This is a repository copy of *Horizontal transfer of parasitic sex ratio distorters between crustacean hosts*.

White Rose Research Online URL for this paper:
http://eprints.whiterose.ac.uk/1010/

Horizontal transfer of parasitic sex ratio distorters between crustacean hosts

A. M. DUNN[1]* *and* T. RIGAUD[2]

[1] *Department of Biology, University of Leeds, Leeds LS2 9JT, UK*
[2] *Université de Poitiers, Laboratoire de Génétique et Biologie des Populations de Crustacés, 40 Avenue du Recteur Pineau, 86022 Poitiers Cedex, France*

SUMMARY

Parasitic sex ratio distorters were artificially transferred within and between crustacean host species in order to study the effects of parasitism on host fitness and sex determination and to investigate parasite–host specificity. Implantation of *Nosema* sp. to uninfected strains of its *Gammarus duebeni* host resulted in an active parasite infection in the gonad of recipient females and subsequent transovarial parasite transmission. The young of artificially infected females were feminized by the parasite, demonstrating that *Nosema* sp. is a cause of sex ratio distortion in its host. In contrast, we were unable to cross-infect *Armadillidium vulgare* with the feminizing microsporidian from *G. duebeni* or to cross-infect *G. duebeni* with the feminizing bacterium *Wolbachia* sp. from *A. vulgare*.

Key words: artificial infection, *Nosema* sp., *Wolbachia* sp., sex ratio distorter, feminization.

INTRODUCTION

A number of feminizing microparasites have been found in terrestrial and aquatic Crustacea. These parasites are transovarially transmitted from mother to offspring and convert genetic males into phenotypic females (Hurst, 1993; Dunn *et al*. 1995; Rigaud, 1997). In the isopods *Armadillidium vulgare* and *Armadillidium nasatum*, feminization is induced by infection with a bacterium of the genus *Wolbachia* (Martin, Juchault & Legrand, 1973; Juchault & Legrand, 1979; Rousset *et al*. 1992). In amphipods, feminization results from protozoan infection. The haplosporidian *Paramartelia orchestiae* induces intersexuality in male *Orchestia gammarellus* (Ginsburger-Vogel, Carre-Lecuyer & Fried-Montaufier, 1980) and the microsporidian *Octosporea effeminans* causes female or intersex development of *Gammarus duebeni* (Bulnheim & Vavra, 1968; Bulnheim, 1977).

A microsporidian of the genus *Nosema* has recently been described in the host *G. duebeni* (Terry, Dunn & Smith, 1997). The parasite is localized in the gonad and is transovarially transmitted. It is ultrastructurally distinct from *O. effeminans* and also differs in that the parasite burden is relatively high and the infection is pathogenic, causing a reduction in growth and fecundity. Broods raised from infected mothers are strongly female biased, suggesting that

the parasite has a feminizing effect (Terry, unpublished results). However, there is no direct evidence for parasite-induced feminization.

In this study we artificially infected naive *G. duebeni* with *Nosema* sp. to investigate its possible feminizing effect and also investigated the parasite–host specificity of different feminizing parasites in Crustacea by carrying out cross-infections of *G. duebeni* with feminizing *Wolbachia* sp. from *A. vulgare* and of *A. vulgare* with *Nosema* sp.

MATERIALS AND METHODS

Gammarus duebeni were taken from 2 strains which originate from Millport, Isle of Cumbrae, UK: an uninfected strain and a strain infected with *Nosema* sp. Both were maintained in the laboratory in Leeds. *Armadillidium vulgare* were taken from strains maintained at the University of Poitiers: an uninfected strain from Nice (France) and a strain from Celles-sur-Belles infected with *Wolbachia* sp. (Juchault *et al*. 1994).

Parasite implantation

A suspension of microsporidian-infected ovarian material was prepared by dissecting the ovaries from 10 infected donor *G. duebeni* females and homogenizing the material in 100 μl of filtered brackish water (6·5‰, salinity equal to that of the host). A control suspension of uninfected material was prepared from 10 uninfected donors. Recipient

* Corresponding author: Department of Biology, University of Leeds, Leeds LS2 9JT. Tel:+0113 2332856. Fax:+0113 2332835. E-mail: a.dunn@leeds.ac.uk

