ABSTRACT: The aim of this study was to evaluate the influence of preservation method on the results of parasite community studies. Two host species, European perch *Perca fluviatilis* and European bitterling *Rhodeus amarus*, were examined for parasites after having been subjected to 4 different storage treatments: freezing, preservation in 4% formaldehyde or 70% ethanol and transportation of live (fresh) fish as a control. Preservation prior to dissection resulted in a loss of information, leading to incomplete quantitative data (all preservation treatments), qualitative data (ethanol and formaldehyde preservation) and a lowered ability to determine parasites to species level based on morphology compared to dissecting fresh fish. Of the more abundant taxa, only crustaceans and acanthocephalans provided relatively even results between treatments. We conclude that preservation media, such as ethanol or formaldehyde, significantly affects the ability to obtain precise parasite community data; hence, we recommend the use of freshly sacrificed fish for parasite community studies whenever possible. Alternatively, freezing may prove acceptable for evaluating parasite community taxonomic composition.

KEY WORDS: Parasite community · Preservation methods · *Perca fluviatilis* · *Rhodeus amarus* · Methodology · Parasitological examination

INTRODUCTION

Parasite-mediated effects appear to be important drivers of biodiversity as they can shape host population dynamics, alter interspecific competition and influence energy flow (Hudson et al. 2006). A full understanding of the complex relationships between host and parasite diversity is important, therefore, for conservation management and public health (Hatcher et al. 2012). Accurate data on parasite diversity, based on the use of the most suitable methods for parasite collection and identification, are necessary for biological diversity measurement to determine the geographical distribution of individual species (Poulin 2014).

Fish parasites are commonly used as tools for the analysis of many aspects of fish ecology, including host biogeography, host–parasite evolutionary relationships and fish stock discrimination (Lester 1990, Byrnes & Rohde 1992, Brooks & McLennan 1993). Over the last half century, fish parasite communities have commonly been studied as model systems to describe general patterns in host–parasite associations (Kennedy 2009). If such studies are to be comparative (and comparable), however, the parasite community must be described with maximum precision.

Recent studies have shown that the results of parasitological examinations can be significantly affected by a range of methodological issues, such as the length of time fish are held alive prior to dissection (Kvach et al. 2016a) or the method used to sample the fish, e.g. electrofishing, beach seining or gillnetting (Kvach et al. 2016b). Accurate parasite community estimates are of particular importance for such studies, yet some authors have even cast doubt on the reliability of traditional fish parasite surveys (Williams et al. 1991), with one potentially important issue being the method used to preserve dead fish.
prior to dissection (Grutter 1995, Rubtsova & Sarabeev 2006).

Present knowledge suggests that the optimal methods for studying fish parasite communities in freshwater species are the use of electrofishing for sampling and dissection of fresh fish (i.e. stored alive) within 3 d of sampling (Kvach et al. 2016a,b). Occasionally, however, it may be impossible to dissect within the recommended period, especially when sampling at a significant distance from the place of dissection (see Kvach et al. 2017). In such cases, the fish are usually preserved, either by freezing (González & Poulin 2005, Aguirre-Macedo et al. 2007, Alarcos & Timi 2012) or by storage in 10% Kehsol® (Zander et al. 2000, Zander 2003), 4% formaldehyde (Timi & Poulin 2003, Zander 2005) or 96% ethanol (Sokolov et al. 2015). However, such methods have the potential to significantly influence the results of parasite community structure through possible misclassification of some taxa (e.g. see Lepitzki et al. 1994 regarding trematode species). Further, freezing may affect quantitative parasitological results as infection parameters and biodiversity indices are usually lower in frozen fish (Rubtsova & Sarabeev 2006). It has also been shown that small worms, such as monogeneans, can be easily overlooked in preserved fish and that extra effort may be required to find or dislodge specimens (e.g. the monogenean Neoheterobothrium hirame is only 0.3 mm long when newly attached; Ogawa 2000). In such cases, dislodging with a magnetic stirrer has been recommended following formaldehyde or ethanol preservation (Anshary et al. 2001).

The aim of this study was to evaluate the influence of such preservation methods on the results of parasite community studies, including quantitative and qualitative evaluations and the ability to determine parasites to species level based on morphology. To this end, we compared 4 fish storage treatments (transportation of live fish, freezing, preservation in 4% formaldehyde and 70% ethanol), using fish of the same species originating from the same population.

