The effect of ethanol concentration on the preservation of insects for biodiversity studies

Daniel Marquina[1,2], Fredrik Ronquist[1] & Piotr Łukasik[1,3]

[1] Department of Bioinformatics and Genetics, Swedish Museum of Natural History, Stockholm, Sweden.

[2] Department of Zoology, Stockholm University, Stockholm, Sweden.

[3] Institute of Environmental Sciences, Jagiellonian University, Krakow, Poland

Abstract

1. Traditionally, insects collected for scientific purposes have been dried and pinned, or preserved in 70% ethanol. Both methods preserve taxonomically informative exoskeletal structures well. Highly concentrated ethanol (95-100 %), preferred as a DNA preservative for molecular biology, has generally been assumed to make specimens brittle and prone to breaking. However, systematic studies of the correlation between ethanol concentration and specimen preservation are lacking.

2. Here, we tested how preservative ethanol concentration in combination with different sample handling regimes affect the integrity of seven insect species representing four orders, and differing substantially in the level of sclerotization. After the treatments, we counted the number of appendages (legs, wings, antennae or heads) that each specimen had lost.

3. We found that high ethanol concentrations indeed induce brittleness in insects. However, the magnitude and nature of the effect varied strikingly among species. In general, ethanol concentrations at or above 90 % made the insects more brittle and resulted in more shriveling, but insects with more robust or sclerotized exoskeletons actually retained more of their appendages at high concentrations. Surprisingly, neither freezing the samples nor drying the insects after immersion in ethanol had a negative effect on the loss of appendages. However, the morphology of the insects was severely damaged if they were allowed to dry.

4. While higher ethanol concentrations might positively affect long-term DNA preservation, there is a clear trade-off between collecting and preserving insects for morphological examination and genetic analysis, since the optimal ethanol concentration for the latter is detrimental for the former and vice versa. The trade-off needs to be considered in large insect biodiversity surveys and other projects aiming to combine molecular work with traditional morphology-based characterization of the samples.

Keywords: insects, ethanol, DNA preservation, morphology, collections

Introduction

The first records of the use of ethanol for the preservation of animal tissue date back to the mid 1600s, when Robert Boyle mentions that he successfully used the "Spirit of Wine" to preserve blood and soft parts of a human body, as well as a fish, for many months (Boyle, 1664). The knowledge of the preservative properties of ethanol, in combination with the discovery of cheap and effective ways of producing ethanol in high concentration, led to widespread adoption among naturalists, and ethanol has been the fixative most widely used in museum and private natural history collections since the 18th century. The attractiveness of ethanol as a drug has sometimes caused challenges, however. For instance, Carl Linnæus, the father of modern taxonomy, needed to apply for a special permission to import ethanol for preserving his collections after home distilling was temporarily banned in Sweden (von Linné, 1764).

During the last two decades, as the research focus has shifted from the analysis of morphological features to molecular work, including DNA sequencing and amplification, ethanol has remained the preferred preservative liquid. Ethanol is an excellent fixative for DNA for three reasons: it kills decomposing microorganisms; it removes water from the tissue, slowing down enzymatic processes; and it denatures the DNA, making it inaccessible to DNA-degrading enzymes (Srinivasan, Sedmak, & Jewell, 2002).

The standard ethanol concentration employed for preserving insects for morphological examination used to be 70 % (Martin, 1977). However, a higher concentration (95% or higher) is recommended for optimal preservation of DNA (Nagy, 2010). Although storing specimens for short periods in 70 or 80 % ethanol has proven good enough for PCR and sequencing (Carew, Coleman & Hoffman, 2018; Stein, White, Mazor, Miller & Pilgrim, 2013), it has been shown that degradation occurs in the long term, and that at these concentrations the DNA gets more and more fragmented over time (Baird, Pascoe, Zhou & Hajibabaei, 2011; Carew, Metzeling, Clair & Hoffman, 2017). Thus, it would appear advantageous to change the standard practice and preserve insects in higher concentrations of ethanol. This idea has not been widely adopted, however, because of the common assumption, apparently based largely on anecdotal evidence, that high grade ethanol makes the insects brittle and prone to damage during manipulation, most likely due to excessive tissue dehydration. To our knowledge, there is only one published study that has directly addressed the effect of high ethanol concentrations on insect preservation for morphological study: King & Porter (2004) used three species of ants to test the effects of ethanol concentration on the mounting of specimens, concluding that 95 % ethanol made the ants hard to mount and prone to breaking. Our own experience with other insect groups is similar. For instance, we have noted that cicadas (Hemiptera) become brittle when stored in 95% ethanol, while preserving them in 90 % ethanol made them more suitable for mounting and taxonomic characterization.

Increasingly, insect biodiversity inventories and ecological assessments are accompanied by or solely rely on DNA sequencing (e.g. Janzen et al., 2009; Shokralla et al., 2014; see Matos-Maraví et al. (2019) for a comprehensive review on insect genomics applied to biodiversity study). This leads researchers to collect samples in the field in higher concentration ethanol, which could be detrimental for the morphological integrity of the insects collected, potentially causing the loss of morphological characters and decreasing the value of the specimens for taxonomic description or further anatomical or ecological study. When the purpose is to store specimens for both DNA sequencing and morphological study, it now seems common practice to use 80% ethanol (e.g., Hallmann et al. 2017), even though there appears to be no hard evidence that this is optimal. There may well be a trend towards

preserving insects in even higher concentrations of ethanol, on equally shaky grounds.

