Alterations caused by parasitism play a central role in the ecology and evolution of the host. To estimate the intensity and pathogenicity of different parasite species and their impact on the host, identification of parasite species must be reliable, which requires appropriate fixation and preservation. The protocols used in helminthological studies apply different solutions such as formalin, alcohol and Zenker’s fluid (applied hot, with acetic acid or glycerine) to preserve parasites. Furthermore, before identification under the microscope, fixed worms should be mounted on slides in a drop of lactophenol or Hoyer’s solution to improve their transparency or stained (Soulsby 1982, MAFF 1986).

Unfortunately, these conventional methods of preserving adult nematodes for taxonomic purposes have limitations because of the toxicity of some solutions such as formaldehyde, lactophenol and chloral hydrate. These compounds can be irritant, allergenic or even carcinogenic, and so prolonged exposure to them should be avoided. These inconveniences will be eliminated if nematodes can be stored at low temperature using cryoprotectants.

There are a variety of reasons for which many types of biological materials are cryopreserved (Hubálek 2003). Parasitic protists and helminths can be cryopreserved using Me3SO or glycerol to maintain them viable, which is mainly important in species/strains with significant biological characteristics (drug resistance, high or low antigenicity, etc. – see Eckert 1988). Although numerous authors have studied cryopreservation of parasites (e.g. Eckert 1988, Pozio et al. 1988, Gill and Redwin 1995, Hubálek 2003, Miyake et al. 2004, Kopp et al. 2008), there are no reports on cryopreservation of adult nematodes for species identification based on morphological characteristics.

Glycerol is one of the most commonly used additives in cryopreservation of biological materials and was evaluated as cryoprotectant in the preservation of adult small nematodes at low temperature (-20°C) to preserve their morphological characteristics and to enable routine species identification. Glycerol was chosen for its ability to penetrate into cells, to render the cell membrane more plastic, to bind intracellular liquids to prevent excessive dehydration and formation of ice crystals in the cells, and to avoid health risk during its handling. Moreover, the authors have successfully used glycerol for cryopreservation of faeces to preserve whole parasite oocysts and eggs (Beraldo et al. 2004).

The purpose of the present study was to evaluate the effectiveness of glycerol cryopreservation of nematodes of the superfamily Trichostrongyloidea for morphological identification compared to preservation in buffered formaldehyde solution, and refrigeration and freezing of specimens without cryoprotectants.

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MATERIALS AND METHODS

Samples
Abomasal nematodes of the superfamily Trichostrongyloidea found in the abomasum of five roe deer (Capreolus capreolus Linnaeus) from the Julian Alps, northeastern Italy (Friuli Venezia Giulia region), were used. The abomasums were removed after isolation of the extremities by hunters and was stored at +4 °C until examination of their content.

The abomasum was cut open along the greater curvature over a bowl, washed with tap water and the abomasal content was sedimented into 1 litre plastic cone at +4 °C overnight. The supernatant was removed and the sediment was resuspended in a suitable volume, then mixed well and split into subsamples using 500 ml cones while in constant agitation. The second sedimentation took about 2 h at +4 °C. Finally, the supernatant was removed and the sediment was treated according to individual preservation protocols (see below). An aliquot was refrigerated at +4 °C until the parasite collection, which also served as a control (fresh sample, F). Fresh samples were examined within 15 h after reception and approximately 36 h after the death of the animal (carcass was maintained at approximately 12 °C).

Preservation protocols
(1) Glycerol cryopreservation (G50). Glycerol was added to abomasal aliquot into a plastic bottle until a final concentration of 50% was reached. The mixture was blended well and maintained at +4 °C overnight for equilibration. Following this, the sample was cryopreserved at -20 °C for at least one month. Before being used, the chilled mixture was thawed at +4 °C overnight and equilibrated to room temperature for three hours until worm collection.

