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

Daniel Marquina[1,2], Mateusz Buczek [3], 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

Corresponding author: Daniel Marquina. Address: Department of Bioinformatics and Genetics, Swedish Museum of Natural History, Stockholm, Sweden; Department of Zoology, Stockholm University, Stockholm, Sweden. Email: daniel.marquina@nrm.se

Running title: Insect fragility and DNA preservation in ethanol

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 but are suboptimal for preserving DNA. Highly concentrated ethanol (95 – 100 %), preferred as a DNA preservative, has generally been assumed to make specimens brittle and prone to breaking. However, systematic studies on the correlation between ethanol concentration and specimen preservation are lacking.

2. 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 preservation and treatments (various levels of disturbance), we counted the number of appendages (legs, wings, antennae, heads) that specimens had lost. Additionally, we assessed the preservation of DNA after long-term storage by comparing the ratio of PCR amplicon copy numbers to an added artificial standard.

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, but for species with robust, thicker exoskeletons, this did not translate to an increased loss of appendages. Neither freezing nor drying the insects after immersion in ethanol had a negative effect on the retention of appendages. We also found that DNA preserves less well at lower ethanol concentrations when stored at room temperature for an extended period. However, the magnitude of the effect varies among species; the concentrations at which the number of COI amplicon copies relative to the standard was significantly decreased compared to 95 % ethanol ranged from 90 % to as low as 50 %.

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

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. Consequently, ethanol has been the fixative most widely used in museum and private natural history collections since the 18th century. The attractiveness of ethanol for other human uses 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 tissues, slowing down enzymatic processes; and it denatures both the DNA and DNA-degrading enzymes, preventing further enzymatic degradation (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) has been recommended for the optimal preservation of DNA (Nagy, 2010). Although 70 % or 80 % ethanol is known to suffice as a DNA preservative for PCR and sequencing purposes during short-term storage (Carew, Coleman & Hoffman, 2018; Stein, White, Mazor, Miller & Pilgrim, 2013), it has been shown that DNA degradation occurs in the longer term, resulting in the molecule getting increasingly 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 ethanol concentrations. This idea has not been universally adopted, however, because of the common assumption that high grade ethanol makes the insects brittle and prone to damage during manipulation, most likely due to excessive tissue dehydration. However, 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 noted that cicadas (Hemiptera) become very brittle when preserved in 95 % ethanol (repeatedly replaced to prevent its dilution by insect body fluids) and subsequently mounted. Preserving them in 90 % ethanol made cicadas much more suitable for mounting and taxonomic characterization (K. Nazario and P. Łukasik, pers. obs).

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, researchers seem to be using a variety of approaches: from standardizing ethanol concentrations to 80 %, through opting for concentrations closer to 95 %, to starting with a high concentration and not measuring it again after insects are preserved, or relying on sub-zero temperatures as the primary means of preservation. There appears to be no hard evidence or consensus on which approach is optimal.

While it may be challenging to find a sweet spot that is acceptable for all intended uses, there is a clear need for experimental studies of the nature of the trade-offs between the preservation of insects for DNA sequencing and morphological study. Here, we take a few steps towards filling this knowledge gap. Specifically, we examined the effects of increasing ethanol concentrations on

specimen fragility and DNA preservation in seven species of insects, spanning four orders and representing different sizes and levels of sclerotization.

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). For better readability, only the generic names will be used from now on in the article. Adults of these species represent a wide range of body shapes, cuticle hardness levels, and responses to varying ethanol concentrations and treatments based on anecdotal information and prior observations. Specimens of 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 desired ethanol concentration (see below). Specimens of *Aphidoletes* and *Dermestes* were killed by placing them in the experimental tubes at the desired concentrations directly. 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 40 mL of preservative 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.

We used these preserved mock communities for three experiments, where we measured the effect of ethanol concentration on the morphological integrity of specimens subjected to different handling

127 regimes, as described below. Insects used for the first of these experiments were subsequently stored
128 long-term at the same ethanol concentration and used for the DNA preservation study.
129
130 *Experiment 1: Effect of ethanol concentration on specimen brittleness*
131 In the first experiment, we analyzed whether short-term preservation in high concentrations of ethanol
132 alone increased the fragility of the insects. For the main experiment (Figure 1B), we used mock
133 communities preserved in eight ethanol concentrations: 30, 50, 70, 80, 90, 95, 97, and 99 %, with ten
134 replicate tubes for each concentration. The tubes were subjected to two different shaking regimes by
135 manually vortexing them in a horizontal position for either 1 min ("Gentle" shaking, five tubes per
136 concentration) or 2 min ("Vigorous" shaking, the remaining five tubes per concentration). After each
137 treatment, all individuals were inspected under a stereomicroscope and scored for lost appendages.
138 The type of appendages scored for each species varied, and this was decided based on pilot trials (see
139 Supplementary Material). Specifically, we scored the loss of: head, legs, wings, and antennae for
140 *Macrolophus*, *Aphidoletes,* and *Dacnusa*; head, legs, and wings for *Drosophila*; legs and wings for
141 *Calliphora* and *Dermestes*; and legs and antennae for *Formica*. Only forewings were considered
142 (elytra in the case of *Dermestes*).
143
144 *Experiment 2: Effect of transport regimes*
145 In the second experiment, we measured if the sensitivity to transport-induced damage was influenced
146 by the ethanol concentration. For this experiment (Figure 1C), we used two ethanol concentrations: 70
147 and 95 %, with 12 mock community replicates in each. These tubes were transported in three ways,
148 corresponding to treatments. Treatment "Walking" consisted of the experimenter (D.M.) carrying
149 three tubes/concentration in the backpack home-to-work and work-to-home for a total distance of
150 approx. 10 km (three days * 3.2 km), walking only. Treatment "Running" was similar, but the
151 experimenter (P.Ł.) ran, chasing after public transportation, for approximately half of the total
152 distance of approx. 8 km (three days * 2.7 km). The last treatment, "PostNord", consisted of two
153 shipments of 3 tubes/concentration, separated by one week, from the Swedish Museum of Natural
154 History in Stockholm to Station Linné in Öland and back, using the Swedish national post service. We

