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Microsporidian parasites feminise hosts without paramyxean co-infection: support for convergent evolution of parasitic feminisation

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ABSTRACT

Feminisation of amphipod crustaceans is associated with the presence of at least three microsporidian parasites and one paramyxean parasite, suggesting that the ability to feminise has evolved multiple times in parasites of amphipods. Co-infection by a paramyxean with one of the putative microsporidian feminisers, *Dictyocoela duebenum*, has inspired the alternative hypothesis that all feminisation of amphipods is caused by paramyxea and that all microsporidian associations with feminisation are due to co-infection with paramyxea (Short et al., 2012). In a population of the amphipod *Gammarus duebeni*, breeding experiments demonstrate that the microsporidia *D. duebenum* and *Nosema granulosis* are associated with feminisation in the absence of paramyxea. Co-infection of the two microsporidia is no more frequent than expected at random and each parasite is associated with feminisation in the absence of the other. These findings support the original hypothesis that the ability to feminise amphipods has evolved in microsporidia on multiple occasions. Additionally, the occurrence of a non-feminising strain of *D. duebenum* in *Gammarus pulex* suggests that different strains vary in their feminising ability, even within microsporidian species. The presence or absence of feminising ability in a particular microsporidian strain should not therefore be generalised to the species as a whole.

1. Introduction

Feminisation of genetic males by maternally inherited parasites is widespread among arthropods, including butterflies (Hiroki et al., 2002), woodlice (Bouchon et al., 1998) and amphipod crustaceans (Bulnheim, 1978; Ginsburger-Vogel and Desportes, 1979a). Feminising parasites can alter the sex ratios of host populations (Ginsburger-Vogel, 1975; Bouchon et al., 2008), increasing or decreasing population growth rates and hence resilience to perturbations. They can also cause dramatic changes in the genetic sex determination mechanisms of their hosts, including local extinction of sex chromosomes and transitions from male to female heterogamety (Rigaud and Juchault, 1993). Most known feminising parasites are bacteria of the genus *Wolbachia* (Valette et al., 2013). However, among amphipod crustaceans, feminisation is associated with eukaryotic microsporidian and paramyxean parasites (Bulnheim, 1978; Ginsburger-Vogel and Desportes, 1979a; Terry et al., 1998; Ironside et al., 2003). Previous investigations have indicated the existence of multiple species of feminising microsporidia in amphipods (Ironside et al., 2003; Mautner et al., 2007). However, a recent study (Short et al., 2012) suggests that some or all instances of apparent feminisation by microsporidia in amphipods may be due to co-infection with feminising paramyxean parasites.

The production of thelygenous broods containing abnormally high numbers of female offspring is associated with parasitic infection in the amphipod species *Orchestia gammarellus* (Ginsburger-Vogel and Desportes, 1979a), *Gammarus duebeni* (Bulnheim, 1978; Terry et al., 1998; Ironside et al., 2003) and *Corophium curvispinum* (Mautner et al., 2007). In these species, the total number of offspring produced by infected females is not reduced, suggesting that the parasites distort the sex ratio by feminising rather than killing infected male embryos. In the case of *O. gammarellus*, the feminising parasite is a paramyxean, *Paramarteilia orchestiae* (Ginsburger-Vogel and Desportes, 1979b). In *G. duebeni*, feminisation is attributed to two microsporidian parasites, *Nosema granulosis* (Terry et al., 1999) and *Dictyocoela duebenum* (Terry et al., 2004). A further two feminising microsporidia, *Octosporea effeminans* and *Thelohania hereditaria*, have been described (Bulnheim and Vavra, 1968; Bulnheim, 1971) but these are now thought to be synonymous with *N. granulosis* and *D. duebenum* (Jahnke et al., 2013). In *C. curvispinum* a third, undescribed microsporidian appears to be responsible for sex ratio distortion (Mautner et al., 2007). These three microsporidia are distantly related and belong to clades

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consisting predominantly of non-feminisers (Terry et al., 2004), suggesting that the ability to feminise amphipod hosts has evolved on at least four separate occasions, once in paramyxea and three times in microsporidia. *Nosema granulosis* and *D. duebenum* exhibit low pathogenicity (Ironside et al., 2003; Kelly et al., 2003) and efficient vertical transmission (Terry et al., 1998; Ironside et al., 2003), which occurs through female hosts only. They therefore have the potential to obtain significant benefits from feminising male hosts. In addition to the three host species mentioned above, parasitic feminisation by microsporidia has been suggested in a number of other amphipod hosts, on the basis of higher prevalence in adult females than in males (Terry et al., 2004; Ryan and Kohler, 2010). These studies are not supported by experimental evidence from brood sex ratios and so the observed female bias in parasite prevalence might be produced by other causes, such as parasite tissue specificity for the female gonad, killing of infected male embryos or ecological differences between males and females affecting the likelihood of infection.

