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Histological analysis of thelohaniasis in white-clawed crayfish *Austropotamobius pallipes* complex

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ABSTRACT

Key-words: Thelohania contejeani, Austropotamobius pallipes *complex, white-clawed crayfish, north-eastern Italy, histology*

From 2004 to 2006, a parasitological survey aimed at the detection of the microsporidian parasite *Thelohania contejeani* Henneguy was carried out on 177 wild white-clawed crayfish (*Austropotamobius pallipes* complex) captured in six streams and rivers of the province of Belluno in north-eastern Italy. Microscopical examination of the skeletal muscles, and histological analysis applying different histochemical stains to full transverse and sagittal sections of the cephalothorax and abdomen were carried out. Transmission electron microscopy (TEM) was also conducted on the parasites recovered during the survey. Out of 177 crayfish examined, *Thelohania contejeani* (Microsporidia, Thelohaniidae) was present in only one crayfish from the Vena d'oro creek. The parasite was detected in the skeletal muscles in several developmental stages, including mature spores, which represented the most common stage recovered. Sporophorous vesicles were also present. Histological examination revealed that the fibres of the skeletal, cardiac and intestinal muscles were filled with spores. Melanin infiltrations were focally present in the infected striated muscles. The gill phagocytic nephrocytes were engulfed by small masses of spores. Among the staining techniques applied, Crossman's trichrome stain represented the most effective method of detecting *T. contejeani*.

RÉSUMÉ

Analyse histologique de thelohaniasis dans le complexe spécifique des écrevisses à pattes blanches *Austropotamobius pallipes*

Mots-clés : Thelohania contejeani,

De 2004 à 2006, un suivi parasitologique recherchant la détection du parasite microsporidien *Thelohania contejeani* Henneguy a été effectué sur 177 écrevisses à pattes blanches sauvages (complexe *Austropotamobius pallipes*) capturées dans six rivières et torrents de la province de Belluno au Nord-est de l'Italie.

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complexe Austropotamobius pallipes, *écrevisse à pattes blanches, Italie du Nord-est, histologie*

L'examen microscopique des muscles squelettiques, et des analyses histologiques faites avec différents fixateurs histologiques sur des coupes transverses et sagittales du céphalothorax et de l'abdomen ont été réalisées. Des observations au microscope électronique à transmission (TEM) ont été également conduites sur les parasites trouvés pendant ce suivi. Des 177 écrevisses examinées, *Thelohania contejeani* (Microsporidia, Thelohaniidae) était présent sur une seule écrevisse du ruisseau Vena d'oro. Le parasite a été détecté dans les muscles squelettiques à différents stades de développement, dont des spores à maturité, qui sont le stade le plus représenté. Des vésicules sporophores sont aussi présentes. L'examen histologique révèle que les fibres des muscles squelettiques, cardiaques et intestinaux sont remplies de spores. Des infiltrations de mélanine sont ponctuellement présentes dans les muscles striés infectés. Les néphrocytes phagocytaires branchiaux sont envahis par de petites masses de spores. Parmi les techniques employées, la fixation trichrome de Crossman est la méthode la plus efficace pour détecter *T. contejeani*.

INTRODUCTION

Microsporidia are unicellular organisms belonging to the phylum Microsporidia (Balbiani, 1882) Weiser, 1977. The systematic classification and taxonomy of microsporidia has changed over time and is currently still under debate (Wittner, 1999). Initially thought to be protozoans in the kingdom Protista, new molecular biology data has suggested their affinity for fungi (Müller, 1997; Franzen and Müller, 1999). Recent analyses have consistently confirmed this hypothesis (Keeling, 2003; Keeling and Slamovits, 2004; Fischer and Palmer, 2005; Lee *et al.*, 2008).

Microsporidia constitute a group of extremely specialised obligate intracellular spore-producing parasites. The infectious stage is represented by the spore, which is highly resistant, persisting in the environment for long periods of time.

In Europe, during the XIX century, these parasites were recognised for the first time when *Nosema bombycis* was isolated as the agent of an epidemic disease in silkworms (Nägeli, 1857; Pasteur, 1870).

