

# PARASITE INFECTION STATUS OF *POTAMOPYRGUS ANTIPODARUM*

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## ♦ ABSTRACT

Outside its native range, the invasive species *Potamopyrgus antipodarum* (New Zealand Mud snail; NZMS) has seldom been reported to harbor parasites. To test this intriguing observation, 7 sites along the Snake River and Polecat Creek in the Grand Teton National Park/John D Rockefeller Memorial Parkway area (Wyoming, USA) were surveyed for NZMS, native aquatic snails and parasites: digenean trematodes (specialist parasites of snails) and nematormorphs (horse hair worms, generalist parasites of aquatic invertebrates), in July 2005. Non-destructive examination of 96 native snails at each site revealed abundant presence of digeneans (parasites that specialize in snails) at 6 sites. Within 2 hours, up to 12.4% of lymnaeid snails shed furcocercous cercaria or xiphidiocercaria, and up to 50% of physid snails released furcocercous cercaria or echinostome cercaria. A total of ~900 NZMS (collected from 5 of the sites) were investigated for infection with digeneans by overnight shedding. One NZMS yielded amphistome-like metacercariae that had encysted in the observation tray. The dissection of 150 ethanol-fixed NZMS (30/site) revealed several types of internal metacercarial stages of digeneans in 5 snails (all from Polecat Creek). PCR-based assays targeting parasite

rDNA sequences in DNA extracted from pools of ~150 snails each also indicated associations between digenean parasites and NZMS. All parasites were detected in NZMS from Polecat Creek. NZMS were not observed to harbor nematormorph parasites with any of the techniques used. In conclusion, the sites tested contained multiple native snail-digenean parasite associations, and the capacity of invasive NZMS outside the native range to serve as first and second intermediate host for digenean parasites may have been underestimated previously.

## ♦ INTRODUCTION

*Potamopyrgus antipodarum*, the New Zealand Mud snail (NZMS) is an invasive species that has spread far beyond its native range of New Zealand. The success of this invader, also present in the Greater Yellowstone Ecosystem (e.g. Kerans et al., 2005) has been considered in light of the capacity for parthenogenic (asexual) reproduction, tolerance for a wide range of abiotic conditions, and the (virtual) absence of parasitism in introduced ranges (Gerard et al., 2003). Generally, invasive organisms incur fewer (types of) parasites in introduced ranges as compared to the native range. This reduction likely

benefits the fitness of invasive species (Torchin et al., 2003).

Invasive *P. antipodarum* may not have carried parasites, or their native parasites did not establish in the introduced range. However, newly colonized habitats contain a variety of parasitic organisms. Likely, these include digenean trematode parasites that specialize in using snails as intermediate host. Many digeneans exhibit specificity to particular snail species in which they can reproduce, but penetration of non-host snails also occurs. Both incompatible and compatible digeneans are a burden to snails. In the first case, snails mount a defense response to kill and destroy an incompatible invader. Over time, with repeated encounters, some parasites may adapt to previously incompatible snail species. In fact, digeneans have acquired new hosts across large phylogenetic distances (Brant and Loker, 2005), and a novel host-parasite association involving *P. antipodarum* and a sanguinicolid trematode was described from Europe (Gerard and Le Lannic, 2003). In the second case, compatible digeneans avoid or evade the host defenses and modify the physiology of the snail to benefit the asexual multiplication of intramolluscan parasite larvae. Some digeneans exert parasitic castration, severely impact host fitness by causing a snail to cease reproduction (Sorenson and Minchella, 2001).

Certain groups of digeneans provide additional risk of infection to snails. The cercaria from echinostome and plagioclid parasites emerge from the first intermediate host snail to penetrate to encapsulate as metacercaria within a second intermediate host, awaiting predation to reach the next host (e.g. Galaktionov and Dobrovolskij). Metacercariae evoke little host response and can persist in a variety of organisms, including invertebrates and vertebrates. Both native and invasive snails may be infected indiscriminately, at the expense of providing space, and possibly other resources to digenean metacercariae. Other generalist parasites may similarly infect any snail that they encounter. For instance, larval stages of nematomorphs (horse hair worms; parasites of arthropods) use aquatic invertebrates as transport hosts. One field study showed that close to 40% of physid snails harbored one or more nematomorph cysts (Hanelt et al., 2001). Although there was no clear pathology associated with the cysts, infected snails cause parasites to persist such that they may ultimately infect and impact their true host.