(2) Formaldehyde solution (FH). An abomasal aliquot was added with 4% buffered formaldehyde solution (formalin), adjusted to a final concentration of 2% and preserved at room temperature for one month. Before analysis, formalin was removed from the sample by four consecutive sedimentations in water (totally spent time: two hours). This limits the risk of formalin fume inhalation during worm collection, which is a particularly time-consuming procedure. Some nematodes from the abomasum were also evaluated after clearing on glass slides in a drop of two different types of solution: lactophenol (crystals of phenol, lactic acid, glycerol and water) and Hoyer’s solution (arabic gum, glycerine, chloral hydrate and thymol).

(3) Freezing without cryoprotectants (FwC). An abomasal content aliquot was frozen at -20 °C without cryoprotectant for a month. Before using frozen sediments, they were thawed at +4 °C overnight and equilibrated to room temperature for three hours until worm collection.

Nematode collection and identification
The aliquots were made up to a volume of 500 ml by water addition, agitated vigorously and a 100 ml sample was transferred into a beaker, making sure dynamic stirring continued throughout the sampling process. Each 100 ml sample was examined separately by placing small quantities in a Petri dish and the worms were counted and sexed using a stereomicroscope. Further 100 ml samples were examined in a similar manner until at least 60 male nematodes were collected. Male nematodes were identified using different morphological keys (Drozdz 1965, Demolin 1984, Skrabin et al. 1991, Gibbons 2010). Nematode species preserved in different ways were documented by digital camera (Leica ICC50).

Comparative evaluation
Cephalic portion (details of the oesophagus), male genitalia and cuticular bursa (or male copulatory bursa) of nematodes were evaluated using a score protocol (from 1 to 5) based on three different parameters: morphology, namely, visibility of morphological details; transparency, i.e. the passage of light in the microscope without losing the contrast of the details to evaluate; elasticity, which is the specimen property of being handled without damage (this property makes it possible to move the nematodes on a glass slide easily without damaging). Scoring criteria for comparative assessment of the different parameters are shown in Table 1.

The study involved the observation of 378 adult abomasal nematodes on which about 3000 evaluations of the different parameters were performed. Different nematode species (abomasal parasite infracommunity) were assessed in proportional number with respect to their relative abundance. Comparative evaluation was done always by the same person who did not know which preservation methods had been used.

Statistical analysis
Kruskal-Wallis and Mann-Whitney U tests were used to examine the effects of different methods of nematode preservation on the selected parameters. Since multiple comparisons were made, a Bonferroni correction was calculated and applied. A P value ≤ 0.003 (0.05/15) was considered to be statistically significant.

Table 1. Scoring criteria (morphology, transparency and elasticity) applied to evaluate samples of nematodes.

<table>
<thead>
<tr>
<th>Morphology</th>
<th>Transparency</th>
<th>Elasticity</th>
</tr>
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<tbody>
<tr>
<td>1/5 not visible or barely visible</td>
<td>no transparency</td>
<td>extremely delicate specimen handling</td>
</tr>
<tr>
<td>2/5 only morphological profiles</td>
<td>scarce</td>
<td>specimen breaking after slight pressure on the coverslip</td>
</tr>
<tr>
<td>3/5 few morphological details</td>
<td>discrete</td>
<td>specimen breaking after repeated pressure on the coverslip</td>
</tr>
<tr>
<td>4/5 many morphological details</td>
<td>good</td>
<td>specimen resistance to strong and repeated pressure on the coverslip</td>
</tr>
<tr>
<td>5/5 perfectly recognizable morphology</td>
<td>excellent</td>
<td>extremely elastic specimen</td>
</tr>
</tbody>
</table>

RESULTS
The parasite component community of host samples was composed by 11 parasite species (mean 7 species/animal), all belonging to the superfamily Trichostrongyloidea: Haemonchus contortus (Rudolphi, 1803), Ostertagia koichida Popova, 1937, Ostertagia leptospicularis Asadov, 1953, Ostertagia lyrata Sjöberg, 1926, Spiculopteragia mathevossianii Ruchliadeu, 1948, Spiculopteragia spiculoptera (Guschanskya, 1931), Teladorsagia circumcincta (Stadelmann, 1894), Trichostrongylus axei (Cobbold, 1879), Trichostrongylus capricola Ranson, 1907, Trichostrongylus longispicularis Gordon,
1933; and *Trichostrongylus vitrinus* Looss, 1905. Effect of preservation method on the morphology, transparency and elasticity of the nematodes is summarized in Table 2. 