In more recent years, microsporidian species have been reported to infect nearly all the invertebrate phyla, including unicellular organisms, as well as all classes of vertebrates from terrestrial and aquatic environments (Larsson, 1999).

There are currently over 1200 species identified from 143 genera. At least 14 species from 8 genera are known to infect humans (CDC, 2004). Microsporidia are serious pathogens of several decapod crustaceans including crabs, prawns and freshwater crayfish (Sprague and Couch, 1971; Sindermann, 1990). Among the microsporidian genera infecting species of freshwater crayfish, *Thelohania* is a serious pathogen in many countries (Skurdal *et al.*, 1990; Diéguez-Uribeondo *et al.*, 1997; Edgerton *et al.*, 2002; Evans and Edgerton, 2002).

Henneguy and Thélohan (1892) were the first to describe *Thelohania contejeani* Henneguy 1892, which was isolated from the noble crayfish *Astacus fluviatilis* (= *Astacus astacus*) by the French Department of Doubs, where it was identified as the causative agent of a disease inflicting heavy losses in crayfish populations.

Although the host range of most microsporidian species is usually restricted, *Thelohania contejeani* has been reported as a cause of infection in distantly-related freshwater crayfish found in Europe, including *Austropotamobius pallipes* (Vey and Vago, 1973), *Astacus astacus* (Skurdal *et al.*, 1988, 1990), *Pacifastacus leniusculus* (Dunn *et al.*, 2009), *Astacus leptodactylus* and *Cambarus affinis* (*Orconectes limosus*) (Krucinska and Simon, 1968), and in North America in *Orconectes virilis* (France and Graham, 1985) and *Pacifastacus leniusculus* (McGriff and Modin, 1983). *Thelohania contejeani* has also been isolated in Australasia from *Paranephrops zealandicus* (Quilter, 1976).

Thelohaniasis in crayfish is also known as “porcelain disease”, due to the noticeable whitening of the skeletal muscle as a consequence of the microsporidian infection.

The white-clawed crayfish (*Austropotamobius pallipes* complex) is a protected native European species which is considered to be an endangered aquatic organism. As they are important to the biodiversity and richness of European freshwater ecosystems, the monitoring of crayfish health is an essential conservation initiative.

A previous study in Italy has focused on the occurrence and distribution of *Thelohania contejeani* in several white-clawed crayfish populations inhabiting the Liguria region in the northwest (Mori and Salvidio, 2000).

The main purpose of this study was to assess the presence of thelohaniasis in populations of white-clawed crayfish from streams and rivers of the province of Belluno (north-eastern Italy) and to evaluate various histological staining techniques for the detection of *T. contejeani* in infected crayfish.

MATERIALS AND METHODS

From 2004 to 2006, during summer and early autumn, samples of wild white-clawed crayfish (*Austropotamobius pallipes* complex) were catched to detect the microsporidian parasite *Thelohania contejeani* Henneguy. A total of 177 crayfish were captured from watercourses of the province of Belluno (Veneto region, north-eastern Italy) (Figure 1) as reported in Table I.

Gross examination of each specimen was performed, and one subject showing evident lesions referable to thelohaniasis was euthanised and underwent parasitological and histological analysis. Furthermore, 32 of the 177 subjects without clinical signs were sampled in relation to the population consistency in order to carry out the same analyses for epidemiological purposes.

The samples were fixed in 10% buffered formalin (for at least 24 h), trimmed into microcassettes, decalcified, dehydrated in an ethanol series, cleared in xylol and embedded in paraffin wax. Sections of 5 μm were stained with Haematoxylin and Eosin (HE), periodic acid-Schiff’s (PAS), Crossman’s trichrome, Macchiavello stain, Ziehl–Neelsen and Giemsa. Photographs were taken with a Nikon Ds-Fi1 digital camera using Nikon Nis-Elements Imaging Software Version 3.00 SP4.

Histopathological analysis was carried out on full sagittal sections of the cephalothorax and transverse sections of the abdomen. Portions of the gills, legs and antennules were also included.

Fresh and Giemsa-stained smears of the infected skeletal muscles were observed under a light microscope. A total of 100 spores were measured using a drawing tube with a magnification of 1000×.

