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Effects of feminizing microsporidia on the masculinizing function of the androgenic gland in *Gammarus duebeni*

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ABSTRACT

Feminizing parasites enhance their vertical transmission to the host offspring by converting genetic male hosts into phenotypic females. Crustacea are the only invertebrates where sexual differentiation is controlled by a specialised endocrine organ, the androgenic gland, rather than by the gonads. We showed that a feminizing microsporidian *Microsporidium* sp. inhibits androgenic gland differentiation. We investigated the effect of *Microsporidium* sp. and a second feminizing microsporidium, *Nosema granulosis*, on the masculinizing function of the androgenic gland in *Gammarus duebeni*. Androgenic gland implants had a masculinizing effect on the sexual characteristics and sexual behaviour of recipient female hosts, reflecting the masculinizing function of the androgenic gland. Individuals that had received androgenic glands showed changed morphology in comparison with controls; they were bigger overall, they lost their oostegite marginal setae, developed calceoli and acquired a male-like behaviour. This effect was observed in uninfected females, as well as in females infected with the *Microsporidium* sp. The masculinizing effect of androgenic gland implants was smaller in *N. granulosis* infected individuals. *N. granulosis* and *Microsporidium* sp. fall into distinct clades of the Microspora. It appears that these divergent parasites both act by inhibiting the development of the androgenic gland. However, they differ in their ability to inhibit the host's response to the hormone that controls male sexual differentiation.

1. Introduction

Vertically transmitted parasites are transmitted maternally from infected female hosts to their offspring. Male hosts are dead ends for transmission and consequently many such parasites have evolved strategies to enhance the proportion of females (i.e. the transmitting sex) in the progenies of their host (Bandi et al., 2001; Engelstaedter and Hurst, 2009). Sex-ratio distortion in favour of females is induced by a large variety of vertically transmitted parasites, including endosymbiotic bacteria such as *Wolbachia* or *Cardinium*, viruses, and eukaryotic parasites from the phyla Microspora and Haplospora (Ginsburger-Vogel and Desportes, 1979; Dunn et al., 2001; Terry et al., 2004; Werren et al., 2008; Engelstaedter and Hurst, 2009; Cordaux et al., 2011; Short et al., 2012). Sex-ratio distortion can be either achieved by killing the males in the progeny, which decreases competition among the progeny and consequently enhances the females' fitness (and thus the parasite's transmission), or by converting non-transmitting males into transmitting females. Such conversion can result from the inversion of genetic males into genetic females, as seen in haplodiploid insects (parthenogenesis induction), or from converting genetic males into phenotypic females (feminization; Cordaux et al., 2001; Kageyama et al., 2012).

The mechanisms by which feminization is induced are not well known. In insects, some recent studies evidenced manipulations of the sex-determination system itself by reproductive parasites (Beukeboom, 2012; Sugimoto and Ishikawa, 2012), while others suggested possible alterations of the sex-differentiation process through a manipulation of the endocrine signalling pathway (Narita et al., 2007; Negri, 2012). In crustacean hosts, feminizing parasites appear to modulate the endocrine control of sexual differentiation to convert genetic males to phenotypic females (Bulnheim, 1978; Bouchon et al., 1998; Rodgers-Gray et al., 2004).

Crustacea are the only invertebrates where sexual differentiation is controlled by a specialised endocrine organ, the androgenic gland, rather than by the gonads (Charniaux-Cotton and Payen, 1985). The sexual phenotype differentiates relatively late in development when the gland is activated in genetic males, releasing androgenic gland hormone (AGH). AGH suppresses ovarian development and promotes spermatogenesis, and regulates the expression of primary and secondary sexual characteristics (Okuno et al.,

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1997; Sagi et al., 1997). In the absence of the androgenic gland, female differentiation occurs (LeBlanc, 2007). This delay in sexual differentiation may explain the susceptibility of this group to epigenetic factors which override genetic sex determinants; these factors include environmental cues (Dunn et al., 2005), pollutants (Ford et al., 2012) and cytoplasmic parasites (Bouchon et al., 1998; Terry et al., 2004). *Gammarus duebeni* is host to several species of microsporidian parasite of which two, *Nosema granulosis* and *Microsporidium* sp. are feminisers which cause infected female hosts to produce broods that are more than 90% female (Terry et al., 1998; Ironside et al., 2003). The two species fall into discrete branches of the phylum Microspora. *Microsporidium* sp. has been referred to as *Dictyocoela duebenum* and falls into a clade that is basal to the lineage of microsporidia infecting fish (Terry et al., 2004), although the genus awaits formal description.

In the present study, we investigate the mechanism of feminization of these parasites. Previous studies have shown that androgenic gland differentiation does not occur in *N. granulosis*-infected animals (Rodgers-Gray et al., 2004). We first dissected females infected by *Microsporidium* sp. to see whether androgenic gland differentiation occurred in individuals harbouring this feminizing parasite. We then tested whether females infected by feminizing microsporidia were as sensitive to androgenic gland hormone as uninfected ones. In uninfected females, androgenic gland implants have been shown to induce masculinization (Sagi and Khalaila, 2001). Here we compared the effect of androgenic gland implants on the sexual characteristics of uninfected *G. duebeni* females and on females infected with the feminizing microsporidia *N. granulosis* and *Microsporidium* sp.