Evaluation of different methods has demonstrated that the quality of nematodes fixed with glycerol cryopreservation (G50) is generally better than that obtained with other methods, especially the formaldehyde solution (FH) method. In particular, the caudal portion with spicules, gubernaculum, genital conus, caudal cuticular bursa and telamon of G50 nematodes were well preserved, transparent and elastic, and thus these specimens were very easy to identify. This preservation status, although superior in quality, in some cases is comparable to that of fresh (F) nematodes, especially in the cephalic region. However, the possibility of reliable species identification of FH nematodes was improved by their clearing in Hoyer’s solution or in lactophenol. In fact, after the clearing, the observation of FH male genitalia improves significantly, facilitating the identification of the species, but without ever being comparable to G50, as shown in Fig. 1A,B.

The elasticity score was significantly worse for cleared lactophenol specimens (1.3/5) because the worms became very fragile and broke easily when the coverslip was gently moved to orientate them (Fig. 1C). In contrast, the elasticity of nematodes treated with Hoyer’s solution was comparable to that of nematodes processed by FH (2.4 vs 2.2/5). However, clearing by Hoyer’s solution made quickly some nematode structures (cephalic portion and caudal cuticular bursa) too diaphanous.

Freezing without cryoprotectants (Fwc) caused fewer nematode alterations compared to FH, but the morphology and transparency of male genitalia of nematodes fixed with formalin (F) and frozen without cryoprotectant (Fwc) were quite similar (Table 1).

**DISCUSSION**

The present authors (Beraldo et al. 2008) used successfully glycerol cryopreservation of samples of nematodes in a study of parasites of wild boar, but this protocol has not been experimentally validated. The results of the present comparative study clearly show that cryopreser-

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**Table 2.** Mean scores of morphology, transparency and elasticity of the abomasal male nematodes treated with different preservation methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Morphology</th>
<th>Transparency</th>
<th>Elasticity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cephalic region</td>
<td>male genitalia</td>
<td>cuticular bursa</td>
</tr>
<tr>
<td>G50</td>
<td>2.7 (90)a</td>
<td>4.4 (90)a</td>
<td>4.9 (90)a</td>
</tr>
<tr>
<td>FH</td>
<td>2.2 (100)b</td>
<td>2.1 (100)b</td>
<td>2.7 (100)b</td>
</tr>
<tr>
<td>FH+H</td>
<td>2.2 (48)b</td>
<td>2.8 (48)b</td>
<td>2.9 (48)b</td>
</tr>
<tr>
<td>FH+L</td>
<td>2.6 (40)a</td>
<td>3.3 (40)a</td>
<td>3.7 (40)a</td>
</tr>
<tr>
<td>F</td>
<td>3.1 (79)b</td>
<td>4.0 (80)b</td>
<td>4.2 (80)b</td>
</tr>
<tr>
<td>Fwc</td>
<td>1.3 (20)c</td>
<td>3.4 (20)c</td>
<td>4.0 (20)c</td>
</tr>
<tr>
<td>P(H)</td>
<td>0.00 (102.4)</td>
<td>0.00 (251.4)</td>
<td>0.00 (258.4)</td>
</tr>
</tbody>
</table>

Kruskal-Wallis with Bonferroni’s correction for multiple comparison; different superscript letters correspond to P value ≤ 0.003. F – fresh; FH – formaldehyde solution; FH+H – formaldehyde solution + Hoyer’s solution; FH+L – formaldehyde solution + lactophenol; Fwc – freezing without cryoprotectants; G50 – glycerol cryopreservation.
common in parasitological surveys when the size of the host population sampled is large.