Samples of infected muscular tissue were prepared for transmission electron microscopy using the following method: small pieces of muscular tissue from infected wild white-clawed crayfish were divided into small blocks and fixed in 2.5% glutaraldehyde and 0.1 M phosphate buffer (pH 7.4) for 2 h, post-fixed in 1% osmium-tetroxide, dehydrated through graded ethyl alcohols and propylene oxide, and embedded in araldite. The sections were obtained utilising a Reichert Jung Ultracut E Ultramicrotome. One-micrometre semithin sections were stained with 1% toluidine blue and examined under a light microscope. Ultrathin sections of about 70 nm were obtained and were stained with uranyl acetate and lead citrate, and examined under a JEOL JEM-T8 electron microscope.

RESULTS

Thelohania contejeani was only detected in one subject of *A. pallipes*, showing lesions referable to porcelain disease coming from the Vena d’oro crayfish population. With the exception of Vena d’oro creek, all the sampled watercourses were negative for the presence of *T. contejani*.

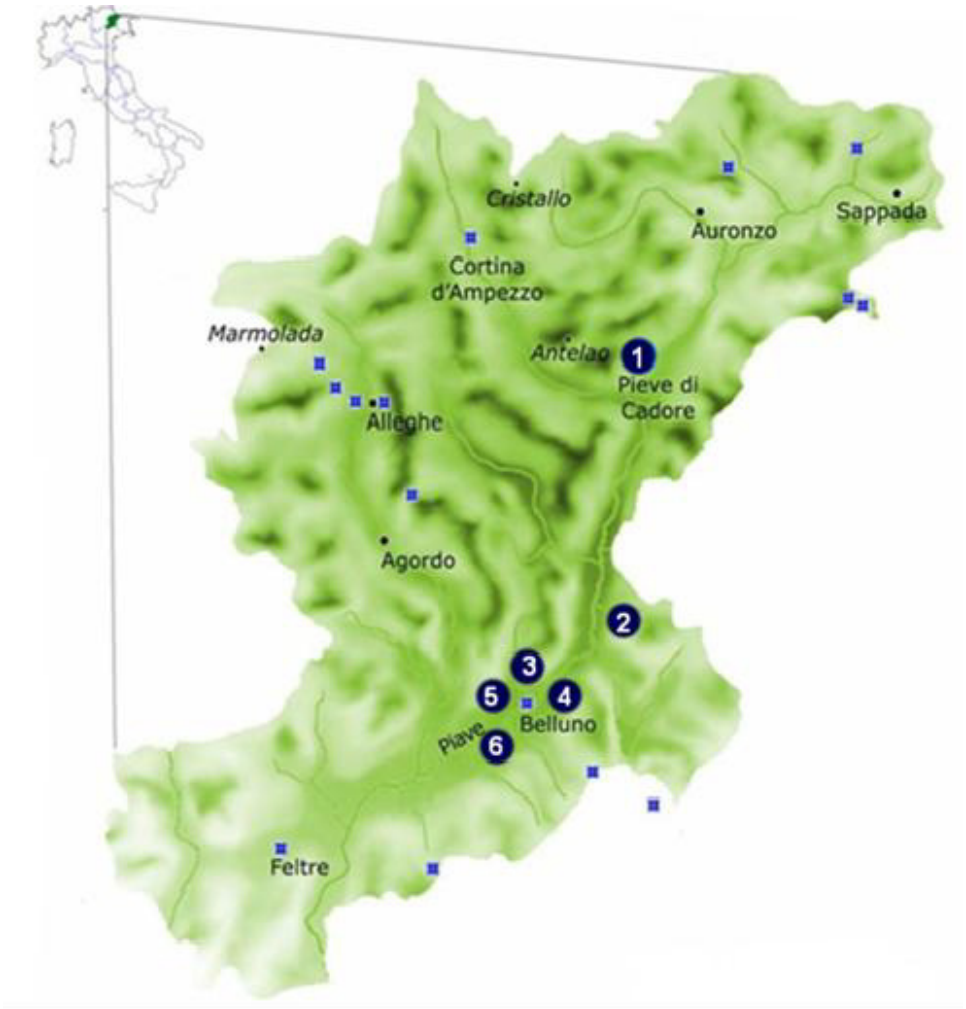

Figure 1
Sampling sites in the province of Belluno: 1- delle Tose Lake; 2- Valturcana; 3- Ardo; 4- Vena d'oro; 5- Morol; 6- Gresal.