**MATERIALS AND METHODS**

Two host species were used for parasitological examination: European perch Perca fluviatilis and European bitterling Rhodeus amarus. Both species were sampled using electrofishing (Kvach et al. 2016b), the perch being sampled from the Hamry Reservoir (49.7326°N, 15.9129°E) and the bitterling from the River Morava (48.7797°N, 17.0692°E), both in autumn 2016. Four fish storage treatments were applied prior to dissection: transportation of live fish, cooling/freezing, preservation in 4% formaldehyde and preservation in 70% ethanol. Immediately after catching, a subsample of each species was preserved either by immersing in (1) 4% formaldehyde, (2) 70% ethanol or (3) by placing it in a plastic bag and storing in ice at or near 0°C in an insulated freezer box for future freezing. These semi-frozen fish were transported (within 1 h) to the Czech Academy of Science’s Institute of Vertebrate Biology (Brno), then immediately placed in a refrigerator at −20°C until dissected. Prior to preservation, each fish was humanely sacrificed by a sharp blow to the cranium, whereupon they were placed immediately into the preservation medium. No chemical anaesthetics were used in order to avoid any potential effect on parasite attachment. For the same reason, no sedatives were used for the transport of live fish. In addition, (4) a sample of live fish (herein termed fresh fish) was gently introduced into a transportation tank containing aerated river water to minimise stress, and then dissected (after humanely sacrificing) within 2 d (see Kvach et al. 2016a). Actual manipulation was kept to the absolute minimum and every effort was made to spare the fish from stress or pain.

In total, 125 perch and 122 bitterling were examined for parasites. The standard length (SL; mm) and total weight (Wt; g) of each fish was measured before dissection and only fish of similar size were examined (see Table 1). Perch were examined for all metazoan parasites. As tissue parasites (Digenea, metacercariae) were absent in perch at our study locality, bitterling, known to be an intermediate host for a range of parasitic species (Dávidová et al. 2011), were additionally collected and examined for tissue parasites, i.e. those found in muscle, mesen-

### Table 1. Mean (±SD) standard length (SL) and total weight (Wt) of fresh, frozen and preserved (formaldehyde and ethanol) fish dissected for parasite community assessment

<table>
<thead>
<tr>
<th>Preservation method</th>
<th>n</th>
<th>SL (mm)</th>
<th>Wt (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perca fluviatilis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>34</td>
<td>51.4 ± 3.4</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>Frozen</td>
<td>30</td>
<td>49.7 ± 3.5</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>30</td>
<td>50.1 ± 2.2</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>Ethanol</td>
<td>30</td>
<td>51.0 ± 3.6</td>
<td>2.2 ± 0.5</td>
</tr>
<tr>
<td>Rhodeus amarus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>32</td>
<td>42.0 ± 1.4</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>Frozen</td>
<td>30</td>
<td>41.3 ± 1.7</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>30</td>
<td>42.6 ± 1.8</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>Ethanol</td>
<td>30</td>
<td>41.1 ± 1.3</td>
<td>3.5 ± 0.4</td>
</tr>
</tbody>
</table>
tery, liver, kidney, gall bladder, swim bladder, gill tissue and eyes. Microparasites were examined immediately using an Olympus CX41 light microscope. Monogeneans were preserved in glycerine-ammonium-picrate (GAP) and prepared as semipermanent slides (Malmberg 1957). Digeneans, cestodes and nematodes from fresh fish were preserved in hot 4% formaldehyde (Cribb & Bray 2010), while acanthocephalans were pressed between 2 slides and then preserved in 70% ethanol. Worms from preserved fish were collected, sorted by taxa and preserved in 70% ethanol for further identification. Glochidia and crustaceans from both fresh and preserved fish were stored in 4% formaldehyde and identified under low microscopy. Cestodes and digeneans were stained using iron acetic carmine, dehydrated in ethanol of increasing concentration and mounted in Canada balsam as permanent slides (Georgiev et al. 1986). Acanthocephalans, nematodes and crustaceans were mounted in glycerol as temporary slides for light microscopy. All parasites were identified to species level or to the lowest possible taxa.