Thus, it is remarkable that invasive *P. antipodarum* are rarely reported to harbor parasites.

However, detection of intramolluscan digenean and nematomorph parasites with traditional methods (shedding and dissection) is labor intensive and may yield false negative results. Detection rates may increase when using PCR-based methods to detect parasite-specific sequences in DNA extracted from (pools of) snails (Rognlie et al., 1994; Hanelt et al., 1997; Hamburger et al., 2004). Previous phylogenetic studies provide (nuclear ribosomal gene) sequences that can be targeted for detection by PCR of digeneans (Olsen et al., 2003), nematomorphs (Bleidorn et al., 2002), and NZMS (*Cytochrome B*, Neiman et al., 2005).

This study applied traditional and PCR-based methods to evaluate the presence of parasites of native snails, and the infection status of invasive *P. antipodarum* at several sites in the Grand Teton National Park/ John D Rockefeller Memorial Parkway area (Wyoming, USA).

## ◆ METHODS AND MATERIALS

### Collection Sites

Several sites were located north of Jackson Lake along the Snake River and Polecat Creek. Also sampled were Oxbow Bend and Jackson Lake Dam, downstream of Jackson Lake. See table 1 for GPS coordinates of the seven locations.

### Native Snails

Ninety-six native snails (independent of species) were collected from each field site and placed individually in wells of 24 well tissue culture plates containing 3 ml of water, and identified to genus level. The snails were kept in a shaded spot along the bank of the collection site for two hours to allow shedding of digenean parasites, and then returned to the field. In the laboratory, the water in each well was inspected using a dissection scope for digenean parasites. The parasites observed were collected, fixed in 100% ethanol, examined microscopically and destructively processed for DNA extraction.

### NZMS

New Zealand mud snails were collected during a 2 hour time interval from each of the sites. Two NZMS were placed in each of 96 wells (3 ml of water) and examined the next day for shedding of parasites. Any parasites and the snails from which these originated were fixed in 100% EtOH. Other

New Zealand mud snails were fixed in 100% EtOH and stored at -30C. For dissection, snails were rehydrated to 25% ethanol. Individual snails (n=30 from each site) were crushed between glass slides and the tissues were examined microscopically for both digenean and nematomorph parasites.

### DNA Extractions and PCR.

DNA was extracted directly from pools of live snails, or of ethanol-fixed snails (rinsed twice with water to remove ethanol), using a CTAB-based method (Winnepenninckx et al., 1993). After microscopical confirmation of species, 150 NZMS from each site were crushed in a DNA extraction buffer containing Hexadecyltrimethylammonium bromide (CTAB), SDS, EDTA and proteinase K. Following incubation at 60C (1 h), the sample was chloroform-extracted, DNA was precipitated from the aqueous phase with isopropanol, rinsed (76% methanol, 10 mM ammonium acetate) and dissolved in 200  $\mu$ l milliQ water. The black color of the resulting samples precluded spectrophotometric measurement of DNA concentration, and 0.5  $\mu$ l volumes were used as template for PCR reaction.

### PCR experiments

PCR reactions were performed to amplify DNA sequences specific for NZMS snails (*Cytochrome C*, positive control for the quality of the sample), digenean parasites (18S and 28S) and *Nematomorpha* (18S). Primers (all shown 5'-3') for a 400 basepair fragment of *Cytochrome C* of *P. antipodarum* were designed from an alignment of 45 haplotypes (Neiman et al., 2005); PaCBF (AAR GTA AAG AAT CGG GTT AAA G) and PaCBR (CAG GAC TAT TTT TAG CAA TGC). PCR primers for detection of digeneia were adopted from Olsen et al. (2003). Digenean 28S rDNA (also designated as large subunit; LSU): LSU-5 (TAG GTC GAC CCG CTG AAY TTA AGC A) and 1500R (GCT ATC CTG AGG GAA ACT TCG). Anticipated amplicons will be about 1400 bps; Also 12S rDNA (small subunit;SSU; about 1800 nt): Worm-A (A/GCG AAT GGC TCA TTA AAT CAG') and Worm-B (ACG GAA ACC TTG TTA CGA CT) with alternative Worm-B (C TTG TTA CGA CTT TAC TTC C). An alignment of 18S sequences of from nematomorpha (Bleidorm et al., 2002) and hydrobiid snails (the gastropod family that includes *P. antipodarum*, Wilke et al., 2001) yielded primers to amplify the 18S gene from the parasite with minimal chance of cross-reactivity with snail sequences. N18F (5'-CCAT GCA TGT VTM AGT ATR AAC-3') and N18R (5'-CAT TCC AAT TAC AGG GTC TC -3').

