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## Effect of 80% ethanol or 10% formalin fixation, freezing at $-20^{\circ}\text{C}$ and staining on *Myxobolus* (Myxosporea) spores to be deposited in parasitological collections

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### ABSTRACT

The preparation of myxosporeans for the description of myxospores and their preservation as type material in parasitological collections show great variations. Most frequently, formalin and ethanol are used for fixation and Giemsa solution for staining spores. In this work, authors studied the effect of 80% ethanol and 10% formalin fixation, freezing at  $-20^{\circ}\text{C}$  and staining on the size and transparency of two *Myxobolus* species of cyprinid fishes, *M. bramae* and *M. bliccae* spore, and recommended a new method for the deposition of type material to parasitological collections in museums. The studies have commended that fresh spores from mature plasmodia are the best material for measuring the size and studying the inner structures, the number of polar tubules in polar capsules and the morphological characters of the intercapsular appendix. The obtained quantitative data suggest that cryo- and chemical preservation do not have a notable negative effect on spores compared to fresh samples but they decrease the transparency of spores. Staining the spores with Ziehl–Neelsen has proved to be a useful method for studying the fine structure without size reduction, while Giemsa staining induced a shrinkage of spores so it seems to be not ideal for description of a new species. When treating spores of *Myxobolus* spp. with Lugol's solution, iodophilous vacuoles in the sporoplasm were not recognised but visualisation of the coils of polar tubules was enhanced. As a type material for newly described species, authors suggest phototypes and spores fixed in 80% ethanol to be deposited into collections, as this preservation method is suitable for subsequent research, such as re-measurements and molecular analysis.

### 1. Introduction

The subphylum Myxozoa Grassé, 1970 harbours a diverse group of metazoan parasites characterised by multicellular spores, with distinct polar capsules and an extrudable polar tubule used in the invasion of the host (Kent et al., 2001; Canning and Okamura, 2004; Lom and Dyková, 2006). The great majority of Myxozoa with spores of hardened shell walls belong to the class Myxosporea Bütschli, 1881.

Among myxosporeans, the genus *Myxobolus* is the most abundant in species. Of them, approximately one thousand species have been recorded in synopses (Landsberg and Lom, 1991; Eiras et al., 2005, 2014, 2021; Vidal et al., 2017) and in some recently published manuscripts. The majority of these species have been described by the shape and size of their myxospores without any deposited material in museums or collections. Others have type material deposited in museums as

stained spores on slides (e.g. Adriano et al., 2009; Kaur and Singh 2010, 2011; Mathews et al., 2016; Milanin et al., 2020). Less frequently the type material is preserved as spores in 5% formalin (Liu et al., 2014) or in a solution of glycerine-alcohol-formalin (Zhao et al., 2008). Following the suggestions of Donec and Schulman (1973), most of the syntype spores of a new species have been preserved in glycerine-gelatine gel (Molnár, 1997; Molnár et al., 2009; Zhang et al., 2010; Batueva, 2020). Nowadays, stained histological slides also serve as type material (Lom and Molnár, 1983; Picon-Camacho et al., 2009; Cech et al., 2012; Cardim et al., 2018). Since Andree et al. (1999) first used molecular techniques for the identification of *Myxobolus* species, this new method has become cardinal tool for the classification of myxosporeans, and a highly recommended requirement when describing a new species. Although sequence analysis is the most reliable way to taxonomic identification of myxosporeans, the morphological examination of

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spores is still an integral and essential part of species descriptions.

For determination of species-specific structure and dimensions, fresh mature spores are the most suitable because their size and transparency has not been modified by fixation and staining. However, there are some situations when interesting and important samples get to laboratories with a delay, so the collected spores need preliminary fixation. For their preservation, fixation in formalin or alcohol, and freezing are the most preferred methods for syntype material, but staining in smears or embedding them in glycerine-gelatine gel are also commonly used. The effect of these methods on the shape, size and structure of the spores is still not clear. Already in the early years of myxosporean research some authors such as Cépède (1906) and Kudo (1921) had pointed out that fixation and staining could change the shape and size of spores. Polyanski (1955) also remarked that fixation and staining, especially Schaudinn's fluid shrink spores of *Sphaeromyxa minuta* Thelohan, 1892. The effect of fixation, dehydration and staining on dimensions of myxosporidian and microsporidian spores were studied on a *Myxobolus* sp. and *Pleistophora ovariae* Summerfelt, 1964 by Parker and Warner (1970). The problem of fixation and long-term preservation of the material was studied in detail by Donec and Schulman (1973). They found that spores embedded in glycerine-gelatine gel under a coverslip preserved their shape and size the best. However, in some of our long time prepared preparations spores preserved in glycerine-gelatine tended to fade and it was difficult to observe their structure and prepare photographs (unpublished data). Besides fixation, Lom (1969) concluded that microphotography is the most reliable solution for studying spores.

