Pattern and potential pathophysiological effects of myxosporean infections in the gills of Tilapia species (Teleostei: Cichlidae) from Bénin

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Abstract

In order to fill the gap between natural fish catches and estimated needs of populations in animal protein consumption, aquaculture required suitable fish. Indeed, much interest has focused worldwide on tilapia species. In this study, three parasites previously described, Myxobolus zillii, M. dossoui and M. beninensis were recorded on Tilapia zillii and Sarotherodon melanotheron melanotheron from Lake Nokoué (Bénin) from November 2011 to december 2012. The pathological investigation by electron microscopy revealed that the spores of M. zillii and M. dossoui were in direct contact with the cartilage of gill arch or filament but M. beninensis developed cysts within the connective tissue of gill arch. The mass of spores and continuous growth of cysts spark hollows in cartilage and connective tissues offering them the capacity to destroy these structures so that the physiological pivotal role of gill in respiratory function and ions balance might be disrupted. Among the 180 examined specimens, 27 T. zillii were infected by M. dossoui or M. zillii and 6 S. melanotheron melanotheron took in M. beninensis. No significant difference was observed within sexes and classes of length in regard to the rate of infection except the prevalence of M. dossoui which varied significantly within the seasons.

Keywords: Gill, Histopathology, Myxosporea, Prevalence, Tilapia, Lake Nokoué.

Introduction

Aquaculture of fish is an important socio-economic sector, especially for rural community, contributing to livelihoods, food security and poverty alleviation [1]. Currently, the trend in fish aquaculture development is towards increased intensification and commercialization of fish production [2]. FAO [3] put special emphasis on tilapia species that are easy to grow, unique in the role of small livestock animal for subsistence fish farmers in developing countries and on word tilapia production that has been booming, with output increasing from 830,000 tonnes in 1990 to 1.6 million in 1999 and to 3.5 million in 2008; this production would reach 3.7 millions tonnes in 2010 and 4.6 millions in 2015. According to Kamal and Mair [4] while the overall proportion of aquaculture production taking place in brackish water has sometimes decreased, there has been a significant increase in the tilapia production in brackish water.

Tilapias (Teleostei: Cichlidae) are euryhaline fish that can live and thrive in a wide range of salinity from fresh water to full seawater although some species tolerate wider range of salinity than others [5]. These species are able to tolerate a broad range of environmental ions conditions by using osmoregulation and ionoregulation mechanisms to maintain homeostasis; those mechanisms are mainly accomplished by the skin, gills, kidneys, urinary tract and intestine. Among these organs, the gills are the major site for ion transport [6,7,8]. In addition to be the major respiratory organ the gills play a pivotal role in ionic balance and adaptive changes following an osmotic challenge; in the branchial epithelium, chloride cells are the ionocytes responsible for ion exchange [9-12].

Among the tilapias species, *Tilapia zillii* Gervais, 1852 and *Sarotherodon melanotheron melanotheron* Rüppel, 1853; Trewavas, 1983 are two important species in

aquaculture [13,14]. They can reach market size of minimum 400g within few months [15] and are suitable for farming in the brackish water of lagoons and estuaries in West Africa where salinity may vary throughout the year from 0 to 90 g/l [16,17]. Specially, *S. melanotheron melanotheron* is known to be very resistant to wide salinity variations [18,19].

In regard to the increasing interest in fish culture, the awareness of parasites that affect fish health, growth and survival is more growing up [20], because disease is nowadays a primary constraint to the culture of many aquatic species. Therefore, the contribution to the knowledge of fish parasites should be a prerequisite for correct monitoring of disease; the best way to reduce out-breaks of disease consisting in preventive measure based on early diagnosis of their agents [21,22]. Myxosporean parasites are known to infect fresh and brackish water fishes [23] with significant negative impact [24-33]. Respiratory capacity has been found to decrease in case of heavy infection of the gill by myxosporidia causing asphyxia, serious loss in length and weight and mortality [34-37].