Clearly, there is a need for experimental studies of the nature of the trade-off between the preservation of insects for DNA sequencing and for morphological study, so that an optimal strategy can be chosen depending on the intended use of the material, if it is difficult to find a sweet spot that is acceptable for all intended uses. Here, we take a first step towards filling this knowledge gap. Specifically, we examined the effects of increasing ethanol concentrations on the morphological fragility of seven species of insects, spanning four orders and representing different sizes and levels of sclerotization. We assessed the effects under three different sample handling regimes, which were chosen to emulate standard sample processing steps. In the first experiment, we subjected the samples to either gentle or vigorous agitation. In the second experiment, we transported samples manually by a walking or a running agent; we also shipped samples using a standard postal service. Finally, we subjected samples to freezing or drying.

Materials and Methods

Mock communities

For the experiments, we constructed artificial (mock) communities made up of seven species of insects (Figure 1A): *Macrolophus pygmaeus* (Hemiptera, Miridae), *Aphidoletes aphidimyza* (Diptera, Cecidomyiidae), *Drosophila hydei* (Diptera, Drosophilidae), *Dacnusa sibirica* (Hymenoptera, Braconidae), *Calliphora vomitoria* (Diptera, Calliphoridae), *Formica rufa* (Hymenoptera, Formicidae) and *Dermestes haemorrhoidalis* (Coleoptera, Dermestidae). In sake of readability, only the generic names will be used from now on in the article. Adults of these species represented a wide range of body shapes, cuticle hardness, and responses to varying ethanol concentrations and treatments based on anecdotal information and prior observations. Some species were commercially purchased and others were manually collected (Table S1). Specimens of *Macrolophus*, *Drosophila*, *Dacnusa*, *Calliphora* and *Formica* were first killed by freezing them at -20 °C for 1-2 h, and then placed in the experimental tubes at the

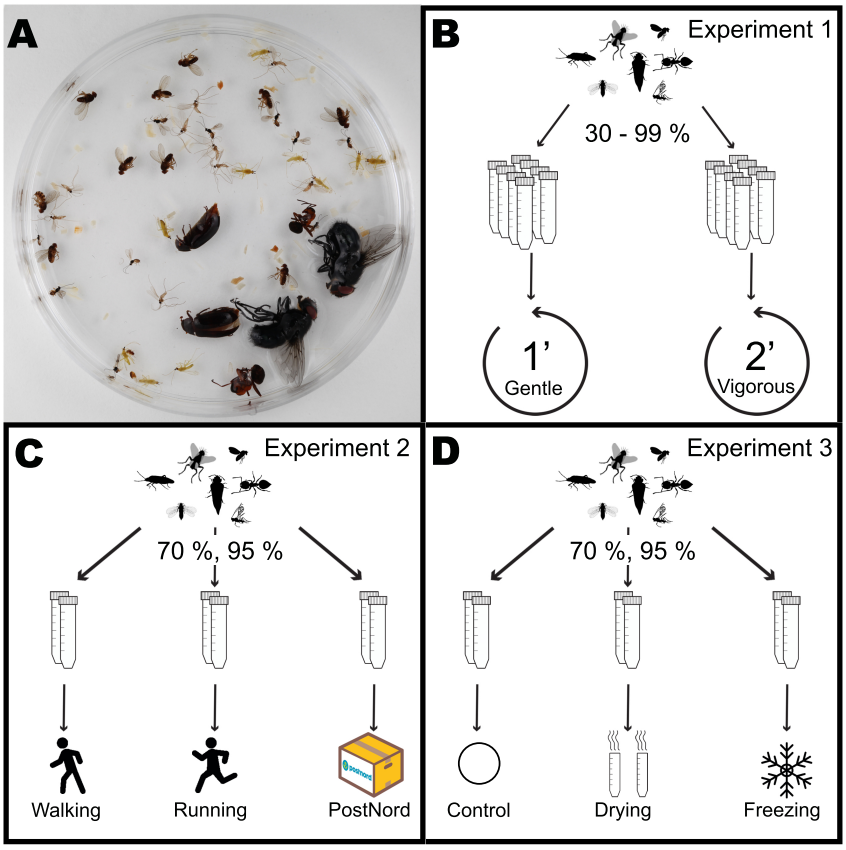


Figure 1. Each mock community sample (**A**) was assigned to an ethanol concentration and experiment. For Experiment 1 (**B**) five tubes per concentration (30 / 50 / 70 / 80 / 90 / 95 / 97 / 99) were subjected to Gentle or Vigorous shaking. For Experiment 2 (**C**) three tubes per concentration (70 / 95) were either carried by walking, running or sent by the Swedish national post service (PostNord) in two different parcels. For Experiment 3 (**D**) four tubes per concentration (70 / 95) were shaken under a Gentle regime after being either dried, frozen or left as control.

desired ethanol concentration (see below). Specimens of *Aphidoletes* and *Dermestes* were killed by directly placing them in the experimental tubes at the desired concentrations. All mock communities consisted of ten individuals of *Macrolophus*, *Aphidoletes*, *Drosophila* and *Dacnusa*, and two individuals of *Calliphora*, *Formica* and *Dermestes*. The specimens were kept in 50 mL Falcon tubes with a volume of 40 mL of ethanol of different concentrations. Communities were prepared over the course of approx. 2 weeks. Once all communities were ready, the ethanol was replaced with a fresh aliquot of ethanol at the same concentration, and kept for a month at room temperature to standardize the incubation before the treatments began.

The mock communities preserved were used for three experiments, where we measured the effect of ethanol concentration on the integrity of specimens subjected to different handling regimes, as described below.