Data for each parasite species/taxon are presented as prevalence, mean intensity of infection and mean abundance (see Bush et al. 1997). The importance of parasites in the community was determined based on their abundance, using the scale of Zander et al. (2000): >2 = core species, 0.6 to 2 = secondary species, 0.2 to 0.6 = satellite species and <0.2 = rare species. Different aspects of parasite community structure were calculated using component community richness and the Shannon (H), Dominance (D) and Evenness (E) indices for fish in each treatment group (Magurran 2004). Diversity index values were calculated using PAST software (Hammer et al. 2001). The same software was also used to compare index values between parasite communities using permutation tests generating 1000 random matrices with 2 samples, the p-value being computed as for the bootstrap test.

Larval trematodes were pooled as soft cysts (including metacercariae of Bucephalus polymorphus and Petasiger radiatus) and hyaline cysts (including Cyathocotyle prussica, Holostephanus spp., Metorchis xanthosomus and Clinostomum complanatum). In the case of Bunodera sp. (cf. luciopercae), Acanthocephalus sp. (cf. lucii) and Holostephanus spp., some individuals could not be determined to species level; hence, all individuals of these taxa were treated as belonging to a single taxon (Bunodera sp., Acanthocephalus sp. or Holostephanus sp.) for community description/analysis, irrespective of whether determination to species was possible or not. Later, we determined the possible effect of treatment on the ability to determine these 3 taxa to species level (herein referred to as precision). Inter-treatment differences in parasite community composition (based on both binary Jaccard and quantitative Bray-Curtis similarity) and taxa richness were examined at both the component community (i.e. pooled for all fish in a treatment group) and infracommunity levels. While visual comparisons only were undertaken at the component community level (i.e. no tests performed), inter-treatment community composition differences at the infracommunity level were tested using permutational multiple analysis of variance (PERMANOVA; Anderson 2001), with p-values obtained using 999 permutations and visualised on the first 2 axes of a non-metric multidimensional scaling (NMDS) model. As the sample size of fresh fish was larger than that in other treatments (32 vs. 30 fish for bitterling, 34 vs. 30 fish for perch), we also provided values based on a rarefied sample of fresh fish (based on 1000 randomly chosen samples of 30 fresh fish) for each community composition comparison.

Differences in parasite abundance, infracommunity taxa richness and precision of species determination between the 4 treatments was tested using generalised linear models (GLM), with Poisson distribution (corrected for over-dispersion, i.e. quasipoisson) detected for abundance and taxa richness and binominal distribution for precision. Abundance was only tested in parasites/groups found in at least 10 fish. Analysis of precision was only undertaken on fish parasitised by Bunodera sp., Acanthocephalus sp. or Holostephanus spp. The Benjamini-Hochberg procedure (Benjamini & Hochberg 1995) was applied in order to control for false discovery rate in multiple comparisons between the 4 treatments (6 comparisons per response variable). All analyses were conducted using R statistical software v.3.2.4 (R Core Team 2015).

RESULTS

General results

In total, 12 parasite taxa were registered in perch (Table 2A). The genera Bunodera and Acanthocephalus were each represented by 2 groups, including individuals identified to species level (B. luciopercae and A. lucii) and those termed as Bunodera sp. (most likely B. luciopercae) and Acanthocephalus sp. (most likely A. lucii) only. The parasite community of
frozen fish had a significantly higher H index than fish preserved in formaldehyde or ethanol, and a significantly lower D (p < 0.01 for all comparisons) than all other treatments. There was no difference in E between treatments (Table 3).

A total of 8 parasite taxa were registered in bitterling, all occurring in fresh fish (Table 2B). Specimens of Holostephanus metacercariae unidentifiable to species level (termed Holostephanus spp.) most likely included the species H. cobitidis, H. dubinini and/or H. luhei. Differences in parasite community composition between treatments resulted in significantly higher D and a lower H index in formaldehyde-preserved fish compared to fresh and frozen fish, and higher E compared to fresh fish (all p < 0.05). Fresh fish displayed a higher H index and lower D than frozen fish (all p ≤ 0.001). Parasite community diversity in ethanol-preserved fish was comparable to that for all other treatments (all p > 0.05; Table 3).