155 reasoned that these three treatments would represent the range of handling regimes that insect samples
156 collected in the field might experience in practice. Damage induced to the individual insects was
157 assessed as above.
158
159 *Experiment 3: Effect of storage and processing factors*
160 In the third experiment, we tested if other types of treatment can magnify the effects of high ethanol
161 concentrations on insect brittleness (Figure 1D). For each of the two ethanol concentrations tested (70
162 and 95 %), we used 12 replicate mock communities, four tubes/concentrations, for each of the three
163 pre-treatments. The first pre-treatment, "Freezing", was chosen to represent the effects of repeated
164 freeze-thaw cycles that some Malaise trap samples may be experiencing during long-term storage.
165 Specifically, we subjected our samples to three cycles of 16 h at -20 °C and 8 h at room temperature.
166 The second pre-treatment, "Drying", tests the effects of specimen drying, included in some non-
167 destructive DNA extraction protocols (*e.g.*, Nielsen, Gilbert, Pape, & Bohmann, 2019, Vesterinen et
168 al., 2016) prior to digestion in the lysis buffer. Samples may also dry up accidentally, especially in
169 difficult field conditions. In this treatment, we carefully poured away the ethanol from the tube, dried
170 the insects for 24 h in the tube at room temperature, and afterwards, added 40 mL of ethanol at the
171 original concentration. The last treatment was a control: the communities remained in ethanol at room
172 temperature. After the pre-treatment, all tubes were manually vortexed in a horizontal position for 1
173 min, corresponding to the "Gentle" treatment described in Experiment 1. The damage was assessed
174 and scored as in Experiments 1 and 2.
175
176 *Experiment 4: Effect of ethanol concentration on DNA preservation*
177 In the last experiment, we used insect samples from the first experiment to test the effects of ethanol
178 concentration on DNA preservation (Figure 1E). After the brittleness assessment, the insects were
179 placed back in the tubes with their corresponding ethanol concentration and stored at room
180 temperature, in shaded boxes, for twelve months. Then, for each of the seven experimental species
181 preserved in eight ethanol concentrations (30, 50, 70, 80, 90, 95, 97, and 99 %), we took a single
182 insect specimen (*Dacnusa*) or a single insect leg (other species) from each of the five tubes from the

183 “Gentle” treatment. We reasoned that in specimens stored at lower ethanol concentrations after
184 extended storage, DNA is likely to be more degraded, containing fewer amplifiable copies of target
185 genes in the same amount of insect tissue. To test this, we quantified differences in PCR template
186 amounts across ethanol concentrations by adding the same amount of an artificial target to each
187 sample of a given species prior to DNA extraction. Subsequently, we used the extracted DNA to
188 prepare cytochrome oxidase I (COI) amplicon libraries that were then sequenced and assessed the
189 ratios of an artificial target to insect COI gene in the resulting amplicon datasets.
190
191 *Amplicon library preparation and bioinformatic processing (Experiment 4)*
192 Each sample (one entire specimen for *Dacnusa* or a single leg for the rest of species) was placed in a
193 2 mL tube, containing 190 µL lysis buffer (0.4M NaCl, 10mM Tris-HCl, 2mM EDTA, 2 % SDS), 10
194 µL of proteinase K and ceramic beads (2.8 mm and 0.5 mm). Samples were homogenized on an Omni
195 Bead Ruptor 24 homogenizer during two 30 s cycles at the speed of 5 m/s and then incubated at 56°C
196 for 2 hours. From this homogenate, 40 µL (corresponding to 20 % of the total lysate volume) was
197 mixed with a predefined amount of a linearized spike-in plasmid carrying an artificial COI target. The
198 plasmid contained a 471-base insert developed based on the *Calliphora vomitoria* barcode region
199 sequence, where outside of the conserved primer regions, approximately 40 % of bases were
200 substituted. Plasmid quantities were determined for each species during pilot experiments and ranged
201 from 11,000 to 317,000 copies per sample (Supplementary Material, Table S7). DNA was purified by
202 mixing 42 µL of each sample (40 µL of homogenate and 2 µL of plasmid) with 80 µL of home-made
203 Sera-mag SpeedBead solution, separating the beads on a magnetic plate, washing with 80 % ethanol
204 twice, and finally, resuspending the DNA in 20 µL of TE buffer.
205 COI amplicon libraries for 278 samples, plus six controls (two for lysis buffer, two for PCR, and
206 two for indexing), were prepared following a two-step PCR library preparation protocol (Method 4,
207 Glenn et al., 2011). In the first PCR, the 458-bp target region was amplified using template-specific
208 primers with variable-length inserts and Illumina adapter tails. After the bead-purification of products,
209 products were completed with Illumina adapters unique for each library in the second, indexing PCR.
210 The oligonucleotide sequences and reaction conditions are provided in Supplementary Material S5.