From a practical point of view, the nematode recovery from abomasal content appears to be facilitated by glycerol as this substance improves nematode elasticity. This positively affects manipulation, especially in positioning the nematode on a slide for identification under the optical microscope. Indeed, the male caudal portion of nematodes fixed with glycerol cryopreservation (G50) is easily oriented in a dorsoventral plane by moving the coverslip to observe the arrangement of bursa the caudal ray of copulative bursa and genital accessories (Fig. 2). In contrast, nematodes fixed with formaldehyde solution (FH) are more fragile and their positioning on the slide is more labourious as they are rigid, often coiled, and their caudal bursa does not open adequately (Fig. 2).

The results have indeed shown that clarification by Hoyer’s solution and lactophenol facilitates the observation of morphological details of the FH nematodes. However, both solutions have drawbacks: Hoyer’s solution makes some worm structures overly transparent (cephalic portion and caudal cuticular bursa), whereas lactophenol dramatically reduces their elasticity (phenol is corrosive and dissolves soft tissues), thus hindering identification. Moreover, the main disadvantage of these solutions is that they are toxic.

Even though 36 hours had passed from the death of some animals, in general the nematodes stored at +4°C during sedimentation, appeared to be well preserved. Visibility of tiny morphological structures, transparency and elasticity of the tissues of fresh nematodes facilitate taxonomic identification, although the final scores were generally lower than those obtained with G50. These parameters seem to be adversely affected by the increase of the time between the recovery of the animal host and parasite collection and identification. Furthermore, the natural colour of nematodes (pinkish or reddish in the case of bloodsucking nematodes) was perfectly preserved in G50 nematodes, unlike those preserved in FH.

As previously mentioned, in ecological studies, in which a considerable number of animals are to be sampled, the carcasses are often frozen before necropsy and collection of parasites, which are then preserved in formaldehyde or alcohol. This condition was partly simulated by freezing the abomasal sediment with parasites without using a cryoprotectant (FwC), which was then compared to G50 and FH. This comparison indicated that FwC is a better preservation method than FH (in term of the parameters assessed), although the appearance and quality of FwC nematodes is not excellent and, in general, worse than that fixed with G50. Indeed, light microscopy shows the negative effects of the formation of ice crystals during nematode freezing, especially in small nematodes (5–6 mm long, species of the genus Trichostrongylus Looss, 1905), showed degeneration of the cuticular bursa membrane.

Fig. 2. Male caudal portions of Spiculopteragia spiculoptera, Ostertagia leptospicularis and Ostertagia kolchida; comparison between specimens preserved in formaldehyde (FH) and cryopreserved in glycerol (G50).
Moreover, glycerol cryopreservation does not preclude other types of investigation. In fact, G50 nematodes could potentially be used for histological and ultrastructural analysis (data not shown), and, as the glycerol should not damage DNA integrity, genetic studies (DNA sequencing) should also be possible (not yet tested by the present authors). However, further investigation is needed to fully understand the real influence of this cryopreservation method on nematode DNA integrity, particularly regarding the long-term preservation of specimens.

Cryopreserved samples remain unchanged through time. However, although, there are some problems, yet minor and easy to resolve, with the facilities and microbiological risk of the cryopreservation method. In contrast to formaldehyde preservation, cryopreservation requires the availability of a refrigerator (phase equilibration) and a freezer. Secondly, some microorganisms such as bacteria present in the abomasum/intestinal content remain viable in the glycerol solution and this represents a potential health hazard. However, this disadvantage can be solved by adopting protective equipment or by performing repeated washing of samples by sedimentation, thus eliminating most of the contaminants.

Glycerol cryopreservation method can be considered a safe option for ecoparasitological surveys, eliminating toxic substances that may endanger the operator (especially one who is inexperienced), and making all the activities up to worm identification easy. Developing a protocol for the morphological identification of large worms (such as ascarids and cestodes) cryopreserved using this method is now a priority.

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