Experiment 1: Effect of ethanol concentration on specimen brittleness

In the first experiment, we analyzed whether short-term preservation in high concentrations of ethanol alone increased the fragility of the insects. For the main experiment (Figure 1B), we used mock communities preserved in eight ethanol concentrations: 30, 50, 70, 80, 90, 95, 97 and 99 %, with 10 replicate tubes for each concentration. The tubes were subjected to two different shaking regimes by manually vortexing them in horizontal position for either 1 min ("Gentle" shaking, 5 tubes/concentration) or 2 min ("Vigorous" shaking, remaining 5 tubes/concentration).

Experiment 2: Effect of transport regimes

In the second experiment, we measured if the sensitivity to transport-induced damage was influenced by the ethanol concentration. For this experiment (Figure 1C), we used two ethanol concentrations: 70 and 95 %, with 12 mock community replicates in each. These tubes were transported in three ways, corresponding to treatments. Treatment "Walking" consisted of the experimenter (D.M.) carrying 3 tubes/concentration in the backpack home–to–work and work–to–home for a total distance of approx. 10 km (three days * 3.2 km), walking only. Treatment "Running" was similar but the experimenter (P.Ł.) ran, chasing after public transportation, for approximately half of the total distance of approx. 8 km (three days * 2.7 km). The last treatment, "PostNord", consisted of two shipments of 3 tubes/concentration, separated by one week, from the Swedish Museum of Natural History in Stockholm to Station Linné in Öland and back using the Swedish national post service. We reasoned that these three treatments would be representative of the range of handling regimes that insect samples collected in the field might experience in practice.

Experiment 3: Effect of storage and processing factors

In the third experiment, we tested if other types of damage can magnify the effects of high concentrations of ethanol on insect brittleness (Figure 1D). For each of the two ethanol concentrations tested (70 and 95 %), we used 12 replicate mock communities, four tubes/concentrations for each of the three pre-treatments. The first pre-treatment, "Freezing", was chosen to represent the effects of repeated freeze-thaw cycles that some Malaise trap samples may be experiencing during long-term storage. Specifically, we subjected our samples to three cycles of 16 h at -20 °C and 8 h at room temperature. The second pre-treatment, "Drying", tests the effects of drying. Drying of specimens is included in some non-destructive DNA extraction protocols (*e.g.* Nielsen, Gilbert, Pape, & Bohmann, 2019, Vesterinen et al., 2016) prior to digestion in the lysis buffer. Samples may also dry up accidentally, especially in difficult field conditions. In this treatment, we carefully poured away the ethanol from the tube, dried the insects for 24 h in the tube at room temperature, and afterwards added 40 mL of

ethanol at the original concentration. The last treatment was a control: the communities stayed in ethanol at room temperature during the time the other two treatments took place. After the pre-treatment, all tubes were manually vortexed in horizontal position for 1 min, corresponding to the "Gentle" treatment described in Experiment 1.

Scoring specimen damage

After each treatment, all individuals were inspected under a stereo microscope and scored for the number of lost appendages. The type of appendages scored for each species varied, and this was decided on the basis of pilot trials (see Supplemental Material). Specifically, we scored the loss of: head, legs, wings and antennae for *Macrolophus*, *Aphidoletes* and *Dacnusa*; head, legs and wings for *Drosophila*; legs and wings for *Calliphora* and *Dermestes*; and legs and antennae for *Formica*. Only forewings were considered (elytra in the case of *Dermestes*).

Data analysis

All analyses were conducted in R v3.3.3 (R Core Team, 2017) using the packages 'glmmTMB' (Brooks et al., 2017), 'DHARMa' (Hartig, 2019) and 'emmeans' (Lenth, 2019), and visualizations were generated with package 'ggplot2' (Wickham, 2016). For the analysis, all types of appendages were considered together (Appendages = Legs + Wings + Antennae + Head), and each species was analysed separately for each experiment. If the head was lost, the antennae were also scored as lost. A generalized linear mixed effects model was fitted to the data with the number of appendages lost as a function of Treatment and Concentration, and their interaction, as fixed effects, and Tube (= replicate) as random effect, assuming a log-link and Poisson distribution (function *glmmTMB* from package glmmTMB). As some species did not lose any appendages at all in certain treatments, zero inflation in the model was tested by simulating scaled residuals with the function *simulateResiduals* (package DHARMa) with the model without considering zero inflation, and checking the presence of zero inflation (function *testZeroInflation* from package DHARMa). If significant, a term for controlling zero inflation dependent on Concentration was included in the model. Subsequently, an analysis of variance (ANOVA) was used to determine whether Treatment, Concentration and their interaction had a significant effect on the number of lost appendages (function *Anova.glmmTMB* from package glmmTMB), and Tukey-adjusted pairwise differences between treatments were calculated (function *emmeans* from package emmeans).

Results

We observed large differences in the numbers of lost appendages between the experimental species, as well as between experiments, treatments and ethanol concentrations. *Aphidoletes* was the species that was most prone to lose appendages, followed by *Macrolophus* and then *Calliphora* and *Drosophila*. *Dacnusa* suffered the loss of fewer appendages than *Dermestes,* and *Formica* rarely lost any appendages. The results are discussed in more detail below; the raw counts for each experiment can be found in tables S2-4 of the supplemental material.

Experiment 1: Effects of ethanol concentration on specimen brittleness

For most insect species tested we found a significant effect of ethanol concentration on the number of broken or lost appendages, but the nature of the effect differed among species (Figure 2). For most species, the number of broken appendages rose at high concentrations of the preservative ethanol, although the magnitude of the effect varied dramatically, and only in some cases it was significant. The lowest concentrations of ethanol (30 or 50 %) were also associated with an increased loss of appendages compared to medium concentrations.