During our continuous survey on fish Myxosporea parasites, three species belonging to the genus *Myxobolus* were found in the gills of *S. melanotheron melanotheron* and *T zillii*. Considering their morphology features, these species are deeply alike those reported by Sakiti et al. [38], namely *M. beninensis*, *M. zillii* and *M. dossoui*. Traditionally, the taxonomic classification of myxosporean parasites species is based on characteristics of spore morphology and other criteria [39,40,41]. Since the late 1990s, molecular analyses have been however use widely in the studies of these parasites [42,43,44] in morphological differentiation of similar species or study of host and tissue specificity [45-48]. Several workers have utilized the combination of the traditional method and the molecular approaches for the identification of parasite [47,49-52].

Using molecular approaches the process of the accurate identification of the present species of *Myxobolus* is still running though they look alike those dealing with our previous occurrence report [53] considering the morphological characteristics.

Light and electron microscopy were utilized to investigate and report herein their morphology features and the pathology that they induced.

Materials and methods

In Benin located between latitude 6°15' and 12°25' north and between longitude 0°45' and 4°00' east, fish samplings of *Tilapia zillii* Gervais, 1852 and *Sarotherodon melanotheron melanotheron* Rüppel 1853, Trewavas 1983 were done from November 2011 to December 2012 in Lake Nokoué (Figure 1). The climate in the sampling zone is subequatorial and characterized by two dry seasons and two wet seasons; the long dry season (LDS) running from December to March, the long wet season (LWS)

from April to July, the short dry season (SDS) from August to September, and the short wet season (SWS) from October to November. Every two months, three physicochemical parameters of water samples were measured including pH, salinity expressed in g/l and temperature (T°C).

In total, 180 specimens (90 *T. zillii* and 90 *S. melanotheron melanotheron*) were collected. At least, 30 individuals of fishes were purchased every two months from the fishermen and transported alive in appropriate tanks to the laboratory at Abomey-Calavi where their total size were measured. Then, fish were dispatched up into size classes with a regular interval of 50mm. Sex determination was made on the basis of external morphology and observation of the gonads after dissection. Macroscopic examination at the naked eye and under the low power stereomicroscope was performed for detection of any visible cysts on the gills which are in round or ovoid form and whitish and doughy aspects. Later the examinations were proceeded on with detection of spores under a phase-contrast light microscope. The morphological and morphometric studies of the spores were based on fresh spores specimens (n=30) according to the guideline provided by Lom and Arthur [39]. The dimensions of spores were given as arithmetic means expressed in micrometers (μm) followed by the minimum and maximum values in parentheses. The prevalence of infection was calculated and presented as a function of the host size and sex and the seasons. The possible influence of physicochemical parameters on the prevalence of parasites was tested using the Pearson correlation coefficient. The chi-square test (χ^2) was used to assess the effect of the host size and sex on the prevalence of the parasite and its variability between the seasons. All analyze were performed with the statistical program MINITAB 14 Demo.

Results were considered significant at the 95% level (p<0.05).

For histological analysis, samples of infected tissue parts were fixed in 10% phosphate-buffered formalin and dehydrated in an ascending ethanol series, before being embedded in paraffin. Serial histological sections (7μm in thickness) of these samples were performed in the microtome and stained with Hematoxylin-Eosin.

For ultrastructural studies, samples of infected gills were safe kept and fixed in absolute ethanol and in formalin 5% or in glutaraldehyde 2.5% in 0.1M sodium cacodylate buffer (pH=7.2). Using appropriate tanks they were carried to the Institut de Modelisation et d'Analyses en Géo-Environnement et Santé (IMAGES), University de Perpignan Via Domitia (UPVD) at Perpignan (France) where they have been kept cold at 4°C as long as necessary for further investigations.

For scanning electron microscope (SEM), the 5% formalin-fixed spores were dehydrated through a series of ethanol concentrations; critical point dried in Biorad, coated with gold in an Emscope SC 500 Sputter coater and viewed using a Hitachi S-4500 SEM operated at 15kV at the Centre de Caractérisation de la Matière (PROMES).
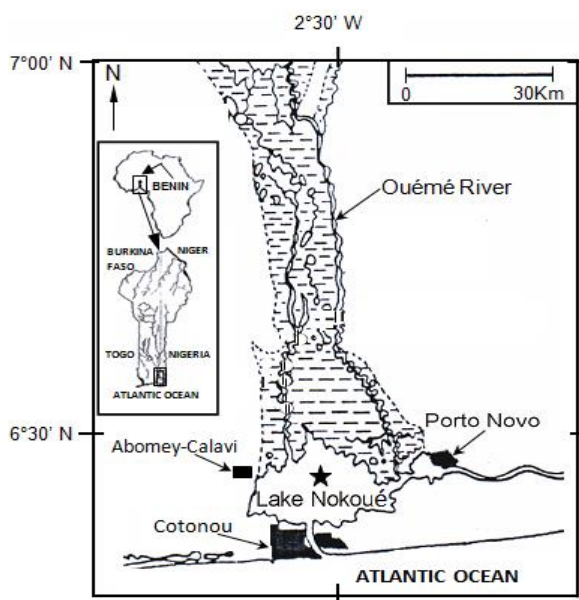
Fig.1 Sampling site in Lake Nokoué (star), Benin, West Africa