(uninfected) *G. duebeni* were anaesthetized in carbonated water and a fine needle was used to make a hole in the carapace in segment 5. Similarly a hole was made in segment 5 of recipient *A. vulgare*. Using a Hamilton syringe with a fine glass needle, 1·5 μl of microsporidian infected ovarian suspension was implanted into the body cavity of 16 female and 11 male *G. duebeni* and into 10 female and 9 male *A. vulgare*. Ten female and 10 male *G. duebeni* controls were implanted with 1·5 μl of ovarian suspension from uninfected females. A suspension of *Wolbachia* sp. infected tissue was made by homogenizing the ovaries from 10 infected *A. vulgare* females in 100 μl of Ringer solution. Ten female and 9 male *G. duebeni* were injected with 1·5 μl of the homogenate as described above. As a control, 5 *A. vulgare* males were inoculated with the same extract.

After injection, *G. duebeni* were maintained in individual containers in 150 ml of brackish water. Rotted sycamore leaves and *Enteromorpha* (a marine alga) were provided as food. Animals were maintained for 6 moults and the weight of each individual was measured after each moult. To investigate possible feminization of males, the following external morphological characteristics were observed after each moult for control and implanted animals: penial papillae (for sperm deposition, present only in males); brood plates (found only in females); calceoli (sensory organs on antennae 2, found only in males). *A. vulgare* were maintained in boxes of moistened soil containing lime leaves and carrots as food. Animals were maintained for 9 moults and the external sexual characters measured after each moult.

Parasite transmission and host feminization

Possible vertical transmission of the parasites was investigated by breeding from implanted male and female *G. duebeni*. After 6 moults, an uninfected male was placed with each *G. duebeni* female and an uninfected female was placed with each male. *G. duebeni* females lay their eggs into the brood pouch where they are fertilized and brooded for 3–4 weeks. After mating, the early stage embryos (2–32 cell stage) were flushed from the brood pouch and screened for *Nosema* sp. or *Wolbachia* sp. as appropriate.

To screen for the microsporidian, the eggs were fixed in acetone, squashed and stained with DAPI (4,6-diamidino-2-phenyl-indole diluted 1/500 in 0·2 M NaH$_2$PO$_4$, pH 7·5), a fluorescent dye for DNA (Dunn & Hatcher, 1997). Slides were screened for parasites using a Zeiss Axioplan fluorescent microscope. The efficiency of transovarial parasite transmission was measured and the parasite burden was recorded for each embryo. Eggs were screened for *Wolbachia* sp. by PCR. Specific primers for *Wolbachia* 16S rDNA were used to detect the parasite (O'Neill *et al.* 1992), using the PCR

protocols described by Rousset *et al.* (1992). When the parasite was present, the specific primers resulted in the amplification of an rDNA partial sequence of 900 kb, visible on an agarose gel after staining with ethidium bromide. When no amplification was obtained with *Wolbachia*-specific primers, the quality of cytoplasmic DNA was checked by amplification of fragments of mitochondrial 16S rDNA with 16Sar and 16Sbr primers (Kocher *et al.* 1989).

To investigate possible parasite-induced feminization of the transovarially infected offspring, those females found to transmit the microsporidian were re-mated and a second brood was allowed to develop in the brood pouch. After release from the mother, juveniles were maintained for 6 months (3–5 animals/pot to minimize competition) and then sexed. Similarly, the sex ratio was measured for the young of control females implanted with uninfected ovary.

Infection in adults

At the end of the experiment, *G. duebeni* implanted with the microsporidian were fixed in formalin, embedded in paraffin wax, sectioned and stained with Geimsa. The internal morphology, in particular the morphology of the gonad and, in males, the androgen gland was examined and the parasite distribution and burden were recorded. The reproductive systems of 6 microsporidia-implanted female *A. vulgare* were dissected and the oocytes (*n* = 419) stained with DAPI and screened as described above. In addition, gonadal tissue, nerve cord and fat tissue from 4 males and 2 females was dissected, examined for gross changes in morphology, in particular of the androgen gland, and prepared for electron microscopy following standard procedures of fixation as described by Martin (1981). *Wolbachia* sp.-implanted *G. duebeni* and *A. vulgare* were screened for infection by PCR as described above.

RESULTS

Microsporidia implantation into G. duebeni *and* A. vulgare

Injection of the microsporidian caused an infection in 3 out of 10 surviving *G. duebeni* females but caused no change in their external or internal morphology. The weight of infected and control females did not change over time (F$_{5,40}$ = 0·37, *P* > 0·05). There was no significant difference in the weight of microsporidian-implanted females in comparison with controls implanted with uninfected extract (F$_{1,36}$ = 4·1, 0·05 < *P* < 0·1).