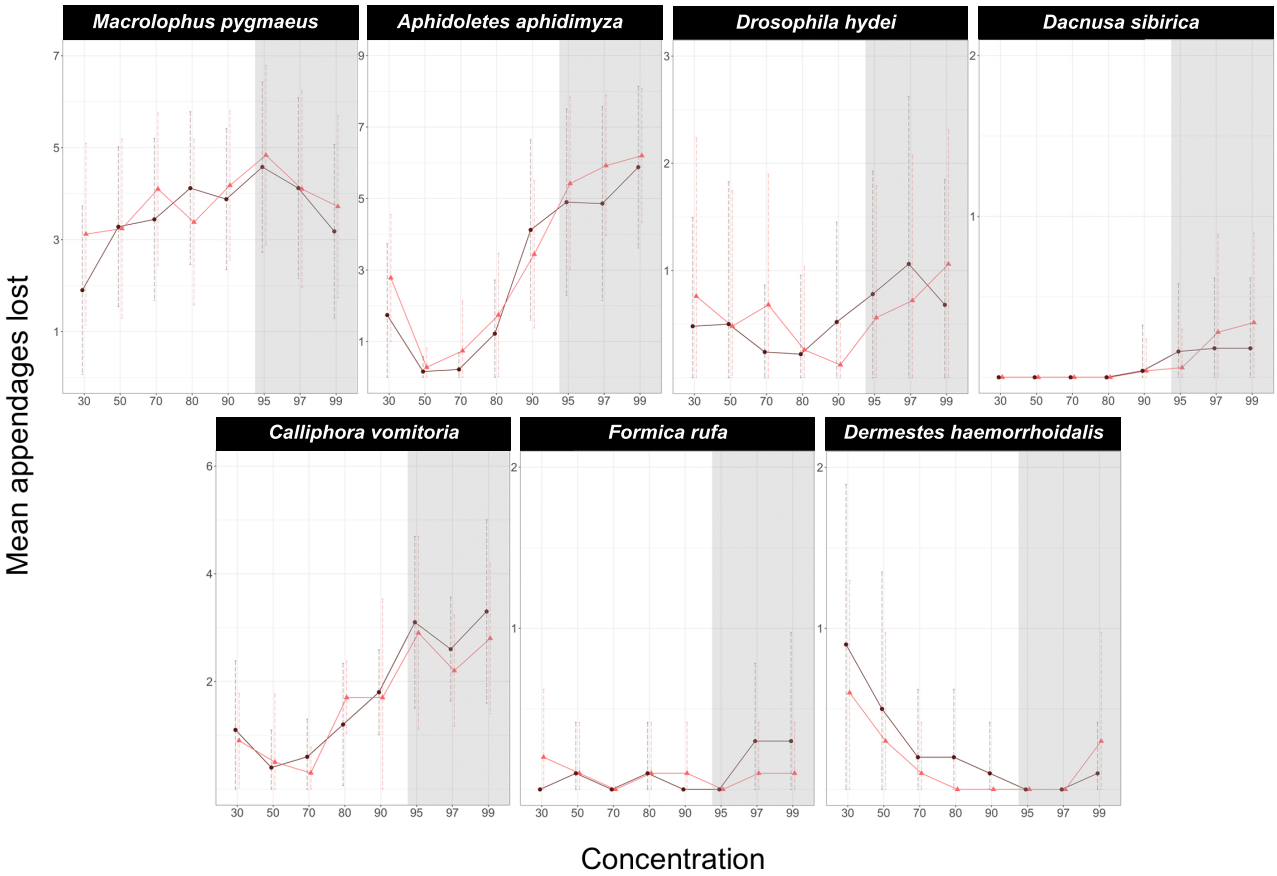


Figure 2. Effect of ethanol concentration on the number of appendages lost by each species. Dark purple circles represent the Gentle shaking regime while bright red triangles represent the Vigorous regime. The shadowed area corresponds to the ethanol concentrations in which DNA is optimally preserved according to literature.

However, the difference between our "Gentle" and "Vigorous" treatments, with a two-fold difference in vortexing time, was relatively small.

When fitted to a model, ethanol concentration alone had a very significant effect on the number of lost appendages in *Macrolophus*, *Aphidoletes* and *Calliphora* but not in *Dacnusa* or *Dermestes* (*Formica* did not lose enough appendages to fit a model; Table 1, Table S5). In both *Aphidoletes* and *Calliphora*, intermediate concentrations of ethanol were optimal for the preservation of appendages, whereas the pattern in *Macrolophus* was less clear. In *Aphidoletes* and *Drosophila*, the shaking regime as well as the interaction of concentration and shaking also had a significant effect on the number of appendages lost. Specifically, Vigorous shaking was more damaging at low and high concentrations of ethanol in both species. The same trend, although not significant, could also be seen at high ethanol concentrations in *Dacnusa* and *Dermestes* (Fig. 1).

The remaining three species turned out to be very durable regardless of the ethanol concentrations. In the case of *Formica*, only a few individuals lost one of the antennae. For *Dacnusa*, a small proportion lost antennae or in one case a wing, and only at ethanol concentrations > 90 %. *Dermestes* specimens sometimes lost elytra, particularly at low ethanol concentrations. However, for these three species, no significant differences were found based on concentration, shaking regime or the interaction.

Table 1. Effect of Concentration, treatment and their interaction in the number of lost appendages for each species in Experiment 1. *F. rufa* did not produce enough data points to fit a model. Shown are Type III test of fixed effects if the model described. Asterisks indicate levels of significance: * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001.