The objective of this work was to evaluate the effects of different preservation methods on myxospore morphology, to determine which has the least impact on morphology relative to fresh material, and therefore what could be recommended for depositions in collections as type material. The authors studied the size and shape of fresh and frozen at  $-20^{\circ}\text{C}$  (cryopreserved) myxospores of *Myxobolus bramae* Reuss, 1906 and *M. bliccae* Donec and Tozzyakova, 1984 by comparing them with spores of the same plasmodium after fixation in 80% ethanol (ethyl alcohol) and 10% formalin or staining with Giemsa and Ziehl–Neelsen as smears.

## 2. Material and methods

Myxospores were obtained from plasmodia infecting two cyprinid fishes, the common bream *Abramis brama* (L.) and the vimba bream *Vimba vimba* (L.). Gills of a three-year-old, 21-cm-long common bream from Lake Balaton, and a three-year-old vimba bream from the River Ipoly were checked for *Myxobolus* plasmodia in April 2019. Mature plasmodia containing *Myxobolus* spores were found in both fish specimens. The common bream was infected with 17 plasmodia, each containing 5 to 10 thousand myxospores of *Myxobolus bramae* Reuss, 1906, while in the vimba bream three large plasmodia of *M. bliccae* Donec et Tozzyakova, 1984 were found, containing more than 20 thousand myxospores each. Three plasmodia of *M. bramae* from the same fish and a single plasmodium of *M. bliccae* were selected for further study. Plasmodia were opened with a needle and the spores released from each plasmodium were divided into 5 equal volumes and transferred to 1.5-ml Eppendorf tubes.

A portion of the spores was examined by microscopy in fresh state at a magnification of 1,000 (100 x objective lens and 10x ocular lens), another part was transferred into a microcentrifuge tube, the final volume adjusted to 500  $\mu\text{L}$  with saline solution, then frozen at  $-20^{\circ}\text{C}$ , and the third and fourth parts were preserved in 80% ethanol and in 10% formalin, respectively. For permanent preparation - from the remaining spores - smears were prepared on slides, air dried, fixed in absolute methanol for 15–30 s then Giemsa solution (Fluka, No. 48900, Buchs, Switzerland) was dropped directly on it for 1 min at RT; or stained according to Ziehl–Neelsen method (Krutsay, 1980). The cryopreserved spores and the samples fixed in 80% ethanol and 10% formalin were examined and photographed three months later. Fresh spores were

placed under a coverslip, gently flattened and microphotographs at immersion magnification were taken of about 100 spores. Similarly, Giemsa- and Ziehl–Neelsen-stained spores were photographed without coverslip but using immersion oil. In addition, some fresh spores of both *M. bramae* and *M. bliccae* were soaked with Lugol's solution under a coverslip for checking the presence of an iodophilous vacuole. The structure of the spores (size, shape and polar capsule) as well as the thickness of the wall with intercapsular appendix were studied and measured on original pictures magnified 200-fold. In each sample, the length, width and the thickness of the spore wall were measured on 50 spores. To record the data of the polar capsule, 25 spores were selected where the two polar capsules of the same size are located in one plane. The presence and visibility of the intercapsular appendage were evaluated in the spores especially where their triangular shape could be observed.

Statistical tests were executed only on the measurement dataset of *M. bramae* spores obtained from three cysts. The length and width of the differently treated (five levels of treatment: fresh, fixed in 80% ethanol, fixed in 10% formalin, frozen at  $-20^{\circ}\text{C}$ , and stained with Giemsa stain) spores of the three plasmodia were compared with repeated measures ANOVA tests. To account for non-independence of spores of each plasmodium, plasmodium ID was included as random effect. All statistical analysis were carried out in R 3.6.3 (R Core Team, 2020) at a significance level  $\alpha = 0.05$ .