Parasite burden and distribution were examined in detail in the infected females by light microscopy. The infection was concentrated in the gonadal tissue, although small numbers of parasites were observed

Table 1. *Parasite infection and burden in female* Gammarus duebeni *6 moults after implantation with microsporidian infected ovary*

(The mean proportion of cells infected (data arc sine transformed) and mean parasite burden±s.e. are given for follicle cells and for stage 3 oocytes of implanted females. Transmission efficiency to embryos was calculated as the proportion of embryos which inherited the parasite.)

	N	Proportion infected	Burden/cell
Follicle cells	449	0·16±0·05	2·11±0·26
Oocytes	47	0·89±0·03	73·36±18·50
		Transmission efficiency	Burden/embryo
Embryos	40	0·86±0·04	147·9±37·90

Table 2. *The effect of microsporidian implantation on female fecundity, brood survival and brood sex ratio*

(Fecundity was measured as the mean number of eggs/brood±s.e. Brood survival is given as the proportion of young surviving to sexual maturity, sex ratio as (number of males/total number of offspring).)

	Female implanted with microsporidia	Female implanted with uninfected extract	Test	
Fecundity	16·5±2·9	15·7±0·81	t-test,	$P > 0·05$
Survival of young	0·59, $n = 39$	0·55, $n = 60$	χ^2 1 d.f. = 0·13,	$P > 0·05$
Sex ratio	0·39, $n = 23$	0·66, $n = 33$	χ^2 1 d.f. = 4·6,	$P < 0·05$

in the adjacent muscle of 2 of the females. No parasites were observed in the blood cells, fat tissues or nerve cord of the host. Examination of the gonad revealed vegetative stages of the parasite in developing and mature oocytes and both vegetative and spore stages in the follicle cells (Table 1). Parasite distribution in the gonad was heterogeneous and parasites were concentrated in the region of segments 4–6, close to the site of implantation, although a few were found in the anterior region of the gonad.

The infected females transmitted the parasite transovarially to their young with a high efficiency (Table 1). Examination of 40 early stage, DAPI-stained embryos from parasite-implanted females revealed binucleate parasites in the perinuclear cytoplasm of the host cells. Broods from control-implanted females were all uninfected. The infection caused no reduction in fecundity. The mean number of eggs per brood was similar for infected and control females (Table 2). Survival of the young to sexual maturity was also unaffected by the parasite. However, the parasite had a feminizing effect on the young. The sex ratio of young from *Nosema* sp.-implanted females was female biased and differed significantly from that of controls (Table 2).

None of the 8 surviving males implanted with the parasite became infected. Light microscopical examination revealed no evidence of microsporidia in the gonadal tissue or in any other tissue and there was no evidence for vertical transmission to the offspring (110 young from 8 males were all uninfected). The external and internal morphology of these males did not differ from that of control males.

None of the *A. vulgare* implanted with the microsporidian became infected. DAPI staining provided no evidence of microsporidian DNA in the cytoplasm of *A. vulgare* oocytes. In addition, electron microscopical examination of gonads, nerve cord and fat tissues provided no evidence of the microsporidian. There was no evidence of feminization or other phenotypic change in the 10 implanted males.

Wolbachia *sp. implantation into* G. duebeni

None of the *G. duebeni* implanted with *Wolbachia* sp. became infected. Seven surviving females and 8 males were uninfected. In addition, 13 broods (191 eggs) taken from these individuals were uninfected. The PCR reaction with *Wolbachia*-specific primers was always negative, whereas an amplification product was obtained using mitochondrial primers, indicating that DNA extraction had been successful. The implant caused no changes in the morphology of recipient *G. duebeni*.

All 5 *A. vulgare* males implanted with the same extract developed an intersex phenotype (oviducts and female genital apertures differentiated after 3

moults and the androgen gland hypertrophied) and the PCR tests showed that all were infected. The failure of *Wolbachia* sp. inoculation in *G. duebeni* was not, therefore, due to an inactive bacterial extract.

DISCUSSION

We were unable to cross-infect *A. vulgare* with feminizing microsporidia from *G. duebeni* or to cross-infect *G. duebeni* with feminizing bacteria from *A. vulgare*. The failure of interspecific parasite transfer may be due to the inability of parasites to live in a different habitat (e.g. the osmotic pressure differs between terrestrial and marine crustaceans; Martin (1981)), or may result from parasite–host specificity.