Core/satellite species

Three core species were registered in fresh perch (Dermocystidium sp., Proteocephalus sp. plerocercoid and B. lucioperca) and one core species in formaldehyde-preserved perch (B. cf. lucioperca; Table 2A). B. cf. lucioperca and Dermocystidium sp. were recorded as secondary species in frozen and formaldehyde-preserved fish and Ergasilus sieboldi in fresh and frozen fish. All other parasites occurred as satellite or rare species (Table 2A).

In bitterling, Bucephalus polymorphus metacercariae were recorded as core species in fresh, frozen and formaldehyde-preserved fish, but as secondary species in ethanol-preserved fish. In fresh and frozen fish, Metorchis xanthosomus were registered as secondary species, together with Holostephanus spp. in fresh fish. All other parasite species occurred as satellite or rare species (Table 2B).
Eight parasite taxa were recorded in fresh bitterling (rarefied to 7.9 ± 0.021), 6 in frozen fish, 3 in formaldehyde-preserved fish and 3 in ethanol-preserved fish. There was a relatively high component community similarity (both Jaccard and Bray-Curtis) between fresh and frozen fish (Table 4), with both groups showing low similarity to formaldehyde- and ethanol-preserved samples (Table 2). While the parasite species list of formaldehyde-preserved fish matched that of ethanol-preserved fish (100% Jaccard similarity), the very low number of parasites recorded in ethanol samples resulted in a low Bray-Curtis similarity to all other treatments, including formaldehyde (Table 4).

**Parasite infracommunity**

For perch, mean infracommunity richness was significantly higher in fresh fish, compared with the 3 preservation methods (GLM, p < 0.05; degrees of freedom for all tests except the ‘Precision’ tests [see below] can be derived from Table S1 in the Supplement at www.int-res.com/articles/suppl/d127p213_suppl.pdf; Fig. 1), with no difference between the preservation methods (GLM, all p > 0.05; Fig. 1). For bitterling, fresh and frozen fish displayed significantly higher infracommunity richness than formaldehyde- and ethanol-preserved fish (GLM, all p < 0.05). There was no difference between frozen and fresh fish (GLM, p > 0.05); however, infracommunity richness was significantly higher in formaldehyde-preserved fish compared with those preserved in ethanol (GLM, p < 0.05; Fig. 1).

For all comparisons, the same results (i.e. significant or non-significant similarity) were obtained using Bray-Curtis quantitative or Jaccard binary measures of infracommunity similarity. For perch, the parasite infracommunity composition for fresh fish differed significantly from both frozen and ethanol-preserved fish (PERMANOVA, both p < 0.05; Fig. 2), but not from formaldehyde-preserved fish (PERMANOVA, p > 0.05; Fig. 2). No significant difference was observed between the 3 preservation methods (PERMANOVA, all p > 0.05; Fig. 2). For bitterling, fresh and frozen fish parasite
infracommunity composition differed significantly from that of fish preserved in formaldehyde or ethanol (PERMANOVA, all p < 0.05; Fig. 2). While there was no significant difference between fresh and frozen fish (PERMANOVA, p > 0.05; Fig. 2), formaldehyde-preserved fish infracommunity composition was significantly different from that in ethanol-preserved fish (PERMANOVA, p < 0.05; Fig. 2).

Abundance

No significant decrease was observed in the abundance of any parasite species or group when dissecting fresh fish (GLM, all p > 0.05 or < 0.05; Fig. 3). In most cases, however, at least one of the preservation methods resulted in a significant decrease in species and/or group abundance compared to fresh fish. Only 3 species (E. sieboldi, Argulus foliaceus, Acanthocephalus sp.) showed no significant difference between the 4 treatments (GLM, all p > 0.05; Fig. 3).