For transmission electron microscope (TEM), cysts fixed in glutaraldehyde were washed with buffer 0.1M three times (10 minutes each), post-fixed in 1% OsO_4, washed again with the same buffer three times for 3hours, dehydrated in increasing concentrations of acetone. Finally, samples are embedded in Epon resin. Leica ultratome was used for semithin and ultrathin sections. The semithin sections were made with glass knives, stained with toluidine blue and examined in Microscope Epifluo: Olympus BX-UCB. The ultrathin ones were cut with diamond knives, double-contrasted with uranyl acetate and lead citrate Sections were examined in Hitachi 7500 and Camera high resolution AMT advantage operating at 80kV. Those operations have been done at the Observatoire Océanographique de Banyuls-sur-mer (OOB).

Results

Parasites, water quality and infection rate as a function of host sex and size and season

The total number of fish specimens collected was 180 (90 *T. zillii* and 90 *S. melanotheron melanotheron*). The characteristics of the morphology of the three parasites recorded from the gill of the two tilapia species are closer to those previously reported (Table 1). According to the traditional criteria of determination of species, it can be assumed that the present parasites are *Myxobolus dossoui*, *M. zillii* and *M. beninensis*.

The physicochemical parameters data showed that during the period of the study mean water salinity varied from 0.82 to 21.83 g/l while temperature varied from 25.2 to 30.03°C and the pH varied between 7.26 and 8.00 (Fig. 2). The Pearson correlation coefficients (r) and associated probabilities (p) for the prevalence with the physicochemical parameters displayed that the infection rate of the fish was not correlated to the physicochemical

parameters (Table 2). Two parasites, *M. dossoui* and *M. zillii* were found in *T. zillii*. The data of Table 3 exhibited a prevalence of 13.33% for *M. dossoui* (12 specimens infected). The difference of prevalence between host sex was not significant (χ^2 = 2.60, df = 1, p > 0.05). For *M. zillii*, 15 specimens (16.66%) were infected and no significant difference between host sex prevalence appeared (χ^2 = 1.14, df = 1, p > 0.05). In *S. melanotheron melanotheron*, only the one parasite, *M. beninensis,* was found; its prevalence was 6.66% (6 infected specimens). There is no significant difference between host sex prevalence (χ^2 = 7.29, df = 1, p > 0.05).

The prevalence distribution according to host size (Fig. 3) showed that *T. zillii* specimens which length ranged from 200 to 250 mm were the most infected. Neither the prevalence of *M. zillii* nor those of *M. dossoui* were significantly different (χ^2 = 0.64, df = 3, p > 0.05 for *M. zillii* and χ^2 = 16.24, df = 3, p > 0.05 for *M. dossoui*). *Myxobolus beninensis* did not infect *S. melanotheron melanotheron* sized between 200 and 250 mm. Its highest occurrence was recorded in the class of 100 to 150 mm and no significant difference was observed within the size classes (χ^2 = 3.14, df = 3, p > 0.05).

The prevalence pattern according to the seasons (Fig. 4) showed that the highest infection rates were 24.55% for *M. dossoui* in LWS-12 and 27.89% for *M. zillii* in SWS-12. *M. dossoui* was not found during SWS-12 and its occurrence varied significantly from one season to another throughout the study period (χ^2 = 18.02, df = 4, p < 0.05). The seasonal occurrence of *M. zillii* did not vary significantly (χ^2 = 9.76, df = 4, p > 0.05). During LWS-12 and SWS-12 respectively, the prevalence was 9.3% and 7.13% for *M. beninensis*.