211 The pooled libraries were sequenced in a multiplexed Illumina MiSeq v3 lane (2 x 300 bp reads) at
212 the Institute of Environmental Sciences of Jagiellonian University (Kraków, Poland).
213 The amplicon data was processed using Mothur v1.44.2 (Schloss et al., 2009). Reads were merged
214 into contigs, had primers trimmed, were dereplicated, and singleton genotypes were removed. These
215 filtered data were then used for 97 % OTU picking. The commands used are provided in the
216 Supplementary Material. Next, the dominant OTUs corresponding to target insects and the artificial
217 target were identified, and for all samples, the insect OTU to artificial target OTU ratio was
218 calculated. Additionally, because for three species (*Drosophila*, *Formica*, and *Macrolophus*)
219 additional COI OTUs (likely corresponding to nuclear mitochondrial pseudogenes) were relatively
220 abundant, we repeated the analyses after summing up the reads of all OTUs that were at least 90 %
221 identical to the dominant sequence. We then translated the abundance ratios to an estimated number
222 of insect COI copies in each sample and standardized the values relative to the median value for 95 %
223 ethanol concentration for that same species.
224
225 *Statistical analyses*
226 All analyses were conducted in R (R Core Team, 2017) using the base package and the packages
227 'glmmTMB' (Brooks et al., 2017), 'DHARMa' (Hartig, 2019), and 'emmeans' (Lenth, 2019).
228 Visualizations were generated with package 'ggplot2' (Wickham, 2016). For the morphological
229 preservation analysis, all types of appendages were considered together (Appendages = Legs + Wings
230 + Antennae + Head), and each species was analyzed separately for each experiment. If the head was
231 lost, the antennae were also scored as lost. A generalized linear mixed effects model was fitted to the
232 data with the number of appendages lost as a function of Treatment and Concentration, and their
233 interaction, as fixed effects, and Tube (= replicate) as a random effect, assuming a log-link and
234 Poisson distribution (function *glmmTMB* from package glmmTMB). As some species did not lose any
235 appendages at all in certain treatments, zero inflation in the model was tested by simulating scaled
236 residuals with the function *simulateResiduals* (package DHARMa) with the model without
237 considering zero inflation, and checking the presence of zero inflation (function *testZeroInflation*
238 from package DHARMa). If significant, a term for controlling zero inflation dependent on

239 Concentration was included in the model. Subsequently, an analysis of variance (ANOVA) was used
240 to determine whether Treatment, Concentration, and their interaction had a significant effect on the
241 number of lost appendages (function *Anova.glmmTMB* from package glmmTMB), and Tukey-
242 adjusted pairwise differences between treatments were calculated (function *emmeans* from package
243 emmeans).

244 For the DNA preservation analysis, a linear model was fitted to the data with a logarithm-
245 transformed estimated number of COI target copies (relative to 95 % ethanol concentration median)
246 as a function of two factors: Species and Ethanol Concentration, and their interaction. As both factors
247 and their interaction were statistically significant (Species: df = 6, F = 88.31, $p < 0.001$; Ethanol
248 concentration: df = 7, F = 41.66, $p < 0.001$; Species x Ethanol Concentration: df = 42, F = 3.81, $p <$
249 0.001), data from each species were analyzed separately. In cases where the estimated number of COI
250 target copies depended significantly on ethanol concentration, contrasts were used to test for
251 differences between each ethanol concentration and 95 % ethanol concentration treatment, assumed as
252 the reference. The same analyses were conducted on the second dataset, containing all OTUs at > 90
253 % identity to the reference sequence.

254

255

256 **Results**

257 *Experiment 1: Effects of ethanol concentration on specimen brittleness*

258 For most insect species tested, we found a significant effect of ethanol concentration on the number of
259 broken or lost appendages, but the nature of the effect differed among species (Figure 2). In general,
260 the number of broken appendages rose with the concentration of preservative ethanol. However, the
261 magnitude of the effect varied dramatically, and only in some cases, it was significant. The lowest
262 ethanol concentrations (30 or 50 %) were also associated with an increased loss of appendages
263 compared to intermediate concentrations. However, the difference between our "Gentle" and
264 "Vigorous" treatments, with a two-fold difference in vortexing time, was relatively small.

265 When fitted to the model, ethanol concentration alone had a very significant effect on the number
266 of lost appendages in *Macrolophus*, *Aphidoletes,* and *Calliphora* but not in *Drosophila*, *Dacnusa* or

267 *Dermestes* (*Formica* did not lose enough appendages to fit a model; Table 1, Table S2). In both
268 *Aphidoletes* and *Calliphora*, intermediate concentrations of ethanol were optimal for the preservation
269 of appendages, whereas the pattern in *Macrolophus* was less clear. In *Aphidoletes* and *Drosophila*, the
270 shaking regime, as well as the interaction of concentration and shaking type, also had a significant
271 effect on the number of appendages lost. Specifically, Vigorous shaking was more damaging at low
272 and high concentrations of ethanol in both species. A similar trend, although not significant, could
273 also be seen at high ethanol concentrations in *Dacnusa* and *Dermestes* (Fig. 1).
274 The remaining three species turned out to be very durable regardless of the ethanol concentration.
275 In the case of *Formica*, only a few individuals lost one of the antennae. For *Dacnusa*, a small
276 proportion lost antennae or, in one case, a wing, and only at ethanol concentrations > 90 %. *Dermestes*
277 specimens sometimes lost elytra, particularly at low ethanol concentrations. However, for these three
278 durable species, no significant differences were found based on the concentration, shaking regime, or
279 their interaction.
280
281 *Experiment 2: Effects of transport regimes*
282 In the transport experiment, only three species produced enough data points to fit a model:
283 *Macrolophus*, *Aphidoletes,* and *Drosophila* (Figure 3, Table 2, Table S3). For *Drosophila*, no
284 significant differences were found between any transport treatment, ethanol concentration, or their
285 interaction. This is consistent with the results from Experiment 1, showing that *Drosophila* is quite
286 robust to the two different shaking regimes we tried under a broad range of ethanol concentrations,
287 including the two concentrations (70 % and 95 %) we used in the transport experiment.
288 For the remaining two species, *Macrolophus* and *Aphidoletes*, specimens carried by a careful
289 (Walking) technician sustained less damage than those carried by a reckless (Running) technician or
290 sent by mail (Postnord). When the samples were treated with care (Walking), preservative ethanol
291 concentration did not affect the number of appendages lost by either species. However, in those
292 treated with less care, including Running and Postnord treatments, both species sustained significantly
293 less damage when preserved in 70% ethanol.
294

295 *Experiment 3: Effects of freeze-thaw cycles and drying*

296 As in the previous cases, the responses to freezing/drying treatments varied among species (Figure 4,
297 Table 3, Table S4). The three toughest species (*Dacnusa*, *Formica,* and *Dermestes*) had to be
298 excluded from the analyses, as they did not generate enough data to fit a model. In the remaining
299 species, we did not observe a significant treatment effect, that is, drying the specimens or exposing
300 them to repeated cycles of freezing and thawing did not seem to affect their brittleness (Figure 4). The
301 only exception was *Macrolophus*, where we observed a mild *positive* effect of drying the specimens.
302 Regardless of treatment, *Aphidoletes*, *Drosophila*, and *Calliphora* lost more appendages on average
303 when stored at 95 %, although the effect was only significant in *Aphidoletes*. In *Macrolophus*, there
304 was no clear difference between the brittleness of specimens stored at 70 % and at 95 %. These
305 species-specific responses to different ethanol concentrations closely match those seen in Experiment
306 1 (Fig. 2).