Figure 1
Sites échantillonnés dans la province de Belluno : 1- Lake delle Tose ; 2- Valturcana ; 3- Ardo ; 4- Vena d'oro ; 5- Morol ; 6- Gresal.

Table I
Sampling sites, number of collected crayfish macroscopically examined and number of crayfish from each site undergoing parasitological and histological examinations

Tableau I
Sites échantillonnés, nombre d'écrevisses collectées pour examen macroscopique et nombre d'écrevisses par site pour examens microscopiques et histologiques.

Watercourse	Number of collected and macroscopically examined crayfish	Number of crayfish undergoing parasitological and histological examinations
Gresal	16	3
Morol	1	1
Delle Tose Lake	20	6
Valturcana	90	10
Ardo	27	6
Vena d'oro	23	7
Total	177	33

Figure 2
A. pallipes *infected with* T. contejeani *with typical signs of "porcelain disease".*

Figure 2
A. pallipes infecté par *T. contejeani* avec signes de la maladie de la porcelaine.

The infected white-clawed crayfish from the Vena d'oro creek had opaque, whitish abdominal muscles (Figure 2). The examination of all the skeletal muscles from the infected crayfish revealed that the microsporidian parasite was present in many different stages, including individual oval-shaped spores (3.6×1.9 μm) and sporophorous vesicles containing up to eight spores (4.0×2.2 μm).

The polar vacuole, which occupies more than one-third of the spore body, was visible using light microscopy with high magnification in both fresh and Giemsa-stained smears (Figure 3).

Histological examination of the skeletal muscles revealed that up to 90% of the muscle fibres were heavily infected with microsporidian parasites at different developmental stages (Figures 4A and 4B).

Clusters of microsporidian spores occupied both the centre and the margins of the muscle fibres. The muscular tissue showed marked atrophy around the spores.

No encapsulation of the spores was observed, but a hemocytic response had occurred in some of the infected areas where the spores were less abundant.

Melanin infiltrations were focally present in the infected striated muscles (Figure 4C). The circular and longitudinal gut muscle, stomach muscle and heart muscle fibres were also infected (Figure 4D).

Phagocytotic nephrocytes, which are large vacuolated cells fixed to the septa of the axis and the filaments of the gills, were enlarged and engulfed by small masses of spores, easily detectable by Crossman's trichrome staining (Figure 4F). Furthermore, microsporidian spores were found in the lumen of the nephridial tubules of the antennal glands (Figure 4E).

The results of the histological staining techniques used to detect the various stages of microsporidian *T. contejeani* in the infected crayfish are reported below.

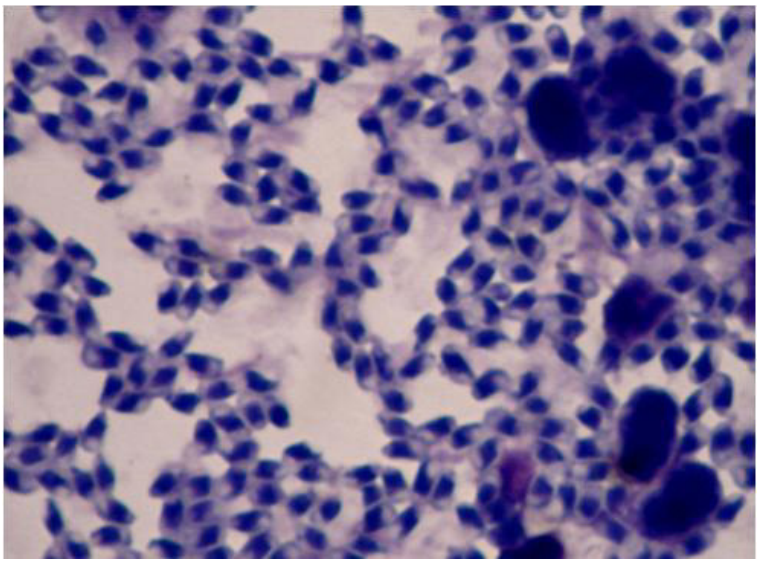

Figure 3
Thelohania contejeani*: Giemsa-stained spores (1000×).*

Figure 3
Thelohania contejeani : spores fixées au Giemsa (1000×).

