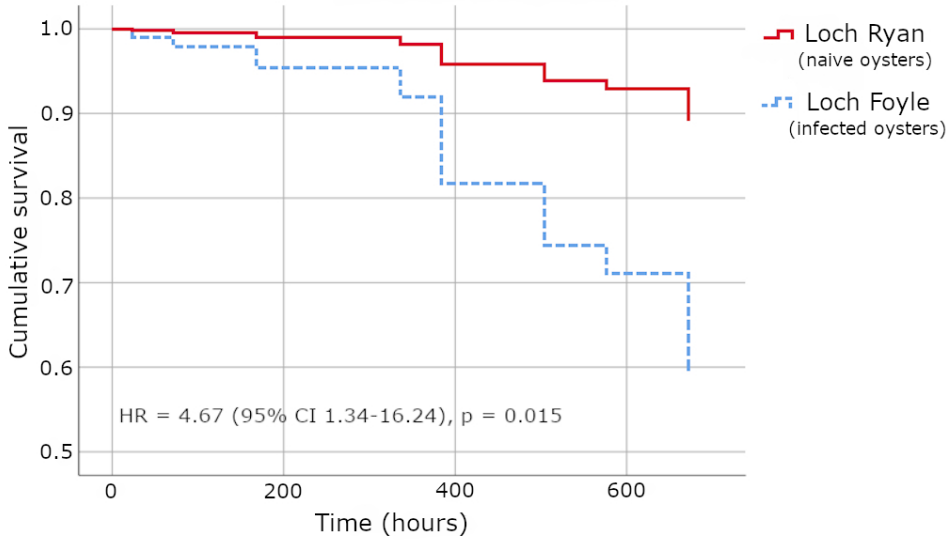
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Haemolymph nitric oxide levels measured in *Ostea edulis* from three sites (Loch Ryan (LR), Rossmore (R) and Loch Foyle (LF)) after having undergone excessive handling pre-treatment and prolonged exposure to a warm dry environment immediately before sampling (n = 10).

85x68mm (400 x 400 DPI)

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Cox regression analysis of overall survival according to oyster group: Loch Foyle (*Bonamia ostreae* infected oysters) vs Loch Ryan (naïve osyters), with immunostimulant treatment as covariate.

67x39mm (400 x 400 DPI)

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Table 1. Overview of results from the *Bonamia ostreae* transmission studies carried out with Loch Ryan (LR) *Ostrea edulis* by University College Cork, Ireland. PCR = polymerase chain reaction; ISH = *in-situ* hybridization. N.A. = Not applicable.

Year	2004	2005	2005 [a]	2007 [b]	2010	2012	2013 [c]
Location	Rossmore	Rossmore	UCC Laboratory	UCC Laboratory	UCC Laboratory	UCC Laboratory	UCC Laboratory
Duration (days (weeks))	120 (17)	49 (7)	109 (15)	180 (25)	21 (3)	180 (25)	28 (4)
LR *O. edulis* (n)	540	240	180	300	50	104	450
Other stocks/ species	Rossmore culture site	Rossmore culture site	Brittle stars, *Ophiothrix fragilis* Beadlet anemones, *Actina equina* Peacock worms, *Sabella pavonina*	Pacific oysters, *Crassostrea gigas*	Loch Foyle *O. edulis* Rossmore *O. edulis*	Loch Foyle *O. edulis* Clew Bay *O. edulis* Galway *O. edulis*	Loch Foyle *O. edulis*
***B. ostreae* screening**							
Heart imprints	0	0	2	0	N.A.	0	N.A.
PCR	2	0	0	7	N.A.	0	N.A.
ISH	N.A.	N.A.	N.A.	N.A.	N.A.	53	N.A.
% Prevalence	0.4%	0%	1.1%	2.3%	N.A.	51%	N.A.

[a] Lynch et al. (2007)

[b] Lynch et al. (2010)

[c] Prado-Alvarez et al. (2015)

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