307

308 *Experiment 4: Effect of ethanol concentration on DNA preservation*

309 For the 284 experimental libraries (278 insects and 6 blanks), we obtained a total of 6,061,251 read
310 pairs. Of these, 5,444,2555 reads, for 276 insect libraries (range 948 – 196,774, median 17,180 per
311 sample), passed all analysis stages and were used for abundance comparisons (Table S7). Across
312 libraries, the median abundance of the artificial target OTU relative to the dominant OTU for the
313 insect species was 0.136 (range 0.003 – 614); the value dropped slightly when all OTUs with > 90 %
314 identity to the reference sequence were combined (0.134). Negative PCR and indexing controls had
315 almost no COI reads (0 – 18), and in the DNA extraction controls, most reads (1760 – 8610, > 98.5 %
316 of the total) corresponded to the spike-in plasmid.

317 In all studied species, we found significant differences in the estimated COI target copy number
318 between ethanol concentrations (Table S8) (Fig. 5). A gradual decrease in the number of COI copies
319 with decreasing ethanol concentration is visible in all species, although the strength of the effect and
320 the concentration at which it sets in varies (Fig. 5). Post-hoc analyses revealed a significant decrease
321 in target copy numbers between at least some of the lower-concentration treatments and the 95 %
322 ethanol reference in all species. Specifically, the target copy number was significantly lower than in

323 the 95% reference in samples preserved in 30 % ethanol (7 out of 7 species), 50 % (7 species), 70 %
324 (5 species), 80 % (3 species) and in one of the species preserved at 90 % (*Macrolophus*). Samples
325 preserved in 97 % and 99 % ethanol did not differ significantly from the 95 % reference. The
326 magnitude of the effect of the ethanol concentration varied among species: for example, in samples
327 preserved at the lowest ethanol concentrations (30 and 50 %), the decrease in the number of COI
328 copies relative to the 95 % reference ranged from 3 to 440-fold.

329 Results obtained from a dataset with all OTUs at > 90 % identity to reference sequence for that
330 species considered do not differ qualitatively from the ones summarized above (Table S8).

331

332

333 **Discussion**

334 It is surprising that there are so few quantitative studies of the effect of ethanol concentration on the
335 preservation of insects for morphological and molecular study. One possible reason for this is the
336 difficulty of quantifying morphological preservation, but also the preservation of DNA for diverse
337 sequencing-based applications. Our approaches allowed us to address both of these challenges.

338 The criterion we used here to evaluate morphological preservation, the number of lost appendages,
339 has the major advantage that it is fast and easy to measure. However, it captures only one aspect of
340 morphological preservation, namely brittleness. Brittleness is important in many contexts, for
341 instance, when handling, examining or mounting specimens, but it is not an ideal measure of the
342 preservation of fine morphological details, or the status of internal anatomy. For instance, we noted a
343 clear discrepancy between brittleness and morphological preservation with the drying pre-treatment.
344 Nevertheless, we believe our approach is a good starting point for further enquiry into the effect of
345 ethanol concentration on the preservation of insects. Undoubtedly, it would be valuable to keep
346 exploring more sophisticated measures of morphological conservation.

347 To assess DNA preservation, we used a quick measure of one simple aspect of DNA degradation:
348 the absolute abundance of a certain 458-base long DNA fragment, part of the mitochondrial genome.
349 It is clearly not a perfect measure of the overall DNA preservation. However, as DNA degradation
350 happens primarily by the accumulation of random breaks within the DNA strands (although other

351 processes play a role, for example, deamination of cytosines), it is unlikely that many longer
352 fragments would remain once the abundance of 458-base targets decreases significantly. Thus, our
353 results are of direct relevance to sequencing approaches that do not require high-molecular-weight
354 DNA, but also informative to researchers that sequence long DNA fragments (*e.g.*, PacBio or Oxford
355 Nanopore sequencing).

356 The use of spike-in plasmids with artificial targets in amplicon sequencing experiments has been
357 gaining popularity as a method for the quantification of microbiomes (Tkacz, Hortala & Poole, 2018;
358 Tourlousse et al., 2017), while spike-in standards consisting of sequences of actual species have been
359 used for abundance estimation in eukaryotic metagenomic studies (Ji et al., 2020). However, to our
360 knowledge, they have not been used to assess DNA degradation. We found the absolute quantification
361 of COI targets using spike-ins to be a quick, inexpensive, and, in our opinion, accurate and
362 biologically informative way of gathering DNA preservation data.

363 In general, our results agree with expectations. They clearly show that ethanol concentration does
364 have an impact on the fragility of insect specimens, even though the effects vary among taxonomic
365 groups. High concentrations tend to increase brittleness, especially in weakly sclerotized insects. We
366 also noted a tendency of more disruptive treatments to have a stronger effect on specimens preserved
367 in high ethanol concentrations. Regarding DNA preservation, the expectations are also met, as we
368 observed a lower proportion of suitable PCR-template DNA fragments in the DNA extracts of those
369 individuals stored at lower ethanol concentrations after only one year.