Effect	χ^2	Df	p-value
Macrolophus pygmaeus			
Concentration	35.0614	7	0.0001 ***
Treatment	1.7642	1	0.1841
Concentration:Treatment	12.4535	7	0.0866
Aphidoletes aphidimyza			
Concentration	170.2500	7	0.0001 ***
Treatment	4.0480	1	0.0442 *
Concentration:Treatment	18.350	7	0.0104 *
Drosophila hydei			
Concentration	10.1877	7	0.1781
Treatment	5.3045	1	0.0212 *
Concentration:Treatment	15.7197	7	0.0278 *
Dacnusa sibirica			
Concentration	4.3189	7	0.7424
Treatment	0.0000	1	1.0000
Concentration:Treatment	3.9310	7	0.7877
Calliphora vomitoria			
Concentration	24.5232	7	0.0009 ***
Treatment	0.6392	1	0.4239
Concentration:Treatment	2.3005	7	0.9413
Dermestes haemorrhoidalis			
Concentration	11.5213	7	0.1174
Treatment	0.3203	1	0.5714
Concentration:Treatment	1.6935	7	0.9748

Experiment 2: Effects of transport regimes

In the transport experiment only three species produced enough data points to fit a model: *Macrolophus*, *Aphidoletes* and *Drosophila* (Figure 3, Table 2, Table S6). For *Drosophila*, no significant differences were found between any transport treatment, ethanol concentration or their interaction. This is consistent with the results from Experiment 1, showing that *Drosophila* is quite robust to the two different shaking regimes we tried under a broad range of ethanol concentrations, including the two concentrations (70% and 95%) we used in the transport experiment.

For the remaining two species, *Macrolophus* and *Aphidoletes*, the effect of the transport treatment was highly significant. Mailing specimens (the Postnord treatment) stored in 95% ethanol resulted in a significant increase in the loss of appendages in both species. The samples of *Macrolophus* stored in 70% ethanol also clearly suffered from mailing, while the samples of *Aphidoletes* at this concentration were not significantly more damaged by mailing than by being carried around by a careful technician (the Walking treatment). The damage induced by a reckless technician (the Running treatment) was on par with or exceeded the damage caused by mailing the specimens when they were stored in 95% ethanol.

Table 2. Effect of Concentration, Transport and their interaction in the number of lost appendages for each species in Experiment 2. Only *M. pygmaeus*, *A. aphidimyza* and *D. hydei* produced enough data points to fit the model. Shown are Type III test of fixed effects if the model described. Asterisks indicate levels of significance: * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001.

Effect	χ^2	Df	p-value
Macrolophus pygmaeus			
Concentration	0.0000	1	1.0000
Treatment	19.3886	2	0.0006 ***
Concentration:Transport	0.8273	2	0.6612
Aphidoletes aphidimyza			
Concentration	0.5238	1	0.4692
Treatment	11.5596	2	0.0031 **
Concentration:Transport	21.5104	2	0.0002 ***
Drosophila hydei			
Concentration	0.9152	1	0.3387
Treatment	3.0386	2	0.2189
Concentration:Transport	1.6271	2	0.4433

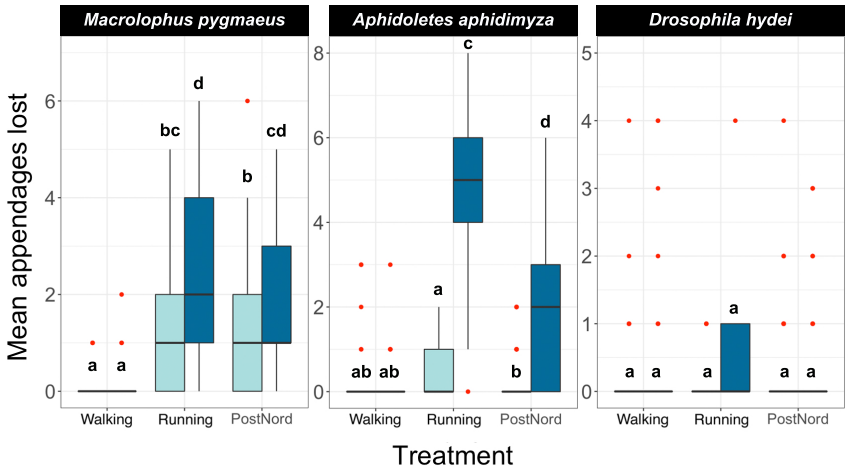


Figure 3. Effect of handling regimes and transport on the number of appendages lost by each species at two different concentrations of ethanol. Light blue boxes represent samples kept in 70 % ethanol and dark blue samples kept in 95 % ethanol. Red dots indicate outliers, and letters indicate groups of the Tukey Test of pairwise comparisons.