PCR reactions consisted of 2.5U of DNA polymerase AmpliTaq Gold (Applied Biosystems), 200mM of each dNTP, 0.5  $\mu$ molar of both forward and reverse primers, 1  $\mu$ liter of DNA template and 4 mM of MgCl<sub>2</sub>. The cycling profile was 10' 95C, 40 cycles of 1'95C, 30" at annealing temperature (between 50 and 65C, depending on primers), 1' 72C, final extension 7' 72C (T-gradient thermocycler, Biometra). Ethidiumbromide-stained PCR amplicons were visualized by UV transillumination following electrophoresis on 1.0% agarose gels (45', 85V).

## ◆ RESULTS

### Native snails and Parasites

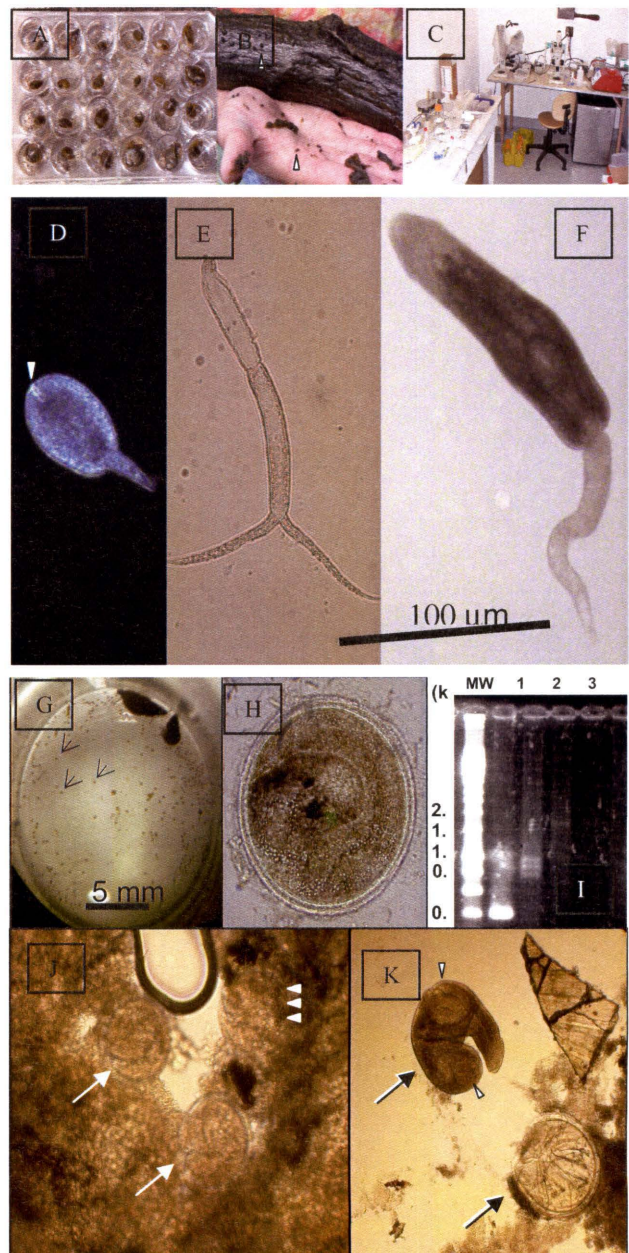


FIGURE LEGEND. **A)** Native snails collected from the field were kept in 3 ml wells of a tissue culture plate to shed parasites. After two hours, these snails were returned to the collection site. **B)** New Zealand Mud Snails were abundantly present in Polecat Creek. Many snails were on this log and easily transferred to hands of snail collectors (arrowheads). **C)** The lab space in the boat house of the UW/NPS research station, set up for examination of parasites, DNA extraction, PCR and DNA gel-electrophoresis. **D-F)** Examples of the main types of digenean trematode cercariae recovered from native aquatic snails, scale bar applies to all 3 figures; **D)** Xiphidiocercaria (phase contrast) collected from a lymnaeid snail (site 1). Note the characteristic short tail and the lancet (arrow head). **E)** Furcocercous cercaria with forked tail and showing two eye spots, recovered from a physid snail, site 5. **F)** Echinostomatid cercaria (note bifurcated intestine) from a physid snail, site 6. **G)** Overnight shedding of two NZMS (site 5), yielded multiple digenean parasite metacercaria (some indicated by arrows) that had encysted in the well. **H)** Close up of a (likely amphistome) metacercaria from the previous photo. Eye spots and a smooth double cyst wall are visible. **I)** Results from PCR with DNA extracted from pooled NZMS collected from site 6. Lane 1: 400 bp PCR amplicon from *cytochrome B* of NZMS (positive control); lane 2: 28S rDNA digeneans, multiple amplicons smaller than the expected product of ~1400nt may be diagnostic for parasites, but this will be confirmed by sequencing.; lane 3; digenean 18S rDNA amplicon is of expected size at ~1800nt; lane 4; 18S rDNA digeneans (alternative primer combo). A weak amplicon of the expected size (~1800nt) is visible on the actual gel, lane 5: 18S rDNA from nematomorphs, expected size ~420nt, no amplicon visible on gel. MW is molecular weight marker, size indicated in kilobases (kb). **J)** The tissue squash of one NZMS (site 6) revealed 3 round echinostome-type metacercariae (white arrows), displaying typical calcareous corpuscles (arrowheads). **K)** Non-identified metacercarial larva expressed from its cyst (black arrows) likely during the squashing of the tissues of one NZMS from site 6. This intramolluscan parasite displays two suckers, typical for digenean trematodes (arrowheads). The triangular shape is a small fragment NZMS shell.