## 3. Results

Spore sizes did not differ between fresh and fixed sample both of *M. bramae* (Table 1, Fig. 1) and *M. bliccae* (Table 2, Fig. 3). Post-hoc analyses with a Bonferroni adjustment (Table 3) revealed a statistically significant ( $p > 0.001$ ) reduction in size in case of staining fresh samples of *M. bramae* with Giemsa stain (Fig. 1e, Fig. 4). At the same time, although we could not include Ziehl–Neelsen-staining in the statistical analyses due to large differences in sample size, no obvious differences could be observed in the size of Ziehl–Neelsen-stained and unfixed or fixed spores (Fig. 1b,c).

The average length of *M. bramae* spores from the three plasmodia both in fresh and fixed state varied between 9.0 and 9.5  $\mu\text{m}$  (Table 1). The spores stained with Giemsa were shrunken significantly, and their length proved to be not longer than 7.8–8.2  $\mu\text{m}$  (Table 1). As regards the differences in the structure of spores, a triangular intercapsular appendix was clearly seen and suitable for measurement in half of the non-fixed and frozen specimens of *M. bramae*. The intercapsular appendix was also recognisable in some spores fixed in 80% ethanol and 10% formalin but its shape was mostly indistinct (Fig. 1b, c).

The size and shape of the polar capsules were appropriate for the identification of spores recorded by different methods, but the contour of the sporoplasms was difficult to distinguish in formalin- or ethanol-fixed and stained spores. Coils of polar tubules were visible only in heavily compressed fresh spores. No well-delineated iodophilous vacuoles were detected in spores treated with Lugol's iodine solution, but surprisingly the coils of polar tubules were clearly visible in them (Fig. 2a).

Although the spores of *M. bliccae* were 3–4  $\mu\text{m}$  larger than those of *M. bramae*, phototypes of their inner structure could be observed more easily. In the fresh spores the number of coils of polar tubules and the elongated triangular intercapsular appendix could be counted and studied, in addition to the shape, size and polar capsules of the spores (Fig. 3a). The majority of the spores were surrounded by a mucous envelope and no iodophilous vacuoles were recorded inside their sporoplasms (Fig. 2b). The size of fresh, frozen, formalin- and ethanol-fixed spores ranged between 12.5 and 13.2  $\mu\text{m}$ , while the Giemsa-stained spores (Fig. 3e) were smaller, and the average length proved to be only 10.4 and 11.7  $\mu\text{m}$  (Table 2). On the other hand, no remarkable size differences were observed between Ziehl–Neelsen-stained (Fig. 3f) and fresh or fixed spores.