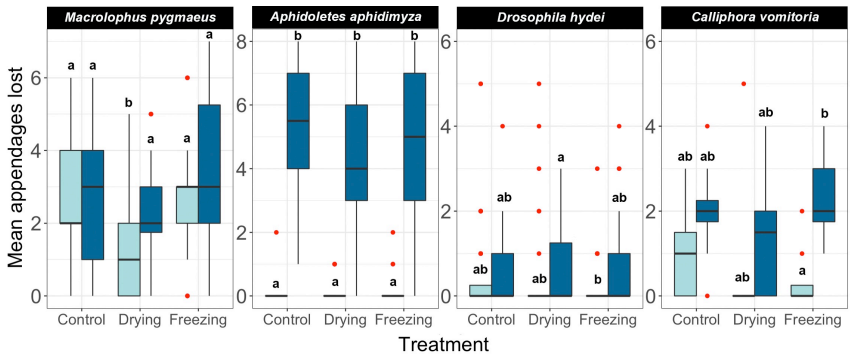
Experiment 3: Effects of freeze-thaw cycles and drying

As in the previous cases, the responses to the different treatments varied among species (Figure 4, Table 3, Table S7). The three toughest species (*Dacnusa*, *Formica* and *Dermestes*) had to be excluded from the analyses, as they did not generate enough data to fit a model. In the remaining species, we did not observe a significant treatment effect, that is, drying the specimens or exposing them to repeated cycles of freezing and thawing did not seem to affect their brittleness (Figure 4). The only exception was *Macrolophus*, where we observed a *positive* effect of drying the specimens. Specimens stored at 95% tended to be more brittle in all species except *Macrolophus*, although the effect was only significant in *Aphidoletes*. In *Macrolophus*, there was no clear difference between the brittleness of specimens stored at 70% and those stored at 95%. These species-specific responses to different ethanol concentrations closely match those seen in Experiment 1 (Fig. 1).

Discussion

It is surprising that there are so few quantitative studies of the effect of ethanol concentration on the preservation of insects for morphological study. One possible reason for this is the difficulty of quantifying morphological preservation. The criterion we used here, the number of lost appendages, has the major advantage that it is fast and easy to measure. However, it captures only one aspect of morphological preservation, namely brittleness. Brittleness is important in many contexts, for instance when handling, examining or mounting specimens. But brittleness is not an ideal measure of the preservation of morphological features in general, or the status of internal anatomy. For instance, we noted a clear discrepancy between brittleness and morphological preservation in the drying treatment, as noted elsewhere. Brittleness is easy to quantify and it is important in many contexts, so we believe our approach is a good starting point for further enquiry into the effect of ethanol concentration on the preservation of

Figure 4. Effect of different pre-treatments on the number of appendages lost by each species at two different concentrations of ethanol. All samples were shaken under a Gentle regime. Light blue boxes represent samples kept in 70 % ethanol and dark blue boxes represent samples kept in 95 % ethanol. Red dots indicate outliers, and letters indicate groups of the Tukey Test of pairwise comparisons.



insects. Nevertheless, it would be valuable with additional studies exploring more sophisticated measures of morphological conservation.

Table 3. Effect of Concentration, Treatment and their interaction in the number of lost appendages for each species in Experiment 3. *D. haemorrhoidalis*, *F. rufa* and *D. sibirica* did not produced enough data points to fit the model. Shown are Type III test of fixed effects if the model described. Asterisks indicate levels of significance: * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001.

Effect	χ^2	Df	p-value
Macrolophus pygmaeus			
Concentration	0.1984	1	0.6560
Treatment	21.4882	2	0.0002 ***
Concentration:Treatment	8.5936	2	0.0136 *
Aphidoletes aphidimyza			
Concentration	40.8062	1	0.0001 ***
Treatment	1.1408	2	0.5653
Concentration:Treatment	1.5768	2	0.4546
Drosophila hydei			
Concentration	0.6375	1	0.4246
Treatment	6.0051	2	0.0496 *
Concentration:Treatment	6.3404	2	0.0419 *
Calliphora vomitoria			
Concentration	0.1544	1	0.6943
Treatment	4.5955	2	0.1005
Concentration:Treatment	6.1628	2	0.0459 *

In general, our results are in line with expectations. They clearly show that ethanol concentration does have an impact on the fragility of insect specimens, even though the effects are not universal. Both low concentrations and high concentrations have a tendency to increase brittleness, especially in weakly sclerotized insects. We also noted a tendency of specimens to be more sensitive to agitation at both low and high concentrations of ethanol.

In general, the effect of both low and high concentrations of ethanol was most pronounced in the most weakly sclerotized species (*Aphidoletes* and *Calliphora* in particular). However, interestingly, the negative effect of low ethanol concentrations was noticeably also in *Dermestes*. These beetles were very robust to damage at high ethanol concentrations, but tended to lose elytra – abdomen and head too – at low concentrations. The effect was not significant but only two individuals of *Dermestes* were included in each tube. If we had included more specimens, we suspect that the difference could have been significant. A possible explanation for this unexpected response could be in the biology of the species. These beetles were obtained from the dermestarium at the Swedish Museum of Natural History, where they are used to clean off the soft tissue of vertebrate carcasses before preparing the skeletal parts for inclusion in the collections. Thus, these beetles presumably harbor a rich biota of decomposing microorganisms on their body surfaces, which could make them more susceptible to decomposition at low ethanol concentrations. However, this will remain speculation until the effects of low ethanol concentrations can be tested on a variety of beetles with different life histories. Our transport experiment clearly shows that the concentration of preservative ethanol has a strong effect on how fragile insect specimens are. Fragile specimens survive transport much better if stored in 70% ethanol than if stored in 95% ethanol (Figure 3). The experiment also demonstrates that it is important to handle samples carefully. A careless technician can cause considerably more damage than shipping the specimens more than 400 km by a much-criticized national mail service. It should be noted, however, that all tubes were filled with liquid to nearly maximum capacity before being shipped, as generally recommended for safe transport (Martin, 1977). The results might have been different if the tubes had been half-empty.