In LWS-12, *M. beninensis* occurrence was nil. There is no significant difference between seasons (χ^2 = 0.92, df = 4, p > 0.05).

Table 1 Comparative description of *Myxobolus* species recorded in the present study from the gill of tilapia species in Lake Nokoué (Bénin, West Africa) with previous species morphologically described according to Sakiti et al. [38]

Myxobolus species	Spore		Polar Capsules		Cyst form
	Length (µm)	Width (µm)	Length (µm)	Width (µm)	
M. zillii (Sakiti et al. 1991)	9.8 (8-11)	7.5 (6-8)	5.1 (4-6)	2.5 (2-3)	*Spherical, oval, elongate*
M. zillii (**present study**)	9.7 (8.1-11.8)	7.7 (4.3-8)	5.2 (4.3-5.8)	2,5 (2.2-3.1)	*Spherical, oval, elongate*
M. dossoui (Sakiti et al. 1991)	9.9 (8.5-11)	9.2 (8-10.5)	5.5 (4.5-6.5)	3.1 (2.5-5)	*Oval, elongate*
M. dossoui (**present study**)	9.9 (8.3-11.1)	9 (8-10.2)	5.7 (4.3-6.4)	3.1 (2.3-4.9)	*Oval, elongate*
M. beninensis (Sakiti et al. 1991)	12.5 (10.5-14)	7.2 (5.5-9)	6.9 (6-8)	2.2 (1.5-3)	*Spherical, oval*
M. beninensis (**present study**)	12.5 (10.5-14.3)	7.4 (5.3-8.9)	6.7 (6.1-7.8)	2.2 (1.5-3.2)	*Spherical, oval*
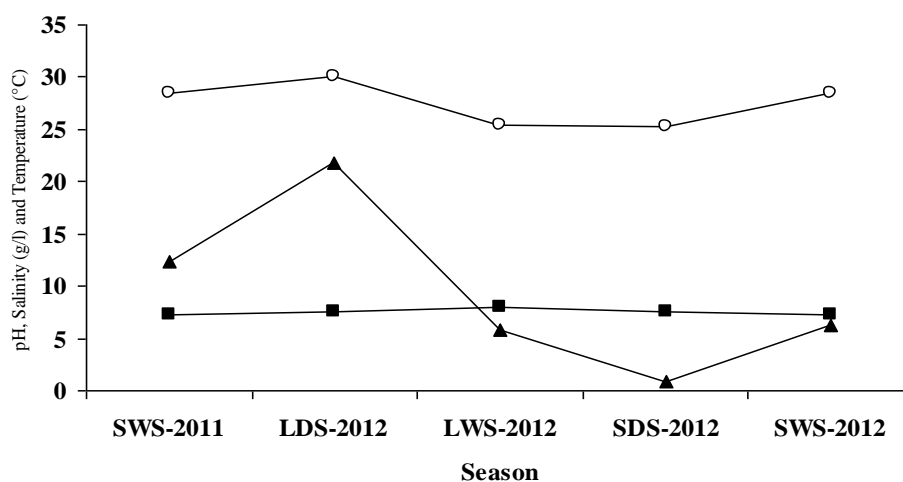
Fig.2 Seasonal changes in the salinity (▲), temperature (○) and pH (■) of Lake Nokoué (Benin, West Africa)
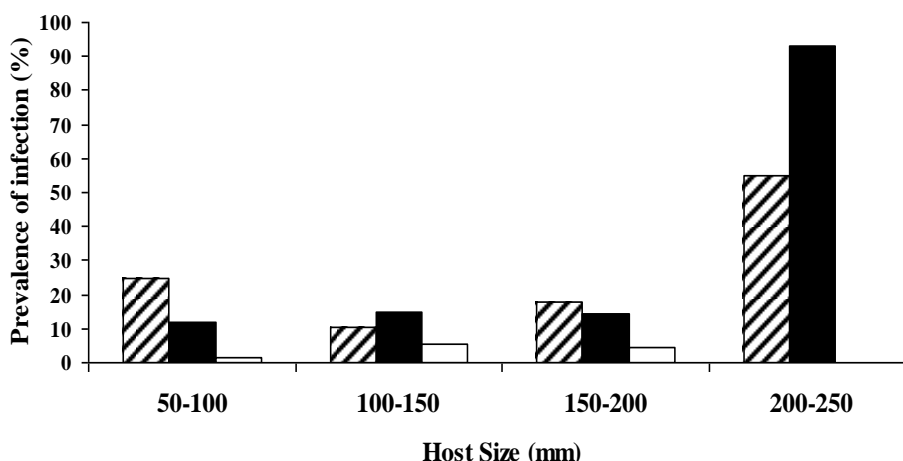
Fig. 3 Prevalence of myxosporean parasites in *Tilapia zillii* and *Sarotherodon melanotheron melanotheron* in Lake Nokoué (Benin, West Africa). *Myxobolus zillii* (hatched bars) and *M. dossoui* (filled bars) in *T. zillii* and *M. beninensis* (open bars) in *S. melanotheron melanotheron*