**Table 1**  
Measurements of fresh, fixed, cryopreserved and stained *Myxobolus brama* spores in  $\mu\text{m}$ .

Spores	Length of spore	Width of spore	Length of polar capsule	Width of polar capsule	Length of intercapsular appendix	Thickness of spore wall
	Mean $\pm$ SD (Min -Max)	Mean $\pm$ SD (Min -Max)	Mean $\pm$ SD (Min -Max)	Mean $\pm$ SD (Min -Max)	Mean $\pm$ SD (Min -Max)	Mean $\pm$ SD (Min -Max)
<b>Cyst A</b>						
<b>fresh</b>	9.0 $\pm$ 0.4 (8.4–9.9)	7.2 $\pm$ 0.2 (7–7.5)	4.6 $\pm$ 0.15 (4.4–4.8)	2.6 $\pm$ 0.13 (2.4–2.8)	1.5 $\pm$ 0.22 (1.3–1.9)	0.6 $\pm$ 0.09 (0.5–0.7)
<b>fixed</b>	9.2 $\pm$ 0.46 (8.7–10.2)	7.5 $\pm$ 0.39 (6.9–8)	4.6 $\pm$ 0.24 (4.2–5.2)	2.6 $\pm$ 0.16 (2.3–2.8)	1.3 <sup>a</sup> (1.1–1.5)	0.6 (0.5–0.7)
<b>fixed in 80% ethanol</b>	9.5 $\pm$ 0.4 (8.9–10.4)	7.5 $\pm$ 0.26 (7.1–8.2)	4.3 $\pm$ 0.38 (3.5–5.0)	2.4 $\pm$ 0.13 (2.2–2.6)	0.9 (0.8–1.0)	0.6 (0.5–0.7)
<b>fixed in 10% formalin</b>	9.2 $\pm$ 0.4 (8.7–10)	7.2 $\pm$ 0.29 (6.7–7.6)	4.3 $\pm$ 0.14 (4.2–4.6)	2.3 $\pm$ 0.19 (2.0–2.8)	1.15 $\pm$ 0.2 (0.9–1.3)	0.61 $\pm$ 0.09 (0.5–0.7)
<b>freezing at –20 °C</b>	8.2 $\pm$ 0.42 (7.8–8.8)	6.4 $\pm$ 0.51 (5.8–7.4)	3.9 $\pm$ 0.3 (3.5–4.3)	2.26 $\pm$ 0.17 (2.0–2.6)	–	0.66 $\pm$ 0.05 (0.6–0.7)
<b>stained with Giemsa</b>						
<b>Cyst D</b>						
<b>Fresh</b>	9.5 $\pm$ 0.43 (8.7–10.4)	.4 $\pm$ 0.31 (7.0–7.8)	4.6 $\pm$ 0.19 (4.4–4.8)	2.6 $\pm$ 0.17 (2.2–2.8)	1.4 $\pm$ 0.17 (1.2–1.7)	0.6 $\pm$ 0.17 (0.4–0.9)
<b>fixed</b>	9.4 $\pm$ 0.45 (8.3–10)	7.3 $\pm$ 0.38 (7.7.8)	4.2 $\pm$ 0.15 (4.1–4.3)	2.3 $\pm$ 0.10 (2.2–2.4)	1.3 $\pm$ 0.2 <sup>b</sup> (1.3–1.4)	0.6 (0.5–0.7)
<b>fixed in 80% ethanol</b>	9.1 $\pm$ 0.4 (8.7–9.6)	7.4 $\pm$ 0.4 (6.5–7.8)	4.3 $\pm$ 0.17 (4.0–4.6)	2.4 $\pm$ 0.18 (2.1–2.6)	1.4 $\pm$ 0.2 <sup>c</sup> (1.1–1.5)	0.5 (0.4–0.6)
<b>fixed in 10% formalin</b>	9.2 $\pm$ 0.4 (8.5–9.9)	7.3 $\pm$ 0.38 (6.7–7.6)	4.25 $\pm$ 0.05 (4.2–4.3)	2.34 $\pm$ 0.08 (2.3–2.5)	– (0.9–1.0)	– (0.6–0.7)
<b>freezing at –20 °C</b>	7.8 $\pm$ 0.49 (7–8.7)	5.7 $\pm$ 0.42 (5–6.3)	3.9 $\pm$ 0.15 (3.5–4.1)	2.3 $\pm$ 0.14 (2.2–2.6)	–	0.5 $\pm$ 0.08 (0.4–0.6)
<b>stained with Giemsa</b>						
<b>Cyst E</b>						
<b>fresh</b>	9.5 $\pm$ 0.23 (9.1–10)	7.6 $\pm$ 0.32 (7–8.2)	4.6 $\pm$ 0.18 (4.4–4.8)	2.7 $\pm$ 0.19 (2.4–3.0)	1.6 $\pm$ 0.18 (1.3–1.7)	0.6 $\pm$ 0.8 (0.5–0.7)
<b>fixed</b>		7.6 $\pm$ 0.3 (7.2–8.5)	4.2 $\pm$ 0.18 (3.9–4.3)	2.4 $\pm$ 0.14 (2.2–2.6)	1.0 $\pm$ 0.2 <sup>c</sup> (0.9–1.3)	0.6 (0.5–0.7)
<b>fixed in 80% ethanol</b>	9.5 $\pm$ 0.31 (9.1–10)	7.4 $\pm$ 0.5 (6.7–8.7)	4.2 $\pm$ 0.18 (3.9–4.5)	2.5 $\pm$ 0.15 (2.2–2.6)	1.3 $\pm$ 0.2 <sup>c</sup> (1.3–1.4)	0.6 (0.5–0.7)
<b>fixed in 10% formalin</b>	9.4 $\pm$ 0.3 (9–10)	7.6 $\pm$ 0.4 (7–8.2)	4.1 $\pm$ 0.18 (3.9–4.5)	2.4 $\pm$ 0.13 (2.2–2.6)	1.2 $\pm$ 0.18 <sup>d</sup> (0.9–1.3)	0.46 $\pm$ 0.07 (0.4–0.6)
<b>freezing at –20 °C</b>						
<b>stained with Giemsa</b>	8 $\pm$ 0.52 (7.8–8.7)	6.3 $\pm$ 0.41 (5.8–7)	3.5 $\pm$ 0.24 (3.2–4.0)	2.0 $\pm$ 0.23 (1.7–2.4)	–	0.66 $\pm$ 0.05 (0.6–0.7)
<b>Ziehl–Neelsen</b>	9.0 $\pm$ 0.35 (8.8–9.8)	7.1 $\pm$ 0.2 (6.8–7.3)	4.1 $\pm$ 0.22 (3.8–4.5)	2.3 $\pm$ 0.16 (2.0–2.4)	–	– (0.5–0.7)