370 The effect of ethanol concentrations on morphological preservation was most pronounced in the
371 most weakly sclerotized species (*Aphidoletes* and *Calliphora* in particular). As the connective and
372 muscular tissues are dried and made brittle by high ethanol concentrations, it is reasonable that insects
373 with less exoskeletal anchorage in their joints will be more severely affected. However, interestingly,
374 the negative effect of low ethanol concentrations was noticeable also in *Dermestes*. These beetles
375 were very robust to damage at high ethanol concentrations, but tended to lose elytra – abdomen and
376 head too – at low concentrations. The effect was not significant, but likely primarily because of lower
377 numbers of *Dermestes* individuals (two per tube) compared to other species. Had we included more
378 specimens, we suspect that this effect would have been significant. It would be interesting to test

379 whether the increased fragility at lower ethanol concentrations is associated with the cuticle hardness
380 that prevents preservative penetration into tissues, or perhaps decomposing microorganisms likely
381 harbored by these carcass-feeding beetles (these beetles were obtained from the dermestarium at the
382 Swedish Museum of Natural History, where they clean off the soft tissue of vertebrate carcasses
383 before the skeletal parts are included in the collections).

384 Our transport experiment clearly shows that the concentration of preservative ethanol has a strong
385 effect on how fragile insect specimens are. Fragile specimens survive transport much better if stored
386 in 70 % ethanol than if stored in 95 % ethanol (Figure 3). The experiment also demonstrates that it is
387 important to handle samples carefully. A careless technician can cause considerably more damage
388 than shipping the specimens more than 400 km by a much-criticized national mail service. It should
389 be noted, however, that all tubes were filled with liquid to nearly maximum capacity before being
390 shipped, as generally recommended for safe transport (Martin, 1977). The results might have been
391 different if the tubes had been half-empty.

392 Subjecting preserved specimens to repeated freezing and thawing cycles did not significantly
393 affect their tendency to lose appendages. Similarly, drying the specimens and then reimmersing them
394 in ethanol had no noticeable negative effect. In fact, drying had a slight positive effect on
395 *Macrolophus* specimens, which lost fewer appendages after drying than in the control treatment.
396 However, these results do not mean that the morphology is preserved intact through these treatments.
397 For instance, we observed that many individuals from most species that had been dried had shrunken
398 heads and abdomens that could complicate their taxonomic examination. It seems likely that also
399 internal anatomy was affected both by the freezing-thawing cycles and by the drying-reimmersion
400 treatment. Thus, it would be valuable to reinvestigate the effects of these treatments using other
401 criteria for morphological preservation than the simple measure of appendage loss that we used.

402 As remarked previously, the effect of ethanol concentration varied strikingly among the different
403 insect species we examined. Although our sample size is small, some results may indicate patterns
404 that apply more broadly to particular taxonomic groups of insects. For instance, it is notable that both
405 hymenopterans we examined, *Dacnusa* and *Formica*, were quite robust regardless of treatment,
406 despite the fact that *Dacnusa* is a small species of relatively delicate build. These results seem to be in

407 concordance with previous studies that show that hymenopteran bodies resist the entrance of ethanol
408 to the point that sometimes small holes must be carved in their exoskeleton to allow DNA
409 preservation (Dillon, Austin & Bartowsky, 1996; Mandrioli, 2008). However, long-term storage in
410 absolute ethanol can nevertheless make ants brittle (King & Porter, 2004). The three dipterans we
411 examined (*Drosophila*, *Calliphora* and *Aphidoletes*) were all quite sensitive to the ethanol
412 concentration, regardless of striking differences in size, body shape, and level of sclerotization. They
413 were also consistently among the insects most affected by the different treatments we exposed them
414 to. Coleoptera was represented by only one taxon in our study (*Dermestes*), but beetles are generally
415 more sclerotized than other insects, and it is no surprise that *Dermestes* belonged to the most robust of
416 the insects we studied. Hemiptera were similarly represented by a single taxon in our study
417 (*Macrolophus*). This species was among the most fragile we studied, but the order does present a wide
418 variety of body types, and future studies will have to show to what extent *Macrolophus* is
419 representative.

420 In general, the opposite pattern to the preservation of morphology was observed for the
421 preservation of the DNA: the higher the ethanol concentration, the better preserved the DNA was. For
422 most species, individuals preserved at concentrations of 80 % ethanol or lower produced significantly
423 fewer copies of the targeted PCR fragment than those preserved at 95 %. This shows that, even when
424 an amplification product can be obtained through PCR, there is, indeed, DNA degradation at these
425 ethanol concentrations. Interestingly, concentrations of 97 and 99 % ethanol never differ significantly
426 from 95 %, albeit in some species like *Dermestes* the average number of COI template copies was
427 visibly higher at both of these concentrations. The concentration at which significant degradation of
428 DNA was first observed varied among species. For four of the seven species, the preservation of the
429 DNA was significantly worse from 70 – 80 % and down, while for *Macrolophus,* even a
430 concentration as high as 90 % underperformed compared to 95 %. On the other hand, the DNA of
431 *Dacnusa* and *Calliphora* was not significantly degraded until the concentration was lowered to 50 or
432 30 %. The mechanisms underlying these differences are unknown to us. One of the possible
433 explanations offered above for the good morphological preservation of hymenopterans (namely, the
434 resistance of their bodies to the entrance of ethanol) is directly at odds with the good preservation of

435 DNA (extremely good in *Dacnusa*, quite good also in *Formica*). This suggests that the morphological
436 solidity of hymenopterans is due to other causes. For instance, the body is more distinctly divided into
437 hard sclerites in Hymenoptera than is the case for many other insect orders, like Diptera. Potentially,
438 this could be associated with an decreased tendency to lose appendages.

439 Regardless of the causes, the variation among taxa in the effects of ethanol concentration on DNA
440 preservation is highly relevant for metabarcoding studies. Metabarcoding involves the amplification
441 of a barcoding gene sequence from DNA templates of many species simultaneously, so differences in
442 the preservation of DNA among taxa could potentially result in significant biases, with the poorly
443 preserved species contributing less than others to the pool of accessible template DNA copies.
444 Minimally, this will cause problems with abundance estimation from metabarcoding data; in the worst
445 case, poorly preserved species might go completely undetected, especially if they are rare.