Compared to fresh fish, frozen fish had a significantly lower abundance in 2 taxa (Ancyrocephalus percae and Bunodera sp.), formaldehyde-preserved fish in 5 taxa and 2 groups (Dermocystidium sp., Proteocephalus cernuae, B. polymorphus, Holostephanus spp., M. xanthosomus; hyalin cysts, soft cysts) and ethanol-preserved fish in 7 taxa and 2 groups (Bunodera sp., A. percae, P. cernuae, Proteocephalus sp. plerocercoid, B. polymorphus, Holostephanus spp., M. xanthosomus; hyalin cysts, soft cysts). Frozen fish displayed a significantly higher abundance in 4 taxa and 2 groups (P. cernuae, B. polymorphus, Holostephanus spp., M. xanthosomus;
hyalin cysts, soft cysts) compared with formaldehyde- and ethanol-preserved fish (GLM, all p < 0.05). *Bunodera* sp. was the only group displaying a significantly lower abundance in frozen fish than the other 2 preservation methods: freezing resulted in a similar abundance to ethanol-preserved fish (GLM, p > 0.05), while both freezing and ethanol-preservation resulted in lower abundances than formaldehyde (GLM, p < 0.05). Finally, fish preserved in formaldehyde resulted in significantly higher abundances of *A. percae* and soft cysts, but significantly lower abundances of *Dermocystidium* sp., than fish preserved in ethanol (GLM, all p < 0.05).

**Precision**

For *Acanthocephalus* sp. (cf. *lucii*) and *Bunodera* sp. (cf. *lucipercae*), dissection of fresh fish resulted in significantly higher precision than any of the preservation methods (GLM, all p < 0.05; df can be derived from sample size provided in Fig. 4), with all parasites determinable to species. While there was no difference between preservation methods (GLM, all p > 0.05) for *Acanthocephalus* sp., *Bunodera* sp. were determined with greater precision in frozen fish than those in formaldehyde or ethanol (GLM, both p < 0.05). *Bunodera* sp. obtained from ethanol-pre-

![Fig. 3. Parasite abundance on live/fresh (Li), frozen (Fr), formaldehyde-preserved (Fo) and ethanol-preserved (Et) (A) perch and (B) bitterling. Bars: mean values predicted by a quasipoisson generalised linear model (GLM); lines: 95% CI. Treatments sharing a lowercase letter do not significantly differ from each other according to the GLM](Fig. 3B on next page)
served fish were determined with greater precision than those from formaldehyde-preserved fish (GLM, p < 0.05). No difference was observed between fresh, frozen or formaldehyde-preserved fish in determination of the genus *Holostephanus* to species (GLM, all p > 0.05; ethanol-preserved fish not included due to low sample size).

**DISCUSSION**

Our results indicate that fish preserved by freezing or storage in formaldehyde or ethanol provide a poorer description of the host's parasite community than fish stored live and sacrificed immediately prior to dissection, i.e. when detecting the maximum number of parasite taxa, collecting the maximum number of parasites of each taxonomic group and identification to the lowest possible taxonomic level. Freezing appears to provide adequate data on parasite species richness, but not abundance. Use of preservation media (i.e. formaldehyde, ethanol) had a significant effect on both quantitative and qualitative parasite community evaluations and species identification.

Parasites remain alive for a period following the host's death; hence, their movement and retention of typical colour and/or shape makes it easier to identify and collect samples. In preserved fish, however, all of
these characteristics are affected to differing degrees. Moreover, extraction of live parasites provides the opportunity to use appropriate methods of preservation prior to identification, e.g. hot formaldehyde for adult cestodes and trematodes (Cribb & Bray 2010) or GAP for monogeneans (Malmberg 1957). When dissecting frozen or media-preserved fish, the recommended methods for parasite preservation are less effective or impossible. On the other hand, storage of fresh fish before dissection has its disadvantages, including the necessity to transport the fish, storage under controlled and appropriate environmental parameters (e.g. temperature, oxygen) and dissection within a short period (Kvach et al. 2016a). This may not always be possible under normal circumstances and freezing or media-preservation provides the opportunity to undertake parasitological dissections at a later, more convenient time. Of the 3 preservation methods examined in this study, cooling/freezing immediately after collection appears to be the most convenient and accurate alternative method to transportation of live fish, with dissection of frozen hosts resulting in a relatively high parasite richness but decreased abundance. Our results correspond with those of Rubtsova & Sarabeev (2006), who recommended freezing for evaluation of parasite community taxonomic composition only (based on a comparison of frozen and fresh fish).