<sup>a</sup> rec. in: 7/50 spores.  
<sup>b</sup> 11/50 spores.  
<sup>c</sup> 9/50 pores.  
<sup>d</sup> 17/50 spores.  
<sup>e</sup> 10/50 spores.

**Table 2**  
Measurements of fresh, fixed, cryopreserved and stained *Myxobolus blicca* spores in  $\mu\text{m}$ .

Spores	Length of spore	Width of spore	Length of polar capsule	Width of polar capsule	Length of intercapsular appendix	Thickness of spore wall
	Mean $\pm$ SD (Min -Max)	Mean $\pm$ SD (Min -Max)	Mean $\pm$ SD (Min -Max)	Mean $\pm$ SD (Min -Max)	Mean $\pm$ SD (Min -Max)	Mean $\pm$ SD (Min -Max)
<b>fresh</b>	12.7 $\pm$ 0.4 (11.9–13.6)	10.2 $\pm$ 0.6 (9.1–10.9)	6.3 $\pm$ 0.45 (5.7–6.5)	3.7 $\pm$ 0.22 (3.5–3.9)	2.6 $\pm$ 0.3 (2.2–3)	0.62 $\pm$ 0.3 (0.5–0.8)
<b>fixed</b>	12.7 $\pm$ 0.4 (11.7–13.0)	10.4 $\pm$ 0.3 (10–10.8)	6 $\pm$ 0.4 (5.2–6.5)	3.6 $\pm$ 1.16 (3.5–4.0)	2.3 $\pm$ 0.28 (2.2.8)	– (0.5–0.7)
<b>fixed in 80% ethanol</b>	13.2 $\pm$ 0.36 (12.6–13.7)	10.6 $\pm$ 0.5 (10.0–13.1)	6.25 $\pm$ 0.15 (5.1–6.5)	3.65 $\pm$ 0.1 (3.5–3.9)	2.4 $\pm$ 0.25 (2.2–2.6)	– (0.6–0.8)
<b>fixed in 10% formalin</b>	12.8 $\pm$ 0.45 (11.9–13.6)	10.7 $\pm$ 0.52 (9.8–11.9)	6.2 $\pm$ 0.17 (5.1–6.8)	3.8 $\pm$ 0.17 (3.6–4.1)	3.2 $\pm$ 0.34 (2.6–3.8)	0.65 $\pm$ 0.1 (0.56–0.72)
<b>freezing at –20 °C</b>	11.4 $\pm$ 0.5 (10.4–11.7)	8.8 $\pm$ 0.36 (7.8–9.5)	5.8 $\pm$ 0.2 (5.6–6)	3.5 $\pm$ 0.06 (3.4–3.6)	–	– (0.6–0.8)
<b>stained with Giemsa</b>	12.1 $\pm$ 0.61 (11.2–12.9)	9.6 $\pm$ 0.40 (8.9–10.2)	6.0 $\pm$ 0.42 (5.3–6.4)	3.6 $\pm$ 0.24 (3.0–3.8)	– (1.7–2.1)	– (0.5–0.8)
<b>Ziehl–Neelsen</b>						

In spores fixed in formalin (Fig. 3c), the intercapsular appendices were mostly detectable but the coils of polar tubules in polar capsules were infrequently observed. In ethanol-fixed spores (Fig. 3b), the intercapsular appendix was difficult to study and the coils of the polar tubules in capsules could not be recognised. In frozen spores (Fig. 3d), the intercapsular appendix and the coils of polar tubules were visible. In some spores stained by the Ziehl–Neelsen method (Fig. 3f), the coils of

polar tubules and the triangular intercapsular appendix could also be observed. No iodophilous vacuoles were recorded in spores treated with Lugol’s iodine solution but surprisingly the coils of the polar tubes inside the polar capsule were clearly discernible (Fig. 2b) and at the posterior end of some spores a piece of the mucous envelope got stained.