Table 2 Results of Pearson correlation of prevalence with physicochemical parameters

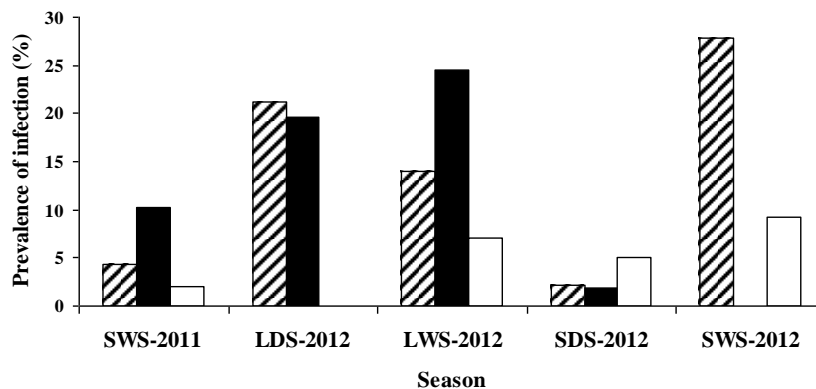|	*Myxobolus dossoui*			*Myxobolus zillii*			*Myxobolus beninensis*		
	T°C	pH	Salinity	T°C	pH	Salinity	T°C	pH	Salinity
r	0.029	0.704	0.487	0.360	0.496	0.734	-0.496	0.130	-0.759
p	0.963	0.185	0.405	0.552	0.395	0.158	0.396	0.835	0.137

[Temperature (T°C), pH, Salinity (g/l)]; *r*: Coefficient of correlation; *p*: Probability

Table 3 Prevalence of myxosporean parasites in *Tilapia zillii* and *Sarotherodon melanotheron melanotheron* in Lake Nokoué (Benin, West Africa)

Parasites	*Tilapia zillii*				
	Males		Females		Chi-2 test (χ^2)
	Examined	Infected (%)	Examined	Infected (%)	
M. zillii	42	6 (14.28)	48	9 (18.75)	χ^2 = 1.14 ; df = 1 ; *p* = 0.649
M. dossoui	42	4 (9.52)	48	8 (16.66)	χ^2 = 2.60 ; df = 1 ; *p* = 0.136
	Sarotherodon melanotheron melanotheron				
M. beninensis	55	4 (7.27)	35	2 (5.71)	χ^2 =7.29 ; df = 1 ; *p* = 0.907

s

Myxobolus zillii and *M. dossoui* in *T. zillii* and *M. beninensis* in *S. melanotheron melanotheron.* [df = degree of freedom]

Fig. 4 Prevalence of myxosporean parasites in *Tilapia zillii* and *Sarotherodon melanotheron melanotheron* in Lake Nokoué (Benin, West Africa). *Myxobolu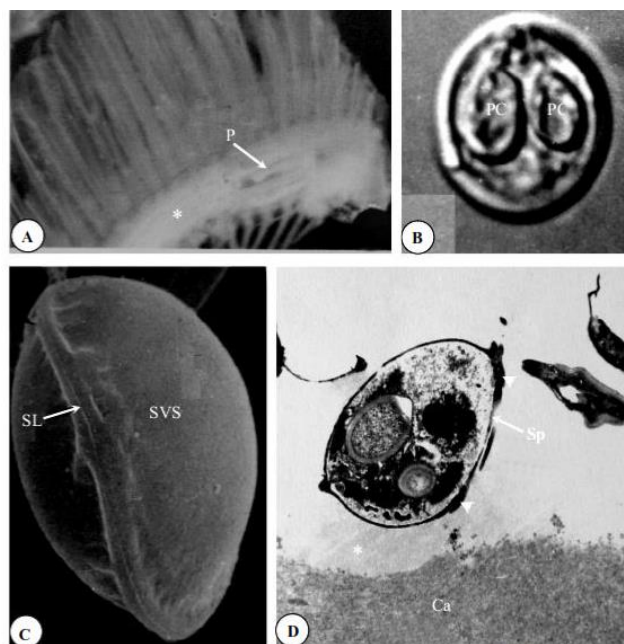s zillii* (hatched bars) and *M. dossoui* (filled bars) in *T. zillii* and *M. beninensis* (open bars) in *S. melanotheron melanotheron*. Sampling season given on x-axis (LDS = long dry season, LWS = long wet season, SDS = short dry season, SWS = short wet season)