Subjecting preserved specimens to repeated freezing and thawing cycles did not have a major effect on their tendency to lose appendages. Similarly, drying the specimens and then reimmersing them in ethanol had no noticeable negative effect. In fact, drying had a positive effect on *Macrolophus* specimens, which lost fewer

appendages after drying than in the control treatment. However, these results do not mean that the morphology is preserved intact through these treatments. For instance, we observed that many individuals from most species that had been dried presented shrunken heads and abdomens that could difficult their taxonomic examination. It seems likely that also internal anatomy was affected both by the freezing-thawing cycles and by the drying-reimmersion treatment. Thus, it would be valuable to reinvestigate the effects of these treatments using other criteria for morphological preservation than the simple measure of appendage loss that we used.

As remarked previously, the effect of ethanol concentration varied strikingly among the different insect species we examined. Although our sample size is small, some results may indicate patterns that apply more broadly to particular taxonomic groups of insects. For instance, it is notable that both hymenopterans we examined, *Dacnusa* and *Formica*, were quite robust regardless of treatment, despite the fact that *Dacnusa* is a small species of relatively delicate build. These results seem to be in concordance with previous studies that show that hymenopteran bodies resist the entrance of ethanol to the point that sometimes small holes must be carved in their exoskeleton to allow DNA preservation (Dillon, Austin & Bartowsky, 1996; Mandrioli, 2008). However, long-term storage in absolute ethanol can nevertheless make ants brittle (King & Porter, 2004). The three dipterans we examined (*Drosophila*, *Calliphora* and *Aphidoletes*) were all quite sensitive to the ethanol concentration, regardless of striking differences in size and body shape. They were also consistently among the insects most affected by the different treatments we exposed them to. Coleoptera was represented by only one taxon in our study (*Dermestes*), but beetles are generally more sclerotized than other insects, and it is no surprise that *Dermestes* belonged to the most robust of the insects we studied. Hemiptera were similarly represented by a single taxon in our study (*Macrolophus*). This species was among the most fragile we studied, but the order does present a wide variety of body types, and future studies will have to show to what extent *Macrolophus* is representative.

In summary, our study may be the first systematic study of the effect of ethanol concentration on morphological preservation of insects. Our measure of morphological preservation, appendages lost, may not be ideal, but it does capture one aspect of preservation, brittleness, which is important in many contexts. Our results largely confirm the commonly held belief that intermediate concentrations of ethanol, around 70-80 % ethanol, are generally the best for morphological preservation, as high concentrations (above 90 %) tend to make specimens fragile. Unfortunately this means that there is a conflict between preserving insects for morphological and for molecular work, as the ethanol concentrations that are ideal for the former purposes result in higher degradation rates of DNA (Baird et al., 2011; Bisanti, Ganassi & Mandrioli, 2009; Mandrioli, 2008). Is it possible to find a treatment that is ideal for both purposes? Stein et al. (2013) showed that if initial preservation is made in 95 % ethanol, good PCR results can be obtained even after storing the insects in 70 % ethanol for an extended period of time. However, this is the exact contrary of what is recommended for morphological study, where a gradual increase in the ethanol concentration is recommended to avoid fast desiccation of the tissues. Whether initial preservation in 95 % and subsequent transfer to 70 % for storage reduces the brittleness is unknown; it is a treatment that should be tested in the future. For now, however, we do not have an ethanol preservation regimen that is ideal for both morphology and molecules. This must be taken into account when planning large collecting campaigns that aim to preserve material for both morphological and molecular work. For instance, we note that catches from Malaise traps, which are frequently used in insect inventories, contain a large portion of Diptera specimens (Hebert et al., 2016; Karlsson et al. 2020;

Ronquist et al., 2019), a group that is heavily affected by high ethanol concentrations. If Malaise trap inventory projects intend to preserve material for molecular *and* morphological study, it may be necessary to store the material at ethanol concentrations that are not ideal either for molecular nor for morphological work.

Acknowledgements

We are thankful to Dave Karlsson from Station Liné for receiving and returning the parcels with the tubes of the PostNord treatment. This project was supported by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska–Curie grant agreement no. 642241 (BIG4 project, https://big4-project.eu), by the Knut and Alice Wallenberg Foundation (KAW 2017.088) and by the Polish National Agency for Academic Exchange (NAWA; Polish Returns project PPN/PPO/2018/1/00015). We acknowledge PhyloPic platform (http://phylopic.org/) for the silhouettes of the insects in Figure 1, as well as the authors of the silhouette corresponding to *Dacnusa sibirica* (Maxime Dahirel (digitisation), Kees van Achterberg et al. (doi: 10.3897/BDJ.8.e49017)(original publication), CC BY 3.0) and to *Macrolophus pygmaeus* (Dave Angelini, CC BY 3.0).

Authors' Contributions

DM, FR and PL conceived and designed the study; DM and PL prepared the communities and conducted the experiment; DM collected and analysed the data, prepared the figures and wrote the first draft of the manuscript. All authors contributed critically to subsequent versions of the manuscript and gave final approval for publication.

Data Availability

All raw appendages counts and provenance of the insects used can be found in the tables of the supplemental material of this article.

References

Baird, D. J., Pascoe, T. J., Zhou, X. & Hajibabaei, M. (2011). Building freshwater macroinvertebrate DNA-barcode libraries from reference collection material: formalin preservation vs specimen age. *Journal of the North American Benthological Society*, 30(1), 125–130.

Bisanti, M., Ganassi, S. & Mandrioli, M. (2009). Comparative analysis of various fixative solutions on insect preservation for molecular studies. *Entomologia Experimentalis et Applicata*, 130, 290–296.

Boyle, R. (1664). *Some considerations touching the vsefulnesse of experimental naturall philosophy.* London: Henry Hall for Richard Davis.