> HEMATOXYLIN AND EOSIN STAIN

Parasites in the tissue appeared as pale pink oval-shaped bodies, and were easily detectable within the muscle fibres but not in other organs (Figure 5A).

> PERIODIC-ACID SCHIFF'S STAIN

Thelohania contejeani spores appeared as refractile bodies, which ranged from pink to translucent in colour but were poorly stained and not well defined. In some cases, a PAS-positive posterior vacuole was visible (Figure 5B).

> ZIEHL–NEELSEN STAIN

The spores appeared either purple or pink, and the sporophorous vesicles were well differentiated (Figure 5C).

> GIEMSA STAIN

The spores appeared pale or dark blue, but the morphology was not well defined. Some of the parasites remained unstained, and there was very poor contrast and differentiation from the background (Figure 5D).

> MACCHIAVELLO STAIN

The spores appeared slightly violet against a green background; they were easily identifiable, even at low magnification (Figure 5E).

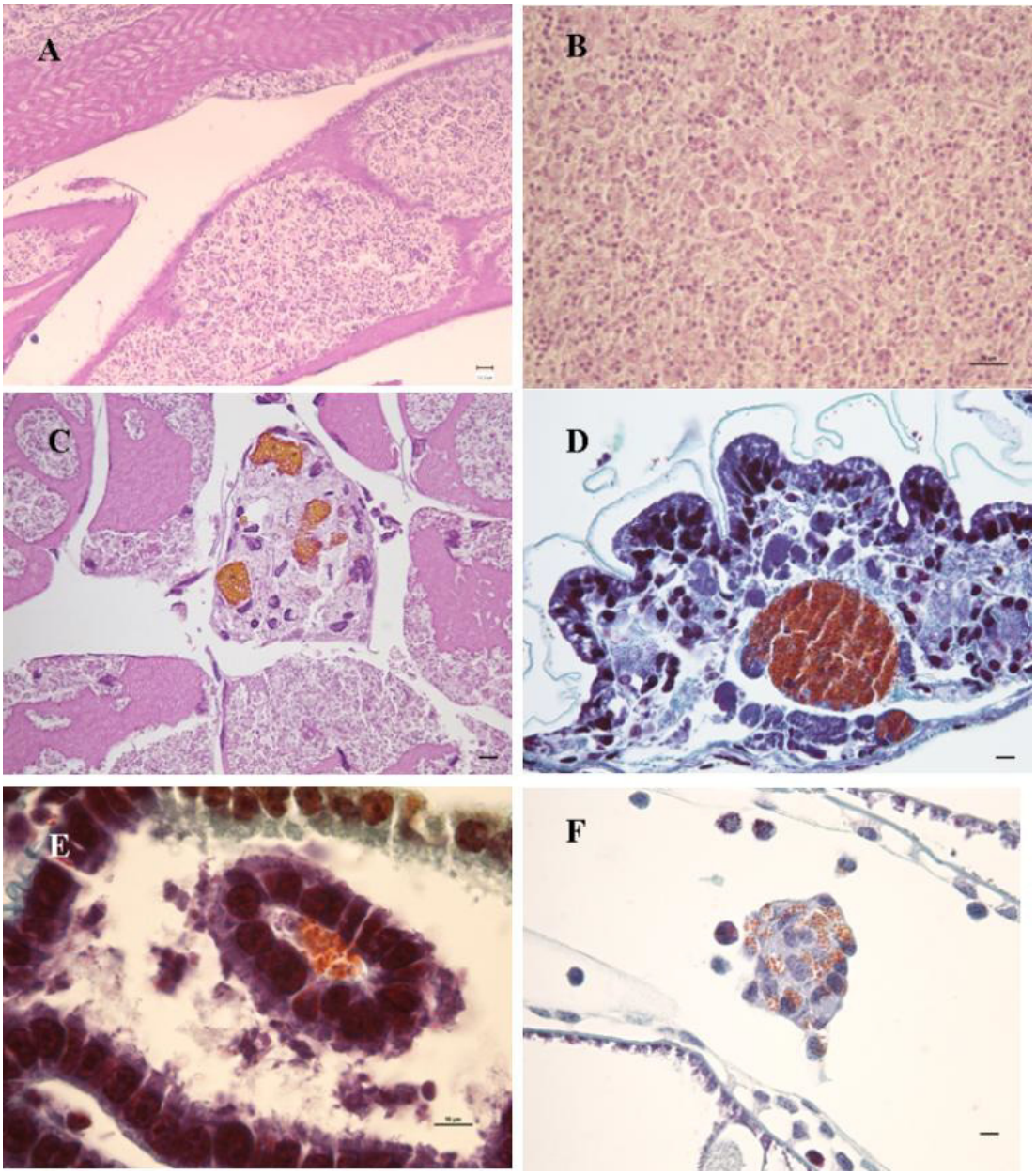

Figure 4
(A, B) Abdominal striated muscle fibres of A. pallipes *filled with spores of* T. contejeani *at various developmental stages (HE); (C) host focal inflammatory reaction with melanin infiltrations in infected striated muscle (HE); (D) large mass of spores in muscle layers of the hindgut (Crossman's trichrome); (E) free microsporidian spores in the lumen of a nephridial tubule of the antennal glands (Crossman's trichrome); (F) phagocytotic nephrocytes containing spores (Crossman's trichrome).*

Figure 4
(A, B) Fibres de muscle abdominal strié d'*A. pallipes* remplies de spores de *T. contejeani* à différents stades de développement (HE) ; (C) réaction locale inflammatoire avec infiltrations de mélatonine dans un muscle strié infecté (HE) ; (D) masse de spores dans des couches musculaires de l'intestin postérieur (trichrome de Crossman) ; (E) spores de microsporidies dans la lumière des tubules népridiens des glandes antennaires (trichrome de Crossman) ; (F) néphrocytes phagocytaires contenant des spores (trichrome de Crossman).