446 Our study may be the first systematic study of the effect of ethanol concentration on morphological
447 and molecular preservation of insects. Our results largely confirm the commonly held belief that
448 intermediate concentrations of ethanol, around 70 – 80 %, are generally the best for morphological
449 preservation, as high concentrations (above 90 %) tend to make specimens fragile, whereas lower
450 concentration in many cases allow some level of degradation. In contrast, the optimal DNA
451 preservation is achieved at ethanol concentrations of > 90 %. Unfortunately this means that there is a
452 conflict between preserving insects for morphological and for molecular work, as the ethanol
453 concentrations that are ideal for the former purposes result in higher degradation rates of DNA. Is it
454 possible to find a treatment that is ideal for both purposes? Stein et al. (2013) showed that if initial
455 preservation is made in 95 % ethanol, good PCR results can be obtained even after storing the insects
456 in 70 % ethanol for an extended period of time. However, this is the exact contrary of what is
457 recommended for morphological study, where a gradual increase in the ethanol concentration is
458 recommended to avoid fast desiccation of the tissues. However, whether initial preservation in 95 %
459 and subsequent transfer to 70 % for storage reduces the brittleness is unknown. Recently, researchers
460 have directed their attention towards preservatives based on propylene glycol (Nakamura, Tamura,
461 Taki & Shoda-Kagaya, 2020; Moreau, Wray, Czekanski-Moir & Rubin, 2012; Robinson, Porter,
462 Wright & Hajibabaei, 2020). Lower flammability, evaporation, and cost make propylene glycol an

463 attractive alternative to ethanol for trapping, transporting, and storing insect samples. Nevertheless,
464 although these results look promising, more studies are needed to determine the quality of DNA and
465 morphological preservation for different insect groups, under a range of conditions, for these
466 preservatives.

467 For now, we do not have a preservation regimen that is ideal for both morphology and molecules.
468 This must be taken into account when planning large collecting campaigns that aim to preserve
469 material for both morphological and molecular work. For instance, we note that catches from Malaise
470 traps, which are frequently used in insect inventories, contain a large portion of Diptera specimens
471 (Hebert et al., 2016; Karlsson et al., 2020; Ronquist et al., 2020), a group whose morphology is
472 heavily affected by high ethanol concentrations. Hymenoptera, on the other hand, is abundant as well
473 in Malaise traps, and not as prone to morphological damage. These differences need to be considered
474 at the stage of experimental design: the relative importance of morphological against DNA data, or
475 perhaps the focus on certain taxonomic groups, should guide the selection of preservative composition
476 and preservation conditions. In any case, if Malaise trap inventory projects intend to preserve material
477 for molecular and morphological study, it may be necessary to store the material at ethanol
478 concentrations that are not ideal neither for molecular nor for morphological work.

479

480 **Acknowledgements**

481 We are thankful to Dave Karlsson from Station Liné for receiving and returning the parcels with the
482 tubes of the PostNord treatment. This project was supported by the European Union's Horizon 2020
483 research and innovation programme under the Marie Skłodowska-Curie grant agreement no. 642241
484 (BIG4 project, https://big4-project.eu), by the Knut and Alice Wallenberg Foundation (KAW
485 2017.088), Polish National Agency for Academic Exchange grant PPN/PPO/2018/1/00015 and Polish
486 National Science Centre grant 2018/31/B/NZ8/01158. Ela Iwaszkiewisz contributed to the design of
487 the artificial target for DNA preservation assessment, and Anna Michalik and Monika Prus prepared
488 the plasmid for use. We acknowledge PhyloPic platform (http://phylopic.org/) for the silhouettes of
489 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 PŁ conceived and designed the study; DM, MB and PŁ prepared the communities and conducted the experiment, collected and analysed the data, and prepared the figures; DM 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, sequence reads counts and provenance of the insects used can be found in the tables of the supplementary material of this article. The amplicon sequencing data was deposited in NCBI Short Read Archive (BioProject PRJNA662328).

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.

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 Resources*, *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.

Glenn TC, Pierson TW, Bayona-Vásquez NJ, Kieran TJ, Hoffberg SL, Thomas IV JC, ... & Faircloth BC. (2019). Adapterama II: universal amplicon sequencing on Illumina platforms (TaggiMatrix) *PeerJ, 7,* e7786.

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.

Ji, Y., Huotari, T., Roslin, T., Schmidt, N. M., Wang, J., Yu, D. W., & Ovaskainen, O. (2020). SPIKEPIPE: A metagenomic pipeline for the accurate quantification of eukaryotic species occurrences and intraspecific abundance change using DNA barcodes or mitogenomes. *Molecular Ecology Resources, 20*(1), 256–267.

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

549 Department of Agriculture.

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

553 Moreau, C. S., Wray, B. D., Czekanski-Moir, J. E., & Rubin, B. E. (2013). DNA preservation: a
554 test of commonly used preservatives for insects. *Invertebrate Systematics*, *27*(1), 81–86.

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

557 Nakamura, S., Tamura, S., Taki, H., & Shoda□Kagaya, E. (2020). Propylene glycol: a promising
558 preservative for insects, comparable to ethanol, from trapping to DNA analysis. *Entomologia*
559 *Experimentalis et Applicata*, *168*(2), 158-165.

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

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

565 Robinson, C. V., Porter, T. M., Wright, M. T., & Hajibabaei, M. (2020). Propylene glycol-based
566 antifreeze as an effective preservative for DNA metabarcoding of benthic arthropods. *bioRxiv*,
567 2020.02.28.970475.

568 Ronquist, F., Forshage, M., Häggqvist, S., Karlsson, D., Hovmöller, R., Bergsten, J., … &
569 Gärdenfors, U. (2020). Completing Linnaeus's inventory of the Swedish insect fauna: Only 5000
570 species left? *PLos One*, *15*(3), e0228561.

571 Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., ... & Sahl,
572 J. W. (2009). Introducing mothur: open-source, platform-independent, community-supported
573 software for describing and comparing microbial communities. *Applied and Environmental*
574 *Microbiology*, *75*(23), 7537-7541.