Brooks, M. E., Kristensen, K., van Benthem, K. J., Magnusson, A., Berg, C. W., Nielsen, A., ... & Bolker, B. M. (2017). glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *The R journal*, 9(2), 378–400.

Carew, M. E., Metzeling, L., St Clair, R. & Hoffmann, A. A. (2017). Detecting invertebrate species in archived collections using next generation sequencing. *Molecular Ecology Eesources*, 17(5), 915–930.

Carew, M. E., Coleman, R. A. & Hoffmann, A. A. (2018). Can non-destructive DNA extraction of bulk invertebrate samples be used for metabarcoding? *PeerJ*, 6(1702), e4980.

Dillon, N., Austin, A. D. & Bartowsky, E. (1996). Comparison of preservation techniques for DNA extraction from hymenopterous insects. Insect *Molecular Biology*, 5(1), 21–24.

Hartig, F. (2019). DHARMa: residual diagnostics for hierarchical (multi-level/mixed) regression models. R package.

Hebert, P. D. N., Ratnasingham, S., Zakharov, E. V., Telfer, A. C., Levesque-Beaudin, V., Milton, M. A., … deWaard, J. R. (2016). Counting animal species with DNA barcodes: Canadian insects. *Philosophical Transactions of the Royal Society of London. Series B,*

Biological Sciences, 371(1702), 20150333.

Janzen, D. H., Hallwachs, W., Blandin, P., Burns, J. M., Cadiou, J.-M., Chacon, I., … Wilson, J. J. (2009). Integration of DNA barcoding into an ongoing inventory of complex tropical biodiversity. *Molecular Ecology Resources*, 9(s1), 1–26.

King, J. R. & Porter, S. D. (2004). Recommendations on the use of alcohols for preservation of ant specimens (Hymenoptera, Formicidae). *Insectes Sociaux*, 51(2), 197–202.

Karlsson, D., Hartop, E., Forshage, M., Jaschhof, M., & Ronquist, F. (2020). The Swedish Malaise Trap Project: A 15 Year Retrospective on a Countrywide Insect Inventory. *Biodiversity Data Journal*, 8, e47255.

Lenth, R. (2018). Emmeans: Estimated marginal means. Aka Least-squares Means, R. https://CRAN.R-project.org/package=emmeans

von Linné, C. (1764). *Museum Sae Rae Mtis Ludovicae Ulricae reginae.* Holmiae: Salvius.

Mandrioli, M. (2008). Insect collections and DNA analyses: how to manage collections? *Museum Management and Curatorship*, 23(2), 193–199.

Martin, J. E. H. (1977). *The insects and arachnids of Canada. Part 1: Collecting, preparing, and preserving insects, mites, and spiders*. Hull: Publication 1643, Research Branch, Canada Department of Agriculture.

Janzen, D. H., Hallwachs, W., Blandin, P., Burns, J. M., Cadiou, J.-M., Chacon, I., … Wilson, J. J. (2009). Integration of DNA barcoding into an ongoing inventory of complex tropical biodiversity. *Molecular Ecology Resources*, 9(s1), 1–26.

Matos-Maraví, P., Duarte Ritter, C., Barnes, C. J., Nielsen, M., Olsson, U., Wahlberg, N., … Antonelli, A. (2019). Biodiversity seen through the perspective of insects: 10 simple rules on methodological choices and experimental design for genomic studies. *PeerJ*, 7(1), e6727–31.

Nagy, Z. T. (2010). A hands-on overview of tissue preservation methods for molecular genetic analyses. *Organisms Diversity & Evolution*, 10(1), 91–105.

Nielsen, M., Gilbert, M. T. P., Pape, T., & Bohmann, K. (2019). A simplified DNA extraction protocol for unsorted bulk arthropod samples that maintains exoskeletal integrity. *Environmental DNA*, edn3.16–11.

R Development Core Team (2017). *R: A language and environment for statistical computing.* Vienna, Austria: R Foundation for Statistical Computing

Ronquist, F., Forshage, M., Häggqvist, S., Karlsson, D., Hovmöller, R., Bergsten, J., … Gärdenfors, U. (2019, July 2). Completing Linnaeus's Inventory of the Swedish Insect Fauna: Only 5000 Species Left. *bioRxiv*, 687392.

Shokralla, S., Gibson, J. F., Nikbakht, H., Janzen, D. H., Hallwachs, W. & Hajibabaei, M. (2014). Next-generation DNA barcoding: using next-generation sequencing to enhance and accelerate DNA barcode capture from single specimens. *Molecular Ecology Resources*, 14(5), 892–901.

Srinivasan, M., Sedmak, D. & Jewell, S. (2002). Effect of fixatives and tissue processing on the content and integrity of nucleic acids. *The American Journal of Pathology*, 161(6), 1961–1971.

Stein, E. D., White, B. P., Mazor, R. D., Miller, P. E. & Pilgrim, E. M. (2013). Evaluating Ethanol-based Sample Preservation to Facilitate Use of DNA Barcoding in Routine Freshwater Biomonitoring Programs Using Benthic Macroinvertebrates. *PloS One*, 8(1), e51273.

Vesterinen, E. J., Ruokolainen, L., Wahlberg, N., Peña, C., Roslin, T., Laine, V. N., … Lilley, T. M. (2016). What you need is what you eat? Prey selection by the bat Myotis daubentonii. *Molecular Ecology*, 25(7), 1581–1594.

Wickham, H. (2016). ggplot2: elegant graphics for data analysis. Springer.

Supplemental Material

Supplemental tables, as well as .csv files with the raw counts can be found in the electronic version of the article.