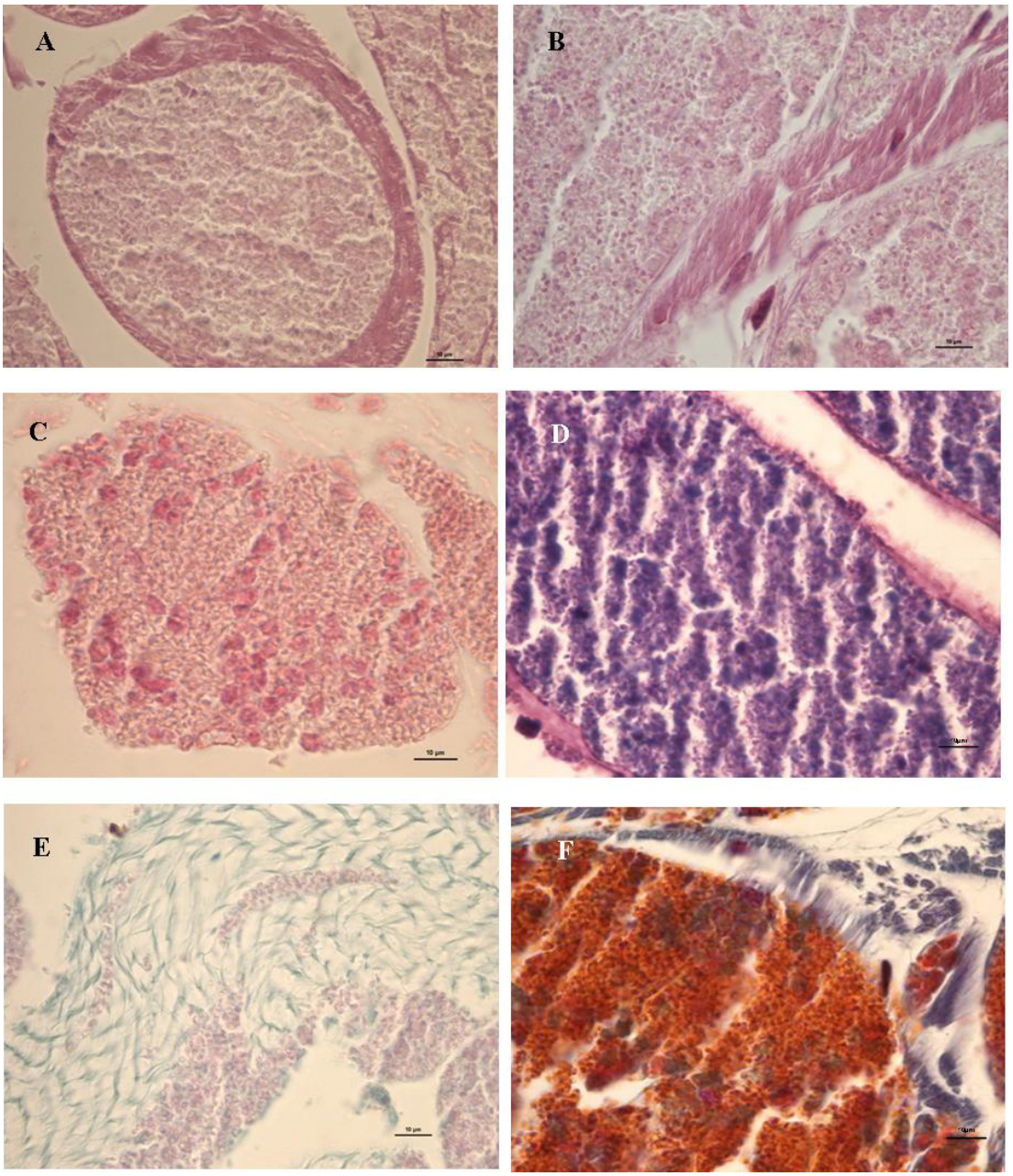

Figure 5
A histological comparison of different staining methods for the detection of a T. contejeani *infection in* A. pallipes*: (A) Hematoxylin and Eosin, (B) Periodic-acid Schiff's, (C) Ziehl–Neelsen, (D) Giemsa, (E) Macchiavello, and (F) Crossman's trichrome.*

Figure 5
Comparaison histologiques des différentes méthodes de détection de l'infection par *T. contejeani* chez *A. pallipes* : (A) hématoxyline et éosine, (B) acide periodique de Schiff, (C) Ziehl–Neelsen, (D) Giemsa, (E) Macchiavello et (F) trichrome de Crossman.