575 Shokralla, S., Gibson, J. F., Nikbakht, H., Janzen, D. H., Hallwachs, W. & Hajibabaei, M.
576 (2014). Next-generation DNA barcoding: using next-generation sequencing to enhance and
577 accelerate DNA barcode capture from single specimens. *Molecular Ecology Resources*, *14*(5),
578 892–901.

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.

Tkacz, A., Hortala, M., & Poole, P. S. (2018). Absolute quantitation of microbiota abundance in environmental samples. *Microbiome, 6*(1), 1-13.

Tourlousse, D. M., Yoshiike, S., Ohashi, A., Matsukura, S., Noda, N., & Sekiguchi, Y. (2017). Synthetic spike-in standards for high-throughput 16S rRNA gene amplicon sequencing. *Nucleic Acids Research, 45*(4), e23.

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.

Supplementary Material

Supplementary material, including the raw counts of lost appendages, can be found in the electronic version of the article.

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

Table 2 Effect of Concentration, treatment 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:Treatment	0.8273	2	0.6612
Aphidoletes aphidimyza			
Concentration	0.5238	1	0.4692
Treatment	11.5596	2	0.0031 **
Concentration:Treatment	21.5104	2	0.0002 ***
Drosophila hydei			
Concentration	0.9152	1	0.3387
Treatment	3.0386	2	0.2189
Concentration:Treatment	1.6271	2	0.4433

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 *

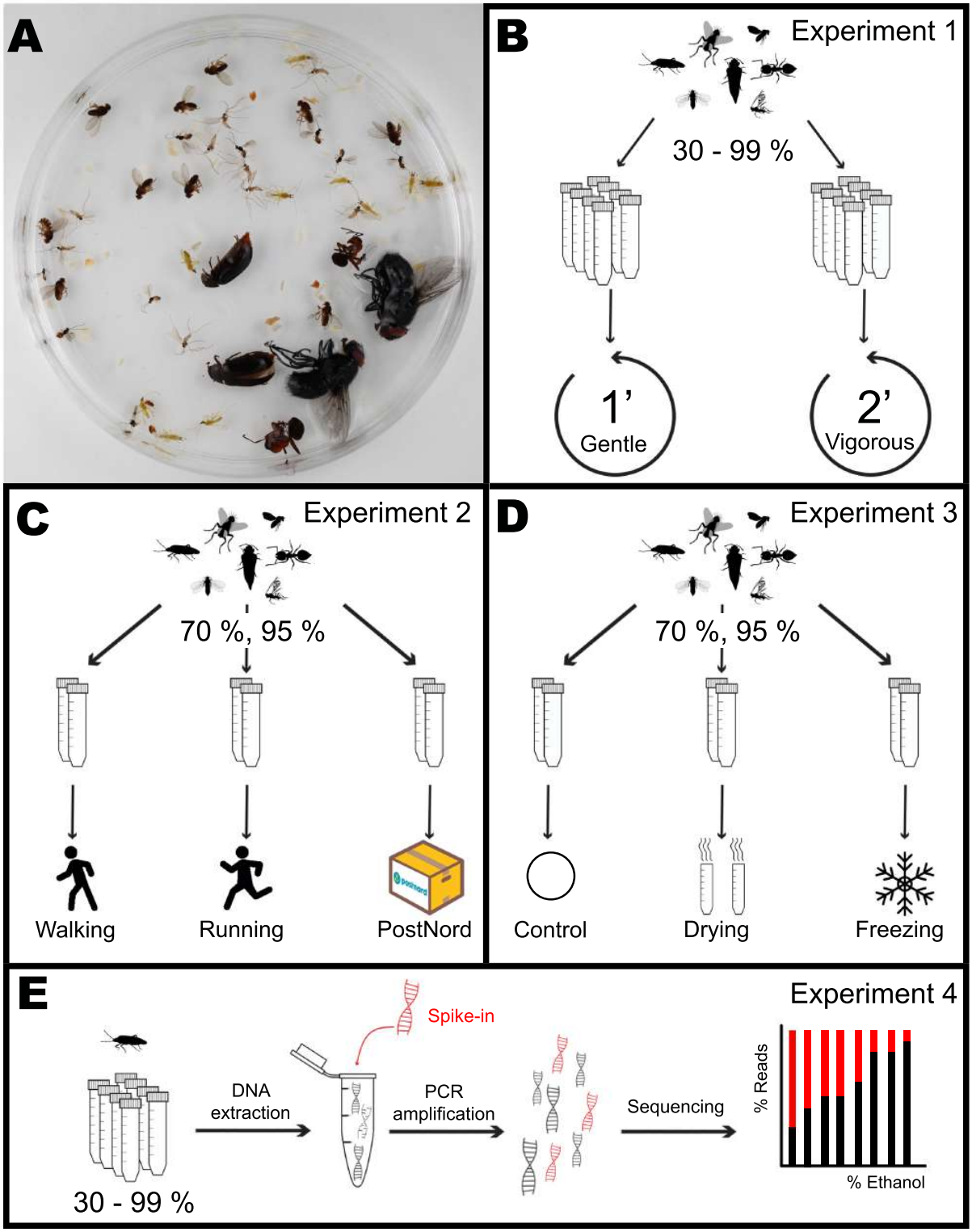

Figure 1. Each mock community sample (**A**) was assigned to an ethanol concentration and experiment. In Experiment 1 (**B**), five tubes for each of eight ethanol concentrations (30, 50,70, 80, 90, 95, 97, and 99 %) were subjected to Gentle or Vigorous shaking. Inn Experiment 2 (**C**), three tubes for each of two ethanol concentrations (70, 95 %) were either carried by a walking or a running experimenter or sent by the Swedish national post service (PostNord) in two different parcels. In

Experiment 3 (**D**), four tubes for each of two ethanol concentrations (70, 95 %) were shaken under a Gentle regime after being either dried, frozen or left as control. After each treatment, the number of appendages lost by every individual was scored. In Experiment 4 (**E**), DNA was extracted from one individual of each species from every tube in treatment Gentle from Experiment 1, mixed with a known quantity of synthetic DNA, PCR amplified and sequenced, and the proportion between insect DNA and synthetic DNA calculated as a measure of DNA degradation.