2. Methods

2.1. Animals

Animals were collected from Budle Bay, Northumberland, UK (55°40′N, 01°43′W) using a fine mesh net. All animals were maintained in brackish water (6.25‰, made from Instant Ocean, Aquatic Systems Inc) and provided with *Enteromorpha* (marine algae) and rotted leaves (*Acer pseudoplanatus*) for food and shelter. Males and females were separated (any intersexes were excluded from the experiments) and the infection status of the females was determined by screening their embryos for vertically transmitted microsporidia (Kelly et al., 2002). Brooding females were anaesthetised, the embryos (1–64 cells) flushed from the brood pouch, permeated with 5 M HCl, rinsed, fixed in acetone and stained with 4,6-diamidino-2-phenyl-indole (DAPI). Eggs were screened for parasites using a Zeiss Axioplan fluorescence microscope. In infected embryos, merogonic stages of *N. granulosis* and *Microsporidium* sp. are found in the perinuclear cytoplasm (Weedall et al., 2006). Based on the size of their nucleus, it is possible to discriminate the two species, since *N. granulosis* meronts are significantly shorter than those of *Microsporidium* sp. (mean length ±SD; *N. granulosis* = 1.51 μm ± 0.17; *Microsporidium* sp. 2.27 μm ± 0.52; *t*-test; $t = -10.02$; df = 62; $p < 0.001$). Such staining-based discrimination fully matches PCR-RFLP-based or FISH-based molecular discrimination ($n = 82$; methods by Hogg et al. (2002) and Dubuffet et al. (2013)).

2.2. Gonad morphology of Microsporidium sp. infected females

Gonads from 21 females infected by *Microsporidium* sp. were dissected and examined for traces of male characters (general aspect, presence of vas deferens).

2.3. Androgenic gland implant treatments

Uninfected females ($N = 64$), females parasitized with *N. granulosis* ($N = 47$), and females parasitized with *Microsporidium* sp. ($N = 92$) were allocated to one of two treatments; implanted with androgenic glands, or a control group that were implanted with muscle tissue from male donors. Two experiments were carried out; in the first, treatment animals were implanted with two androgenic glands or a control muscle implant of equivalent size; in the second trial, treatment animals received four androgenic glands, and control animals received two muscle implants.

Donor males were anaesthetised in carbonated water, decapitated and the vasa deferentia with their attached androgenic glands were dissected in cold van Harreveld crustacean saline (Helly and Thomas, 2003). Dorsal muscle was used for control transplants. Recipient females were anaesthetised, washed in crustacean saline and a hole (150–300 μm diameter) cut in the ventral cuticle of the 5th pereon segment tergite. A glass needle was used to insert the transplants into the dorsal part of the pereon. Recipient animals were kept in individual pots for 3 months and then examined for changes in external sexual characteristics and sexual behaviour.

2.4. Morphometrics

In gammarid Crustacea, the sexes can be identified on the basis of external morphological characteristics (Charniaux-Cotton and Payen, 1985). Males have a pair of genital papillae on the ventral surface of the 7th pereopod segment. Males also have enlarged propodi on the gnathopods which are used to carry the female in precopulatory amplexus, and the male is the larger sex, reflecting the selective pressures of mate guarding (McCabe and Dunn, 1997). *G. duebeni* males also have longer antennae and carry calceoli on the flagellum of the 2nd antennae; the calceoli are sensory organs used in mate detection (Dunn, 1998). In females, genital papillae and calceoli are absent and the gnathopod propodi are not enlarged. The female has on the ventral surface four pairs of oostegites; in sexually mature females these are fringed with setae to form a brood pouch in which embryonic development occurs. Total length of animals was recorded before the implant was given (TLstart) and the following data were collected for each individual at the end of the experiment; total length (TL), gnathopod one length (1GL), gnathopod 1 width (1GW), gnathopod two length (2GL), gnathopod 2 width (2GW), uropod 3 ramus length (Uro), 1st antenna length (1Ant), 2nd antenna length (2Ant). The presence or absence of calceoli and oostegite marginal setae was recorded.

2.5. Behaviour

To test whether the implanted animals developed male mating behaviour, experimental animals were placed with receptive females. Three receptive females (taken from precopula pairs to ensure receptivity, male to female size ratio 1.3 (Dunn et al., 2008) were placed in the pot with each male and the behaviour observed for 5 min.

2.6. Histology

The gonads of 25 androgenic gland and muscle transplant recipients were examined by microscopy of the freshly dissected tissue and of stained sections. Gonads were fixed in 4% PBS buffered formaldehyde, dehydrated, and embedded in paraffin wax. Sections (5 μm) were dehydrated and stained with Harris' haematoxylin and eosin, mounted in DPX and examined using a Zeiss Axiovert microscope.