However, the intraspecific transfer of microsporidia to *G. duebeni* was successful. Six moults after the implantation, active parasitic infection occurred in the gonad of implanted female hosts, as has been reported in naturally infected females (Terry *et al.* 1997). The increase in parasite numbers following infection provided further evidence for parasite multiplication. Furthermore, the parasite was transovarially transmitted to the offspring, multiplied in the early stage embryos and caused feminization of the young. This is the first demonstration of transovarial transmission and host feminization following experimental transfer of the microsporidian. The experiment, at least partially, fulfils Koch's postulates: the parasite was removed from an infected individual and placed into an uninfected host, resulting in female biased sex ratios (Hurst, 1993). In contrast, transfer of uninfected gonad from control females had no effect on either the sexual morphology of recipient animals or the sex determination of their offspring, indicating that feminization could not be attributed to the gonadal implant but was a result of parasitism.

Parasite burden in the oocytes of parasite-implanted females was lower than that reported for naturally infected females ($161 \pm 40 \cdot 5$, Dunn, unpublished results). However, the burden was considerably higher than the initial dose implanted (each recipient animal received approximately 15 % of the parasite burden of a naturally infected donor), providing evidence of parasite replication within the new host. The infection was concentrated in the ovary near the site of implantation and there was no evidence for infection of blood cells, suggesting that there was limited movement of the parasite following transmission. This is in accord with studies of natural *Nosema* sp. infections in *G. duebeni* which show restricted parasite distribution and movement (Terry *et al.* 1997), but contrasts with the *A. vulgare/Wolbachia* sp. system, where parasites may infect blood cells and be transmitted to other tissues (Rigaud *et al.* 1991; Rigaud & Juchault, 1995).

The efficiency of transovarial transmission was similar to that found in natural infections (91 %, Terry unpublished results) and parasite replication occurred in the embryos, although burden was lower than that found in embryos from naturally infected mothers ($288 \cdot 3 \pm 11 \cdot 5$). The efficiency of feminization was also lower in the young of artificially infected mothers than in naturally infected young (mean proportion of males $= 0 \cdot 21$). These data suggest that successful parasite-induced feminization of the host may be dosage dependent.

The infection of male hosts was unsuccessful: we found no evidence for parasitic infection or for changes in host morphology over 6 moults. This is in contrast to artificial infection experiments in *Armadillidium* which demonstrated a feminizing effect of *Wolbachia* sp. on adult males as well as sex ratio distortion in the young of implanted females (Legrand & Juchault, 1970; Martin *et al.* 1973; Rigaud & Juchault, 1995). Bulnheim (1977) also demonstrated partial feminization of male *G. duebeni* after implantation with the microsporidian *O. effeminans* but did not carry out infection of female hosts, nor investigate parasite transmission and its effect on the development of the young.

Successful artificial infection of uninfected *G. duebeni* hosts raises the possibility of horizontal transmission in the field. A number of vertically transmitted microsporidia also have a horizontal transmission stage following parasite-induced host death (e.g. Avery & Undeen, 1990; Koella & Agnew, 1997). Rigaud & Juchault (1995) demonstrated horizontal transmission of *Wolbachia* sp. after blood contact between injured *A. vulgare* hosts. However, *Nosema* sp. is restricted to the gonad of infected hosts and does not cause host death (Terry *et al.* 1997). Therefore, horizontal transmission of this parasite is unlikely in the wild.

REFERENCES

AVERY, S. W. & UNDEEN, A. H. (1990). Horizontal transmission of *Parathelohania anophelis* to the copepod, *Microcyclops varicans*, and the mosquito, *Anopheles quadrimaculatus*. *Journal of Invertebrate Pathology* **56**, 98–105.

BULNHEIM, H. P. (1977). Geschlechtsumstimmung bei *Gammarus duebeni* (Crustaceana, Amphipoda) unter dem Einfluss hormonaler und parasitärer Faktoren. *Biologisches Zeitblatt* **96**, 61–78.

BULNHEIM, H. P. & VAVRA, J. (1968). Infection by the microsporidia *Octosporea effeminans* sp. n., and its sex determining influence in the amphipod *Gammarus duebeni*. *Journal of Parasitology* **54**, 241–248.

DUNN, A. M., HATCHER, M. J., TERRY, R. S. & TOFTS, C. (1995). Evolutionary ecology of vertically transmitted parasites: strategies of transovarial transmission of a microsporidian sex ratio distorter in *Gammarus duebeni*. *Parasitology* **111**, S91–S110.