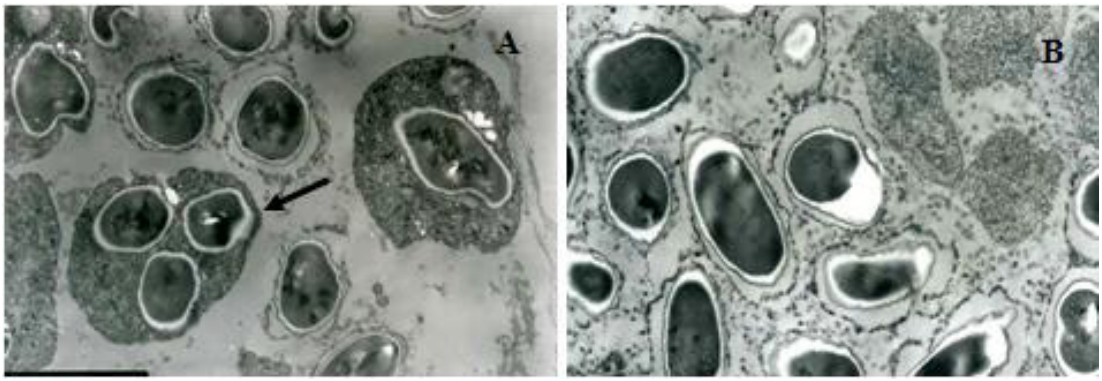

Figure 6
Transmission electron microscopy of A. pallipes *tissue infected with* T. contejeani*: (A) the presence of individual spores and sporophorous vesicles (arrow) containing developing spores and (B) sporoblasts with individual developing and mature spores containing 5–6 polar tube coils.*

Figure 6
Tissus de *A. pallipes* infectés par *T. contejeani* au microscope électronique à transmission : (A) présence de spores seules ou de vésicules sporophores (flèche) contenant des spores développées et (B) sporoblastes avec des spores en développement ou à maturité.