Native aquatic snails were present in such numbers that it required little effort to sample 96 specimens at any of the collection sites. Several (non-determined) snail species of the bassomatophoran gastropod families Lymnaeidae and Physidae were present at all 7 sites. Planorbidae were represented by *Gyraulus* sp. (especially numerous at site 2 downstream of Jackson Lake Dam, but also observed at sites 4 and 6) and *Planorbella* sp. (only site 6, north of Jackson Lake). An amphibious stylomatophoran (*Succinea* sp.) was encountered at site 4 (Oxbow bend). Although not specifically

documented, diverse animal life arthropods, annelids, fish, amphibians, reptiles, birds and mammals, potentially contributing to parasite life cycles, was observed during site visits. As summarized in table 1, the on-site shedding experiments (figure 1A), yielded a variety of digenean cercariae released from lymnaid and physid snails. No parasites were recovered from planorbid snails or from *Succinea*. The snails collected at site 2 did not shed any parasites. The parasites collected encompassed three main morphological types: xiphidiocercaria with or without eye spots (only from lymnaids); furcocercous cercaria with or without eye spots (lymnaeids and physids); and echinostomatid cercaria (physids only), see figures 1 D, E, F. No double infections were evident, individual snails shed only one morphological type of cercaria.

### NZMS and Parasites

*Potamopyrgus antipodarum* was not observed downstream of Jackson Lake Dam or at Oxbow Bend. NZMS were present however, at the 5 collection sites along the north bank of the Snake River and at high numbers in Polecat Creek (Figure 1B). Snails collected during a 2 hour interval were transported to the lab for analysis (Figure 1 C). Overnight shedding of 192 live NZMS from each site (1344 snails total) resulted in one observation of parasites: encysted metacercariae in a well that contained NZMS from site 5. These cysts can only result from cercariae, shed from the NZMS. The morphology of the cysts is consistent with an amphistome digenean (Fig 1 G,H). PCR experiments indicated that DNA isolated from pooled NZMS from site 6 also contained digenean parasites (Figure I). Finally, dissection and microscopical examination revealed intramolluscan metacercarial cysts (one likely echinostome, one unknown) in 5 NZMS from site 6 (Figure 1 J, K), and one snail contained metacercarial cysts of both types. In summary, 6 individual NZMS from sites 5 and 6 (both along Polecat Creek) harbored parasites. This represents 6/(1344 + 150) or 4.01% of all NZMS that were examined individually either by shedding or dissection. The PCR results suggest that additional snails from site 6 carried digenean parasites, but numbers of infected snails can not be derived from this pooled sample.