Freezing mainly appears to affect monogenean and adult digenean parasite abundance, causing them (monogeneans in particular) to detach from the fins and gills (though this might also have been caused by the cooling period prior to freezing applied in this study). Freezing also tends to increase the surface mucus on fish, especially on the gills and skin surface, further increasing the chances of overlooking parasites. Adult trematodes quickly begin degenerating on frozen hosts, probably through both autolysis and bacterial putrefaction. As the host’s internal organs freeze relatively slowly (compared to the outer surface), internal parasites remain alive for some time, allowing them time to change shape and lose structures important for species identification (Miller & Cribb 2008, Cribb & Bray 2010). Indeed, high numbers of damaged helminths were observed on frozen fish in both the present study and that of Rubtsova & Sarabeev (2006), who examined parasites on frozen grey mullet *Liza aurata*. While the use of molecular identification methods, such as DNA barcoding, reduces the importance of inaccurate morphological determination, these new molecular methods are still relatively expensive, hence the older morphological methods are still commonly used.

Unlike fresh or frozen fish, use of formaldehyde or ethanol preservation prior to dissection had a significant effect on parasite community parameters, resulting in a significant decrease in species richness and abundance as well as reduced precision when determining to species level. Formaldehyde and ethanol preservation both resulted in a distinct reduction in monogenean and gut parasite abundance (also noted in frozen fish), along with a significant decrease in the number of metacercariae tissue parasites (Fig. 3). Hence, parasite community studies based on media-preserved hosts may be based on incomplete data, making them incomparable with other studies. As an example, it is almost certain that 2 studies on Chinese sleeper *Percottus glenii* from the same region of Germany demonstrating differences in the parasite community (Sokolov et al. 2015, 4 parasite species; Kvach et al. 2017, 12 species), reflected the method of fish storage prior to dissection (ethanol and fresh fish, respectively) rather than any actual change in parasite diversity.

Isolation of monogeneans from host tissue was highly sensitive to both freezing and media preserva-
tion as dehydration caused the parasites to detach from the gills and skin, thus increasing the chances of overlooking them. As ethanol preservation appeared to cause more intensive dehydration than formaldehyde, the chances of missing parasites is much higher when using ethanol. Anshary et al. (2001) found that the most effective method for sampling monogeneans from ethanol or formaldehyde-preserved fish was by dislodging specimens with a magnetic stirrer and analysing the resultant sediment under a stereomicroscope. Any additional manipulation, however, increases the chances of damaging the parasites, reducing their usefulness for taxonomic studies and/or morphological species identification (Rubtsova & Sarabeev 2006). As such, use of a magnetic stirrer for collecting small monogeneans from the gills and fins of preserved fish would still result in a decline in the number of identifiable species.

None of the pre-dissection storage methods had any significant effect on the detection of gut parasites, though there were some difficulties regarding species identification when using media-preserved fish, with ethanol being the worst option. When necessary, however, molecular methods can be used for identifying ethanol-preserved parasites, though the number of sequenced parasite species in the public database remains limited. As mentioned above, preservation media dehydrate the worms and make them sensitive to manipulation. This is particularly true for digeneans and adult cestodes, which are both sensitive to manipulation under normal circumstances (Cribb & Bray 2010). Our data indicate that these parasites are particularly sensitive to manipulation in frozen and media-preserved fish, with a high proportion of specimens seriously damaged when extracted from the host. There is also the possibility that some parasites may be overlooked due to the level of damage in media-preserved fish. Interestingly, larval cestodes in preserved fish appeared less damaged than adult specimens, with the shape of the scoleces remaining unchanged in larvae (and probably adults) but destrobilation often causing adults to be indeterminable and indistinguishable from larvae. Acanthocephalans and nematodes were easily collected from frozen or media-preserved fish, though a high proportion of acanthocephalans exhibited invaginated proboscises. As the number and structure of hooks on the proboscis is important for acanthocephalan species identification (Amin 1985), worms with invaginated proboscises cannot be used for detailed taxonomic studies. With Pomphorhynchus tereticollis, for example, the main identification feature is the existence of proximal projections on the basal parts of the proboscis hooks located on the posterior proboscis half, which are only visible in live worms (Špakulová et al. 2011).