Fig. 5 *Tilapia zillii* gill arch cartilage infested by *Myxobolus dossoui*. A- Photomicrograph showing the parasite (P) formed by a clump of spores in a hole. Note that the uninfected parts (white star) are not perforated (×10). B- Photomicrograph of phase-contrast light microscopy of the spore of *M. dossoui*: note that the polar capsules (PC) are unequal (x 3,500). C- Photograph of the scanning electron microscopy of the spore of *M. dossoui* in cross view. Note the smooth valvular surface (SVS) and the sutural line (SL) (x 8,000). D- Photograph of the transmission electron microscope of free spore (Sp) of *M. dossoui* in contact with the cartilage (Ca) and surrounded with cell debris (white arrows heads). Note that the cartilage is progressively destroyed (white stars). Moreover, there is no structure like cellular response to infection (× 6,500)
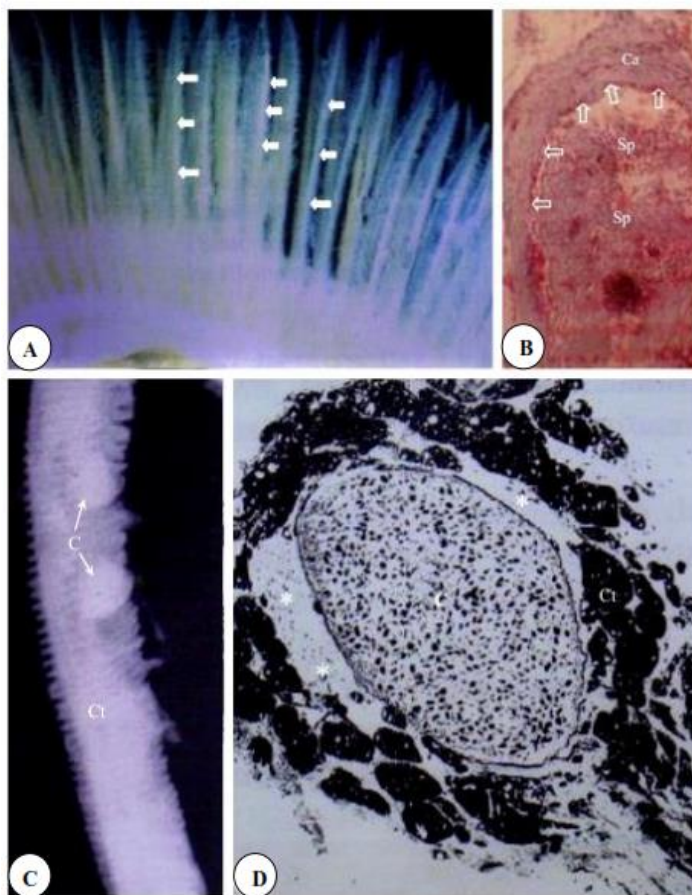
Fig. 6 Infection by *Myxobolus zillii* (A and B) and *Myxobolus beninensis* (C and D). A- Photomicrograph of *M. zillii* (white thick arrows) located along the primary gill filaments of *Tilapia zillii* (×10). Note that numerous filaments harbored cysts. B- Photomicrograph of Haematoxylin-Eosin stained tangential section of cysts in the cartilaginous tissue (Ca) of *Tilapia zillii* showing the destruction (Open arrows) of the cartilage by spores (Sp) (×500). C- Photomicrograph of cysts (C) located in the connective tissue (Ct) of *Sarotherodon melanotheron melanotheron* enveloping the cartilaginous tissue (×15). D- Photomicrograph of toluidine-blue stain transverse section of the connective tissue (Ct) of *Sarotherodon melanotheron melanotheron* showing destruction (white stars) of the neighboring cells surrounding the cyst (C) (×650)