### ◆ DISCUSSION

The cataloguing of native snails and their digenean parasites in the study area ascertained that the environment in the study area was supportive for

maintaining (digenean) parasites-snail host associations in general. Nematomorph parasites were not observed and are not included further in this discussion. The unique failure to detect parasites from site 2 (Jackson Lake Dam) may reflect conditions that are less supportive of parasites, such as fast flowing and cold water. Also detection of parasites by shedding snails, especially for short time intervals, is error prone due to timing issues. Intramolluscan parasites may not yet have developed to the cercarial stage, especially if juvenile snails were collected. Snails may have already shed all available cercariae; parasite emergence can be ruled by circadian rhythms (for discussion see Hanelt et al., 1997; Hamburger et al., 2004). Whatever the cause for the failure to detect parasites, digeneans were again present at Oxbow Bend, downstream of this area.

Regardless, NZMS was not observed at these sites (2 and 4), south of Jackson Lake. The NZMS were present in the Snake River and Polecat Creek, north of Jackson Lake. These sites harbor multiple native snail-parasite associations and the NZMS are likely to repeatedly encounter potential parasites. Under these conditions, the incidence of digenean parasite infection in NZMS was about 4% of individually examined NZMS. This falls in the range of infection frequency of native snails (Table 1). However, the data for the native snails is based on a 2 h shedding assay that likely underestimates parasite incidence. Far greater scrutiny was applied to determine infection in NZMS, using an overnight shedding assay, greater numbers of snails, and multiple detection methods. The PCR-based detection of parasites proved feasible at the station, facilitated by setting up a lab with minimal equipment brought from off-site for DNA extraction, PCR and horizontal agarose gel-electrophoresis.

The combination of different techniques proved valuable. PCR analysis of pooled samples may show presence of parasites (plus/minus result) and although it does not allow distinction of the type of snail-parasite association, or the number of infected snails, PCR takes less effort to study large numbers of snails compared to shedding or dissection methods. Shedding of cercaria (resulting in metacercariae) shows that NZMS serves as a first host for a digenean parasite and helps propagate the life cycle of the parasite by releasing infective parasite larvae. Clearly, if this particular parasite can (adapt to) routinely develop in NZMS, the sheer numbers of potential snail hosts may have considerable impact. Either elevated transmission of the parasite will adversely affect the next host species

in the life cycle, the parasite infection may prove deleterious to NZMS (consider Sorenson and Minchella, 2001) affording some level of control of the invasive nature of NZMS, or both. The intramolluscan metacercaria evidenced by dissection of NZMS (the third and final technique used) may also have different biological significance. These metacercariae resulted from random infection by cercariae that developed in another snail, and NZMS function as a transport host only. If predators or scavengers ingest infected snails, NZMS may again contribute to propagation of a parasite. However, if infected NZMS are not eaten these internal parasites ultimately die with the dead-end snail host. In the latter case, high numbers of NZMS may actually reduce the transmission of some digeneans by sequestering the infective stages of certain digenean parasites away from the environment. Further study will have to be done to clarify how these interactions between NZMS and digenean parasites play out. Finally, the parasite samples from native snails and from NZMS have been destructively sampled for DNA, and if successful, the obtained sequence will be used to identify parasites by comparison with published sequences for known parasites and the results of these efforts will be published elsewhere.

In conclusion, using several complementary methods, digenean parasite infection was documented from invasive *Potamopyrgus antipodarum* in an introduced range. This is one of only few such reports in the literature. Parasite incidence was low and limited to NZMS collected from Polecat Creek. It remains to be determined whether rare parasite infection reflects either highly efficient internal defenses of NZMS, or the recent acquisition of NZMS as a novel snail first intermediate host by an endemic digenean parasite. The monitoring of NZMS as first and as second intermediate host for digenean trematodes may clarify the implications of such interactions for the Grand Teton National Park/John D Rockefeller Memorial Parkway area.

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