Tissue parasites, represented by metacercariae in this study, were not significantly affected by freezing and almost all proved useful for further isolation from cysts and for species identification. On the other hand, metacercariae collection and identification was highly sensitive to both ethanol and formaldehyde preservation, independent of cyst wall thickness. According to Lepitzki et al. (1994), who studied Cyatho cotyle bushiensis and Sphaeridiotrema pseudoglobulus from the faucet snail Bithynia tentaculata, freezing had no physical effect on cysts, though cyst abundance appeared lower in preserved snails than in freshly killed specimens. This was also confirmed by our own results. As the muscle of live and frozen fish absorb and reflect light differently due to alterations to the muscle’s physical structure during freezing and thawing, the average original absorbance spectrum of frozen fish tends to be lower than that for fresh fish (Uddin et al. 2005). Likewise, a similar decrease in absorbance is to be expected in media-preserved fish. Furthermore, both frozen and (especially) media-preserved muscle has a more compact structure than that of fresh fish, making it difficult to collect and identify metacercariae. Last, but not least, the cyst walls of metacercariae preserved in ethanol tend to be softer than those from frozen or fresh fish (Lepitzki et al. 1994), and as such are more easily damaged during the collection process. The extremely low number of metacercariae collected from ethanol-preserved fish in this study may well reflect damage caused during tissue compression.

The mobile ectoparasite group used in this study comprised 2 species, Argulus foliaceus and E. sieboldi (Table 2). Both species can easily leave the host during periods of stress (Grutter 1995) such as during host transportation. As our fish were preserved immediately after catching, this prevented them leaving the host, and as such, we would not expect any change in abundance caused by preservation method. Kvach et al. (2016a) demonstrated that dissection of fresh fish within 3 d of sampling had no significant effect on ectoparasite abundance. Further, as the chitinous external skeleton of crustaceans is relatively resistant to mechanical damage, identification of specimens to species level remains possible in preserved samples.

It should be noted here that, while many significant differences were shown in the parasite fauna of fresh and preserved fish, the actual differences are...
likely to be even larger as we used conservative tests to determine inter-treatment abundance. In particular, the relatively large confidence intervals used in the dispersion parameter estimates (quasi-poisson distribution) and the alpha level correction for multiple comparisons may have caused us to underestimate the negative effects of preservation media in some cases. Of those taxa showing non-significant impacts, only crustaceans and acanthocephalans showed relatively even results between treatments. On the other hand, some parasite groups (e.g. glochidia) were not abundant enough to test, and hence we cannot come to any conclusion on the appropriateness or otherwise of preservation media for studies focused on these species. Nevertheless, our results are strong enough to state that use of any preservation method (i.e. freezing, ethanol, formaldehyde) will result in a biased picture of the fish parasite community.

In conclusion, we provide a comparative analysis evaluating alternative methods of fish storage prior to dissection and discuss their impact on the results of parasite community studies. While parasites isolated from frozen fish can be used to evaluate parasite community taxonomic structure (and remain useful for molecular studies), abundance figures will be reduced. Formaldehyde-preserved fish may also be studied to evaluate the number of particular parasite species and remain useful for both gut parasites and metacercariae. Finally, while ethanol-preserved fish can be used for the study of gut parasites, this method is inappropriate for tissue parasites; though again, parasites remain useful for further molecular study. All preservation methods (freezing and media preservation) proved inappropriate for the study of helminth morphology.

**Ethics statement.** This research was undertaken in line with the ethical requirements of the Czech Republic and was approved by the appropriate ethics committee. All sampling, transportation, maintenance and care of experimental fish, as well as the methods of fish sacrifice, complied with legal requirements in the Czech Republic (§7 Law no. 114/1992 on ‘the protection of nature and the landscape’, and § 6, 7, 9, 10 Regulation no. 419/2012 on ‘the care, breeding and use of experimental animals’ and ‘project of experiments’ no. 030/2015, and approved by the Academy Ethics Committee according to §16, Law no. 246/1992 on Animal Welfare). The use of a sharp blow to the cranium follows the recommended methods for killing experimental animals set out in the above-mentioned laws (§ 9 regulation No. 419/2012), as specified for fish and amphibians. The researchers involved in this study (M.O., M.J. and P.J.) are certified according to Czech legal requirements (§17, Law no. 246/1992 on Animal Welfare) to work with experimental animals.

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