> CROSSMAN'S TRICHROME STAIN

The best results were obtained by using this technique, which allowed for a differentiation between parasite and host cells. The spores appeared deep orange against a blue background (Figure 5F).

> TRANSMISSION ELECTRON MICROSCOPY

Transmission electron microscopy was used to further evaluate and understand the morphology of the parasitic stages found in the muscle tissue. The most common stages observed were immature and mature spores, mostly single or included in sporophorous vesicles (Figures 6A and 6B). Meronts were rarely observed in these samples. The mature spores had an ovoid shape and were nearly identical in size; they were externally surrounded by a crenulated membrane and a capsule (with electron light and electron dense layers). A polar filament, coiled 5–6 times, was present inside the spore and appeared as a tubular structure in a transverse section. Two contiguous nuclei surrounded by a thick nuclear membrane were evident in the inner core of some spores. An electron light area, identifiable as a posterior vacuole, was visible within few spores. Among the mature spores, which was the most common stage identified, sporophorous vesicles containing a variable amount of immature spores were observed. The polar filament in immature spores was coiled 9–11 times. A few sporonts were observed, each containing two nuclei.

DISCUSSION

This is the first report of the sporadic occurrence of *T. contejeani* infection in white-clawed crayfish populations from north-eastern Italy, with heavy tissue damage referable to the parasite.
Infection rates from 0.1 to 50% have been reported in the literature (Schäperclaus, 1954; Sumari and Westam, 1969; Voronin, 1971; Cossins, 1973; Vey and Vago, 1973; Hofmann, 1980; Chartier and Chaisemartin, 1983; O'Keefe and Reynolds, 1983; Cukerzis, 1984; Diéguez

Uribeondo *et al.*, 1997). As stated by Alderman and Polglase (**?**), levels of infection up to 10% are frequent in crayfish populations and values of 30% are not uncommon. The infection rate observed in the Vena d'oro crayfish population during this investigation was 4.3%.

Considering the low infection levels and the long duration of the disease, it is likely that *T. contejeani* has a limited negative impact on these crayfish populations, but it could easily spread to a densely populated environment, such as the Vena d'oro creek. Future periodic surveys of this water course will be necessary to monitor the overall health, and status of *T. contejeani* infections in the *A. pallipes* population.

The histological findings showed that Crossman's trichrome stain is the most effective method for the detection of *T. contejeani*, particularly in the early stages of the parasitic infection, permitting the pathogenesis of the porcelain disease to be better studied.

Crossman's trichome stain allowed us to obtain good contrast between the background and parasites. We observed spores from disrupted muscle fibres both extracellularly, in the interstitium, and intracellularly, within the phagocytotic nephrocytes, which suggests a host immune response. We were able to identify the phagocytised parasite only with the use of Crossman's trichrome stain.

The observed histological lesions were similar to those described in *A. pallipes* by Cossins and Bowler (1974) and in *A. astacus* by Oidtmann *et al.* (1996), where up to 90% of the muscular fibres were infected.

Concerning the results observed by transmission microscopy, the spores were usually found free and a few were inside a sporophorous vesicle as has already been observed by Lom *et al.* (2001).

Indigenous European crayfish species, such as *A. pallipes* complex, now live within a considerably restricted geographical area, largely due to disease, pollution, destruction of habitat and stressed water. To preserve crayfish populations and protect them from the problems of a shrinking habitat, it is necessary to introduce planned restocking programs. In this regard, the restocking activities, when carried out without appropriate health monitoring programs, may introduce crayfish carrying pathogens, such as *Thelohania contejeani*, into unaffected populations.

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