**Table 3**  
Effects of different methods on spore sizes (in  $\mu\text{m}$ ).

Size measurement	Predictor	numDF	denDF	F-value	p-value
Length of spore <i>n</i> = 795	(Intercept)	1	788	37513.93	<0.001
	Treatment	4	788	289.06	<0.001
Width of spore <i>n</i> = 729	(Intercept)	1	722	6831.11	<0.001
	Treatment	4	722	192.49	<0.001

Note: Table shows the results of repeated measures ANOVA tests of spore treatment.

**4. Discussion**

Measurements of fresh, frozen at  $-20\text{ }^{\circ}\text{C}$ , stained, 80% ethanol- and 10% formalin-fixed spores of *Myxobolus bramae* and *M. bliccae* showed a decrease in spore dimensions only during staining with Giemsa. No remarkable change was observed in the size and shape of spores after 3 months of cryopreservation or fixation in 80% ethanol and 10% formalin. So, these results did not support the concerns of Cépède (1906), Kudo (1921), Lom (1969) and Donec and Schulman (1973) about chemical preservation or freezing.

Our data partially correlate with the observation of Parker and Warner (1970) who found that - studying the effect of eight fixatives on the myxosporidian and microsporidian spores - the preservation induced shrinkage in the length of the spores that was less pronounced using formalin. Meanwhile the above-mentioned solutions did not, or slightly affected the width of spores. In our observations, no shrinkage was recorded at the fixation with formalin and ethanol. It should be noted that in our studies we always applied 80% ethanol as a fixative fluid, in contrast to Parker and Warner (1970), who used absolute ethyl alcohol. The differences between the spore dimension data presented here and those of Parker and Warner (1970) can be attributed to the different concentration of ethanol, specifically the stronger dehydrating effect of the absolute ethanol. At the same time, our records showed that the fixation of spores in 80% ethanol or 10% formalin negatively affected the transparency and visibility of polar tubules, intercapsular appendices and the sporoplasm. On the other hand, Giemsa staining resulted in significant differences in the size of spores and polar capsules, and their inner structure was unsuitable for description.

There were differences in morphological parameters among *M. bramae* spores obtained from different cysts (Table 1), correlation

with the findings of Zhai et al. (2016), who frequently observed the existence of intraspecific morphometric variation in myxosporidian species and underlined the importance of measurement ranges besides data on the average size of each species. In addition, Liu et al. (2022) reported a high degree of pleomorphism of *Myxobolus drjagini* (Akhmerov, 1954) myxospores in silver carp (*Hypophthalmichthys molitrix*). In our studies, we also recorded some morphometric variation and pleomorphism among spores of both species, but these deviations concerned only a limited number of the spores examined.

In general, cryopreservation has a negative effect on animal tissues, but in our case the structure of the studied spores remained relatively intact during the freezing. Some publications whose authors prefer to use a staining method other than the most commonly applied Giemsa and Ziehl–Neelsen techniques refer also to spores as type materials. The effect of these stains on the measurements of the spores in most cases were not studied, but some of these procedures have positive effects on the better knowledge of the structure of the spores or their location in the host. For example, Úngari et al. (2021) stained spores carried by the blood stream with eosin methylene blue according to Giemsa (EMB) method, and Kato et al. (2017) successfully detected the undeveloped polar tubules in the rudimentary polar capsules of *M. paratoyamai* using Diff-Quik stain.

Before the genus *Myxosoma* became a synonym of *Myxobolus*, the iodophilic vacuole in the sporoplasm represented a decisive difference between them. The chemical most commonly used for detecting the

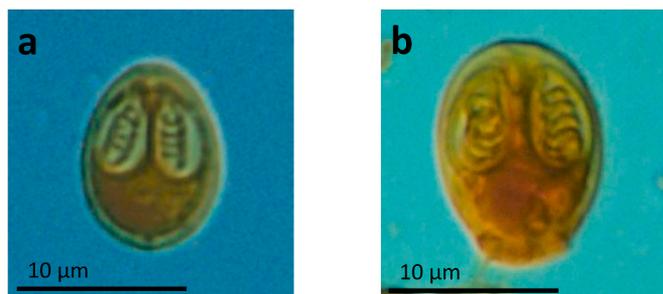


Fig. 2. (a) Myxospore of *M. bramae* treated with Lugol's solution. (b) Myxospore of *M. bliccae* treated with Lugol's solution.