Histopathological and ultrastructural studies

In the present study, myxosporean infections were located in various gill tissues of the Tilapia species. Indeed, infection by *M. dossoui* was found as aggregates spores freely established in holes in the branchial arch cartilage of *Tilapia zillii* (Fig. 5A). The infection was visible as a thick whitish cream that can spread throughout the cartilage. Spores appeared sometimes elongate or oval with two polar capsules unequal in size (Fig. 5B) and showed the very wide sutural line and a smooth valvular surface (fig. 5C). TEM sections revealed that spores were found within the cartilage in a direct contact with host cells and tissues (Fig 5D). This closely contact and the enlarge place covered by the mass of the spores sparks hollows in the cartilage and offers to spores the capacity to erode the cartilage structure which continues to be progressively destroyed.

The infection of *T. zillii* by *M. zillii* was found in the primary gill filament representing by whitish cysts along the filaments (Fig. 6A). The spores which have been described elsewhere were spherical, oval or elongate in shape (data not show). Histological analysis revealed that spores of *M. zillii* were found mostly in the cartilaginous tissue. Tangential section showed that spores led to degenerate the tissue; then, the progression of the development of the spores provoked wastage of the cartilage structure (Fig. 6B). In infected *S. melanotheron melanotheron*, the gill arch of the connective tissue was occupied by large cysts of *M. beninensis*. Cysts were whitish in color and oval or irregular in shape (Fig. 6C). The spores already described elsewhere exhibited spherical or oval form with two polar capsules which are equal in size (data not show). Histological sections of the connective tissue showed that cysts of *M. beninensis* were developed within that tissue (Fig. 6D). Pathological alterations in the tissue were caused by the large mass and continuous growth of the cysts. In the action of the three parasites, no visible structure against development of reaction was observed, but the gill structure was beginning eroded.

Discussion

Infection rate as a function of host sex and size and season

No correlation was observed between seasonal prevalence of the parasites and the measured physicochemical parameters. Similar observations had been reported on *Myxobolus* spp. [26] and *Henneguya* sp [54]. However, prevalence could vary with seasons [55]. That is in agreement with the findings of Molnár [56] who found a major seasonal difference with markedly lower numbers of infected fish specimens in summer months than in spring months and Gbankoto et al. [53] who reported a significant difference between the prevalence of *M. zillii* and the season. The latter authors found that the infection rate of *M. dossoui* varied significantly in function of the host size. Those differences could be due to the short sampling period and the limited examined specimens. Moreover fish habitat is a dynamic environment; later in one or two decades it could not exhibit the same biological aspects or properties.

Despite the lack of significant difference, it appeared that for *T. zillii*, females were more infested than males while males were slightly most infested in *S. melanotheron melanotheron*. That is not in agreement with the observations of Gonzalez-Lanza and Alvarez-Pellitero [57] and Gbankoto et al. [53] who reported that the males are known to be usually more sensitive to myxosporean parasites than females. Poulin [58] claimed that such pattern could be due to testosterone synthesis which may exert an energetic cost on the immune competency. The present results lead to suggest that parasite prevalence in fish could be influenced by its physiological status and maturity.

In this study, *S. melanotheron melanotheron* with large size were not infected whereas large-sized specimens of *T. zillii* displayed high prevalence. Mitchel [59] had reported similar results for myxosporeans species such as *M. muelleri* and *M. dujardini* infecting *Psychocheilus oregonensis*, *P. caurinus* and *Richardsonius blateatus* which exhibited high prevalence among oldest fishes. Such observation might lead to suggest that there exist tolerance between elder host and the concerned parasites [60]. As far as these prevalence fluctuations, Gbankoto et al. [61] thought that any difference based on host size may simply be due to inter-specific differences in the resistance of host species to infection by the parasite.