DUNN, A. M. & HATCHER, M. J. (1997). Prevalence, transmission and intensity of infection by a microsporidian sex ratio distorter in natural *Gammarus duebeni* populations. *Parasitology* **114**, 231–236.

GINSBURGER-VOGEL, T., CARRE-LECUYER, M. C. & FRIED-MONTAUFIER, M. C. (1980). Transmission expérimentale de la thélygenie liee a l'intersexualité chez *Orchestia gammarellus* (Pallas); analyse des génotypes sexuels dans la descendance des femelles normales transformées en femelles thélygenes. *Archives de Zoologie Expérimentale et Générale* **122**, 261–270.

HURST, L. D. (1993). The incidences, mechanisms and evolution of cytoplasmic sex ratio distorters in animals. *Biological Reviews* **68**, 121–193.

JUCHAULT, P. & LEGRAND, J. J. (1979). Analyse génétique et physiologique de la détermination du sexe dans une population du crustacé isopode oniscoide *Armadillidium nasatum* (Budde-Lund). *Archives de Zoologie Expérimentale et Générale* **120**, 24–43.

JUCHAULT, P., FRELON, M., BOUCHON, D. & RIGAUD, T. (1994). New evidence for feminizing bacteria in terrestrial isopods: evolutionary implications. *Comptes Rendus de l'Académie des Sciences de Paris* **317**, 225–230.

KOCHER, T. D., THOMAS, W. K., MEYER, A., EDWARDS, S. V., PAABO, S., VILLABLANCA, F. X. & WILSON, A. C. (1989). Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences, USA* **89**, 6196–6200.

KOELLA, J. C. & AGNEW, P. (1997). Blood-feeding success of the mosquito *Aedes aegypti* depends on the transmission route of its parasite *Edhazardia aedis*. *Oikos* **78**, 311–316.

LEGRAND, J. J. & JUCHAULT, P. (1970). Modification expérimentale de la proportion des sexes chez les crustacés isopodes terrestres: induction de la thélygénie chez *Armadillidium vulgare* (Latr.). *Comptes Rendus de l'Académie des Sciences de Paris* **270**, 706–708.

MARTIN, G. (1981). Contribution à l'étude cytologique et fonctionelle des systèmes de neurosécrétions des crustacés isopodes. Thesis d'Etat, Université de Poitiers, France.

MARTIN, G., JUCHAULT, P. & LEGRAND, J. J. (1973). Mise en évidence d'un micro-organisme intracytoplasmique symbiote de l'Oniscoïde *Armadillidium vulgare* L., dont la présence accompagne l'intersexualité ou la féminisation totale des mâles génétiques de la lignée thélygène. *Comptes Rendus de l'Académie des Sciences de Paris* **276**, 2313–2316.

O'NEILL, S. L., GIORDANO, R., COLBERT, A. M. E., KARR, T. L. & ROBERTSON, H. M. (1992). 16S rDNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proceedings of the National Academy of Sciences, USA* **89**, 2699–2702.

RIGAUD, T. (1997). Inherited microorganisms and sex determination of arthropod hosts. In *Influential Passengers. Inherited Microorganisms and Arthropod Reproduction*, (ed. O'Neill, S. L., Hofmann, A. A. & Werren, J. H.), pp. 81–101. Oxford University Press, Oxford.

RIGAUD, T., SOUTY-GROSSET, C., RAIMOND, R., MOCQUARD, J. P. & JUCHAULT, P. (1991). Feminizing endocytobiosis in the terrestrial crustacean *Armadillidium vulgare* Latr. (Isopoda): Recent acquisitions. *Endocytobiosis and Cell Research* **7**, 259–273.

RIGAUD, T. & JUCHAULT, P. (1995). Success and failure of horizontal transfers of feminizing *Wolbachia* endosymbionts in woodlice. *Journal of Evolutionary Biology* **8**, 249–255.

ROUSSET, F., BOUCHON, D., PINTUREAU, B., JUCHAULT, P. & SOLIGNAC, M. (1992). *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. *Proceedings of the Royal Society of London, Series B* **250**, 91–98.

TERRY, R. S., DUNN, A. M. & SMITH, J. E. (1997). Cellular distribution of a feminizing microsporidian infecting *Gammarus duebeni*. *Parasitology* **115**, 157–163.