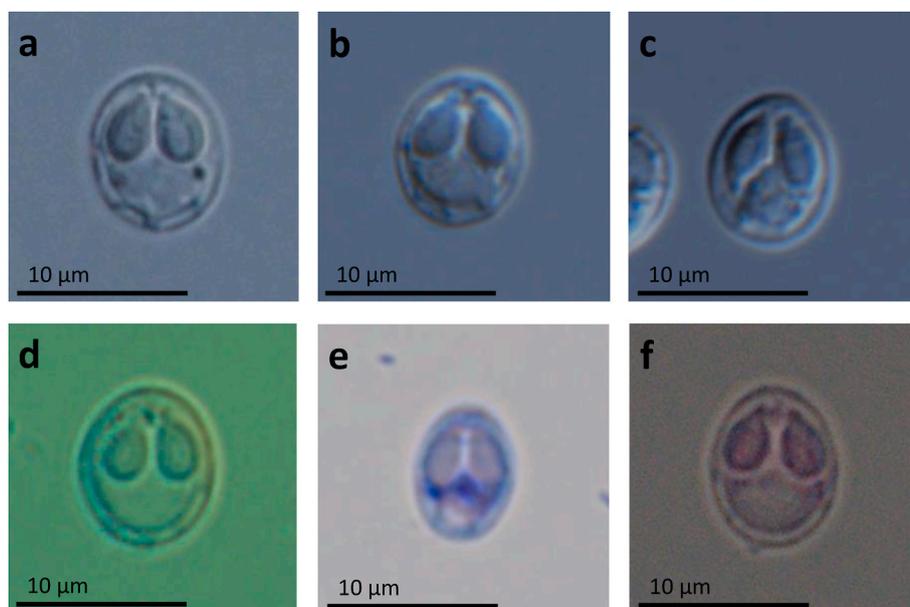
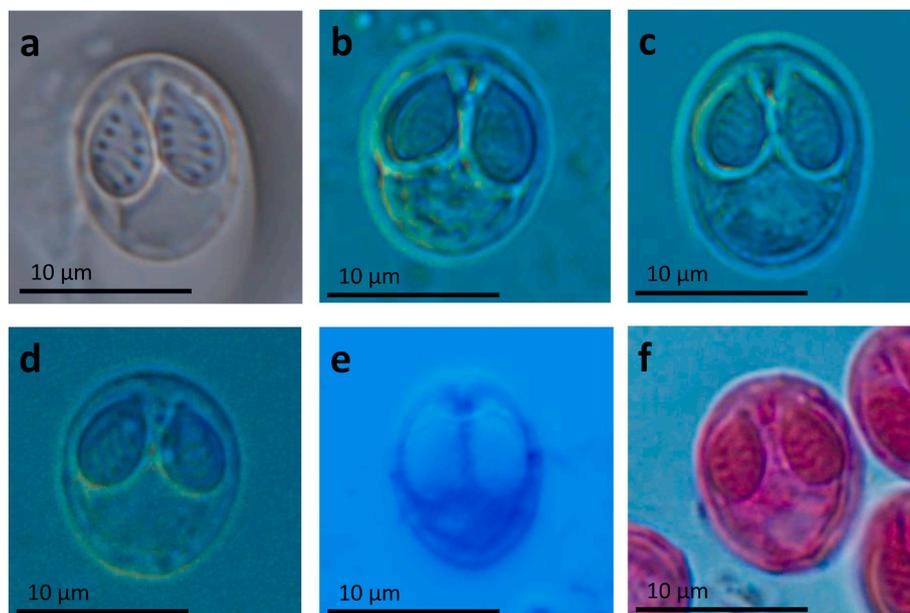
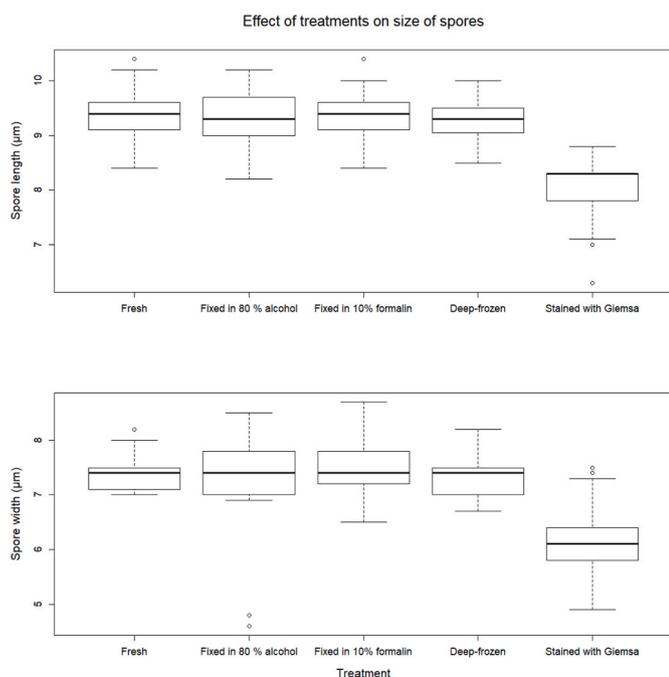