Myxoporidia parasitize invertebrates and lower vertebrates particularly fish, with often fatal consequences for the host [62]. These hosts are representative of many fish families, comprising Cichlidae, Clariidae, Cyprinidae and others as well as in brackish or freshwater all other the world. In Africa, studies dealing with parasites of Cichlidae are mostly based on the genus *Oreochromis* either *O. niloticus* or *O. mossambicus* species [63-67]. Unusually, observations were conducted on *T. zilli* or *S. melanotheron melanotheron* [53,61,68,69]. Parasites could occur in many specific target organs including gills, kidney, and liver which are responsible for vital functions, such as respiration, excretion, accumulation and biotransformation of xenobiotics in fish [70,71]. The gills are signalized to be the preferential location of fish myxosporidia [46] where they can have important negative impact on the complex osmoregulatory mechanisms documented by several reports [6,8,11,12]. The low prevalence of these Myxosporidian parasites in wild fish specimens probably did not reflect the low prevalence of the parasite in farm specimens. This might suggest that the risk of introducing this parasite to fish farms through infected fish could be elevated despite the opposite opinion of Sorour and Al Harbey [72]. Moreover the occurrence of these parasites species in the osmoregulatory system indicates that they are potentially important pathogen, and the presence and dispersion of which need to be monitored closely by commercial fish farmers.

Potential effects of histology and ultrastructure studies

The infections observed in this study could be a major handicap for the respiratory and ionic exchange functions of the gills in fish. Such damages could make gills less functioning by reducing the respiratory surface [73,74]. Myxosporean gill infection can have an adverse effect on the ability of infected individuals to survive [75]. The spores of *M. dossoui* were not encysted in the cartilaginous tissue of the gill arch. The closely contact between parasite and the host cells and tissues created large cavity in the cartilage offering to spores the capacity to erode the cartilage structure which is highly susceptible to be destroyed. The sections in the infected gill arch exhibited massive cysts severely distorting the entire gill arch and its lamellae. Spores pushed and compressed the endothelial cells lining the cartilaginous tissue altering the infected portion. Such observation had been reported by El-Mansy and Bashtar [76]. No cellular structure separated spores and cartilaginous tissue. This type of development was corresponded to the "intrafilamental type" described by Molnár [56]. On this way, available parasites in infected organs are more prejudicial, as they reproduce by asexual way and progressively colonize the organs [61]. Each gill consisted of a large number of gill filaments (primary lamellae) on which a series of alternately arranged secondary lamellae (respiratory lamellae) are projected. While the secondary gill lamellae are lined by pavement cells, below it there are lamellar blood sinuses separated by pillar cells [72]. As early responses, histological changes observed in the primary gill lamellae may lead to disorganization of the structure of the secondary lamellae as well as cellular hyperplasia. Secondary lamellae do not hold on the

primary gill filaments. Myxosporidian parasites can cause damages like vasodilatation of secondary lamellae with breakdown of the pillar cell system; degenerative and necrotic changes of the pillar cells could be registered on the gill lamellae of fish infested as if exposed to a polluted area [72]. Moreover, cysts provoke a lytic action on the muscular fibers surrounding the cartilage. The presence of the cysts might be combined with congestion on gills with excessive sliminess excretion as inflammatory response of irritations on gills caused by movements and fixation of the parasite. The large number of cysts in the covered cartilage might be a significant mechanical barrier to blood flow, leading to more atrophy, sloughing, congestion and perivascular edema [77]. Similar results were observed by Adriano et al. [78,79] who reported that *M. salminus* n. sp. in *Salminus brasiliensis* and *M. cuneus* in *P. mesopotamicus* may compromise blood circulation and gill function. Schwaiger et al. [80] assumed that these gills alterations might interfere with normal respiratory and ionoregulation functions and might lead to impairment of the general health conditions of fish.

Conclusion

In the present study, myxosporean infections were located in various gill tissues of the Tilapia species. The parasites were recorded either in the gill arch cartilage or the connective tissue of the gill arch or the primary gill filament cartilage. Thus, the relationship between the parasites infecting the fish gill tissues is supposed to be high. The infections observed could be a major handicap for the respiratory and ionic exchange functions of the gills in fish. Such damages could make gills less functioning by reducing the respiratory surface. Moreover the occurrence of these parasites species in the osmoregulatory system indicates that they are potentially important pathogen, and the presence and dispersion of which need to be monitored closely by commercial fish farmers.

Acknowledgments

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