In certain populations of the amphipod *Echinogammarus marinus*, a large proportion of intersex individuals occur (Ford et al., 2006). These may be functionally male or female but show secondary sexual characteristics of both sexes. Intersex individuals of both functional sexes exhibit higher prevalence of infection with the microsporidian parasite *D. duebenum* than do normal individuals of either sex. The prevalence of infection in intersexes also appears to be correlated with the prevalence in normal females (Ford et al., 2007). These observations have been used to support the hypothesis that intersexuality in *E. marinus* results from incomplete parasitic feminisation of male hosts by *D. duebenum* (Ford et al., 2006, 2007). Recently, a paramyxean parasite was discovered in *E. marinus* (Short et al., 2012). This parasite, known only from its 16S ribosomal DNA sequence, also occurs at higher prevalence in intersex individuals than in normal individuals and, intriguingly, shows levels of co-infection with *D. duebenum* significantly higher than expected, given the prevalence of the two parasites (Short et al., 2012). Given that the paramyxean *P. orchestiae* is associated with feminisation and intersexuality in *O. gammarellus* (Ginsburger-Vogel and Desportes, 1979a), this discovery opened the possibility that intersexuality in *E. marinus* may be caused by the paramyxean, rather than by *D. duebenum*. In *Orchestia aestuarensis*, Ginsburger-Vogel (1991) observed a stronger association of male intersexuality with paramyxean infection than with microsporidian infection and showed that transplanted *O. aestuarensis* tissue co-infected with paramyxea and microsporidia induced intersexuality in male *O. gammarellus*, even though only the paramyxean cells survived in the new host. Short et al. (2012) used these findings as a basis to speculate that other presumed cases of feminisation by microsporidia, such as those in *G. duebeni* (Ironside et al., 2003) and *C. curvispinum* (Mautner et al., 2007), might be due to undetected co-infection with a paramyxean.

Co-infection with a single paramyxean species offers an attractive explanation for the surprising discovery that several different species of microsporidia are associated with feminisation in *G. duebeni* (Ironside et al., 2003). This co-infection hypothesis might be viewed as more parsimonious in that the trait for feminisation would need to evolve only once (in paramyxea) rather than at least four times convergently (thrice in microsporidia and once in paramyxea). This hypothesis might also explain the finding that morphologically and genetically similar strains of microsporidia are associated with feminisation in some amphipod populations but not in others (Bulnheim, 1978; Terry et al., 2004), (Ironside, R.E., 2003. The diversity and evolution of feminising microsporidia (Ph.D. thesis). University of Leeds, Leeds, UK). This has been interpreted as evidence that microsporidia exhibit heritable variation in their ability to feminise (Bulnheim, 1978) but might alternatively be due to paramyxean co-infection with some microsporidian strains but not others.

However, there remains convincing evidence in support of feminisation by microsporidia, at least in the host *G. duebeni*. The sexual phenotypes of *G. duebeni* infected with different microsporidian species respond differently to hormonal and environmental manipulations such as injections of androgenic hormone and changes in temperature or salinity (Bulnheim, 1977; Rodgers-Gray et al., 2004; Jahnke et al., 2013). These results could only be explained by paramyxean co-infection if each microsporidian species formed an exclusive association with a different paramyxean strain. Furthermore, the co-infection hypothesis is based upon evidence of near-total co-infection of an undescribed paramyxean parasite and a microsporidian described as *D. duebenum* in *E. marinus*, and the association of these parasites with intersex phenotypes in natural populations (Ford et al., 2006, 2007; Short et al., 2012). No direct evidence from breeding or transfection experiments for complete or incomplete sex reversal in infected, genetically male *E. marinus* has yet been produced. It is also not entirely clear that the parasite described as *D. duebenum* by Short et al. (2012) in *E. marinus* belongs to the same strain, or even the same species, as the parasite associated with feminisation in *G. duebeni* (Ironside et al., 2003), since its ssrDNA sequence is only 98.6% similar. If the co-infection hypothesis is to be considered seriously as an alternative explanation for microsporidian-associated feminisation in amphipods then it must be shown to operate in a well-characterised system, such as *G. duebeni*, in which there is convincing experimental evidence that parasite-induced feminisation actually occurs.

Short et al. (2012) have demonstrated that a paramyxean parasite occurs in at least one species of Gammaridean amphipod, that this parasite is associated with intersexuality and that it shows a strong pattern of co-infection with a microsporidian. Given the strong evidence that a paramyxean *P. orchestiae* causes feminisation in *O. gammarellus* (Ginsburger-Vogel and Desportes, 1979a), it is therefore necessary to test directly the hypothesis that some or all feminisation associated with microsporidia in *G. duebeni* results from co-infection with a paramyxean parasite.

2. Materials and methods

2.1. Screening of wild-caught G. duebeni females for parasites

Gammarus duebeni samples were collected using a hand net from beneath rocks in shallow streams where they crossed the tideline on shores consisting of sand with scattered rocks. Sixty-two precopula pairs of *G. duebeni* were collected from Fintray Bay (55°46′05″N, 4°56′16″W) on the Isle of Cumbrae, Firth of Clyde, Scotland in October 2000. Females were separated from males and placed in a freezer at −80 °C. DNA was then extracted from their gonad tissue using two extractions with phenol/chloroform and one extraction with chloroform. The quality of the DNA was tested using a PCR for the host mitochondrial Cytochrome Oxidase 1 (Cox1) gene, with the primers HCO2198 (forward) (5′ TAAACTTCAGGGTGACCAAAAAATCA 3′) and LCO1490 (reverse) (5′ GGTCAACAAATCATAAAGATATTGG 3′) (Folmer et al., 1994). Successful amplification indicated the presence of high quality DNA. Each DNA sample was then subjected to two PCR screens for feminising microsporidia. The first was a single-stage PCR using the primers 285NF (5′ CGGATAACGGTATTACTTT 3′) and 1164NR (5′ CATAACGGACCTGTTTTAAT 3′), which amplifies an 879 bp fragment of DNA from *N. granulosis* but not *D. duebenum*. The second was a two-stage PCR using the primers pairs 254SF (5′ ATCAGTT AGTAAGTAGGGTAAGGGCCTA 3′) and 981R (5′ TGGTAAGCTGTC CCGCGTTGAGTC 3′), followed by 280SF (5′ TTAGACGAATACGGG

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