Fig. 1. Myxospores of *Myxobolus bramae* treated in different ways. (a) Fresh spore, (b) Spore fixed in 80% ethanol, (c) Spore fixed in 10% formalin solution, (d) Spore freezing at  $-20\text{ }^{\circ}\text{C}$  for 3 months, (e) Spore stained with Giemsa stain, (f) Spore stained with Ziehl–Neelsen stain.



**Fig. 3.** Myxospores of *Myxobolus bliccae* treated in different ways. (a) Fresh spore, (b) Spore fixed in 80% ethanol, (c) Spore fixed in 10% formalin solution, (d) Spore freezing at  $-20\text{ }^{\circ}\text{C}$  for 3 months, (e) Spore stained with Giemsa stain, (f) Spore stained with Ziehl–Neelsen stain.



**Fig. 4.** Length and width ( $n = 795$  and  $729$ , respectively) of differently treated spores of *Myxobolus bliccae*. Medians and interquartile ranges are indicated by thick middle lines and boxes, respectively, whereas the whiskers represent maximum and minimum values, and the open circles refer to outliers.

yellow staining starch content in the sporoplasmic vacuoles is Lugol’s solution.

Although the presence of the iodophilous vacuoles has been described for both species studied, we could not display them, which supports the opinion of Lom (1969) that these organelles are not always detectable. On the other hand, treatment of spores with Lugol’s solution has proved to be a useful tool for visualisation of the coils of polar tubules even in small-sized species like *M. bliccae*, in which these structures generally remain invisible or are difficult to observe.

### 5. Conclusions

Considering our results on the testing of fixation methods and depositing spores to collections the following can be suggested. When fresh material is not an option, or samples are to be saved in a collection, the best method, of those we tested, to preserve the size, structure and transparency of spores by preparing high quality microphotographs in different positions directly after opening the plasmodia, then fixing the spores in 80% ethanol and deposit them to collections as type material. Ethanol-fixed spores have some disadvantages compared to fresh ones but they serve two purposes: to present material for (1) further molecular study, and (2) re-examination of the size and shape of the original sample. The suspicion of Cépède (1906), Kudo (1921), Lom (1969), that fixation has some negative effects on the preserved spores was equally confirmed by Parker and Warner (1970), Donec and Schulman (1973) and by our experimental data as well; but we observed a significant decrease in the spore size only when the spores were stained with Giemsa.

In the process of Giemsa staining, the size of the spore and the polar capsule was reduced, while during the fixation in 80% ethanol or in 10% formalin and by freezing at  $-20\text{ }^{\circ}\text{C}$  no obvious shrinking was detected, but the transparency of spores decreased and the chance of observing the intercapsular appendix and tubular coils in the polar capsules reduced. Experiments with Ziehl–Neelsen staining show that it can be a useful method for studying the fine structure of spores. Other methods not studied by us may also contribute to a better knowledge of *Myxobolus* infections; among them, e.i. histological sections deposited to museums also are providing useful data on the location of plasmodia in the infected organ.

The results indicate that, in addition to using the phototypes of fresh spores, the fixation of specimens in ethanol for further molecular analysis is the best preservation method for the original material to be deposited to museums.

### Declaration of competing interest

The authors state that they do not have any conflicts of interest to declare.

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