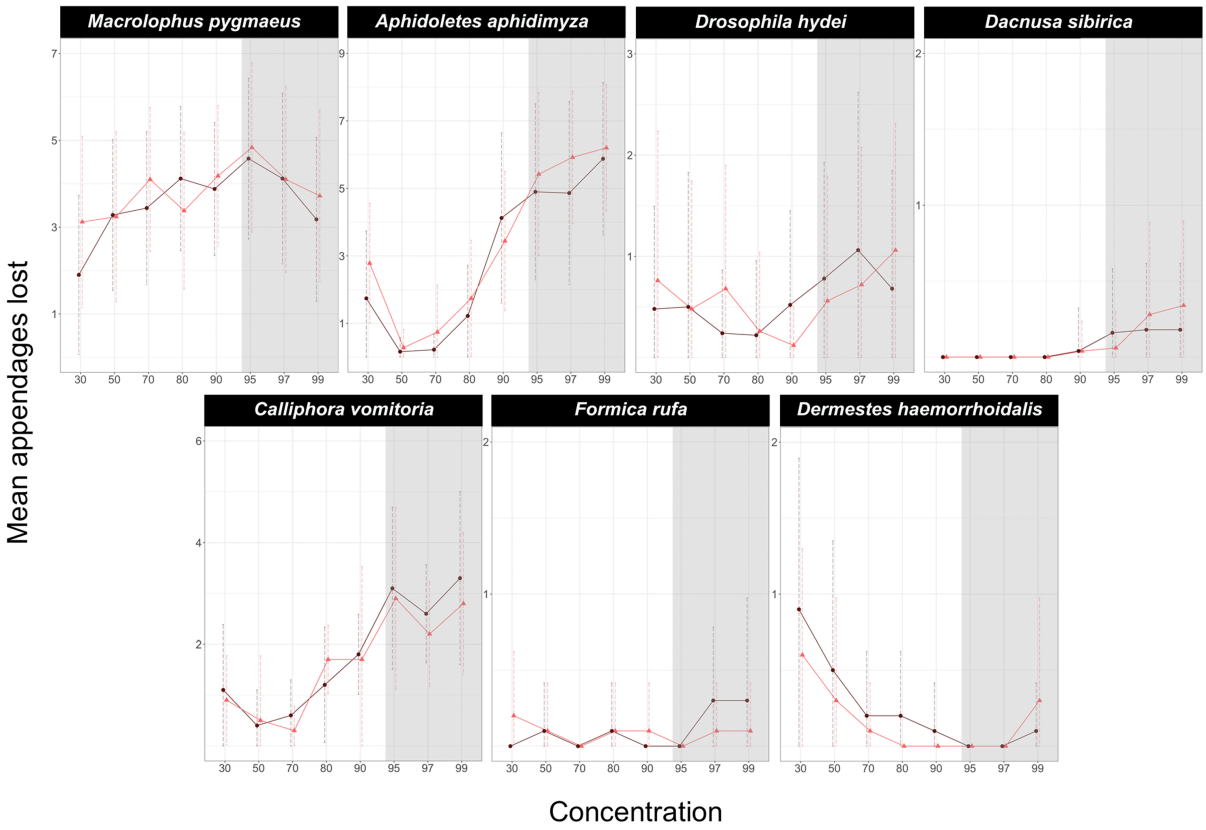

Figure 2 Effect of ethanol concentration on the number of appendages lost by each species. Dark purple circles represent the Gentle shaking regime while bright red triangles represent the Vigorous regime. The shadowed area corresponds to the ethanol concentrations in which DNA is optimally preserved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

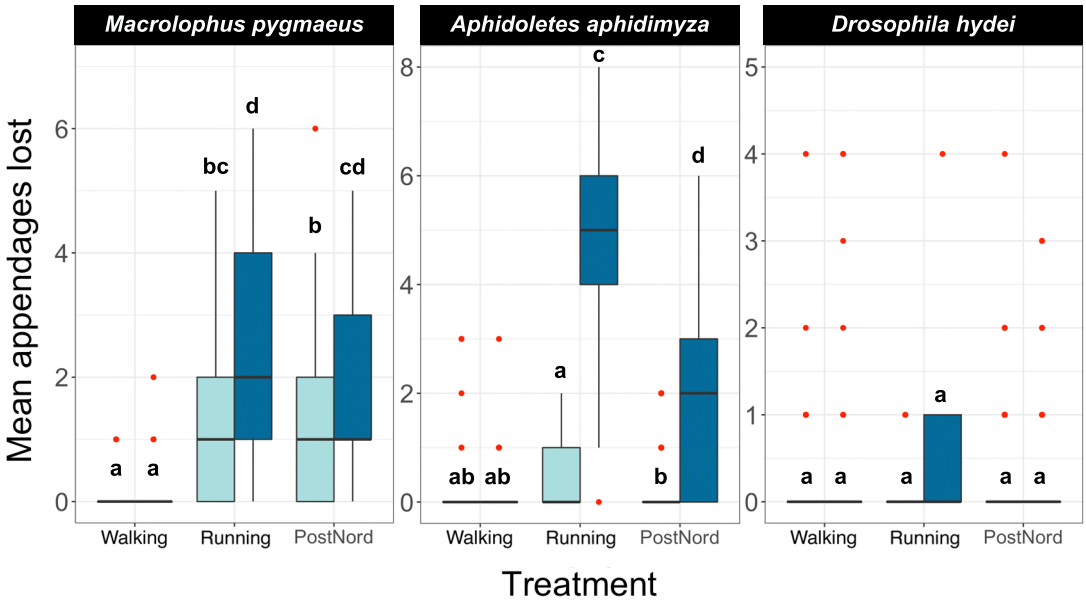

Figure 3 Effect of handling regimes and transport on the number of appendages lost by each species at two different concentrations of ethanol. Light blue boxes represent samples kept in 70 % ethanol and dark blue samples kept in 95 % ethanol. Red dots indicate outliers, and letters indicate groups of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

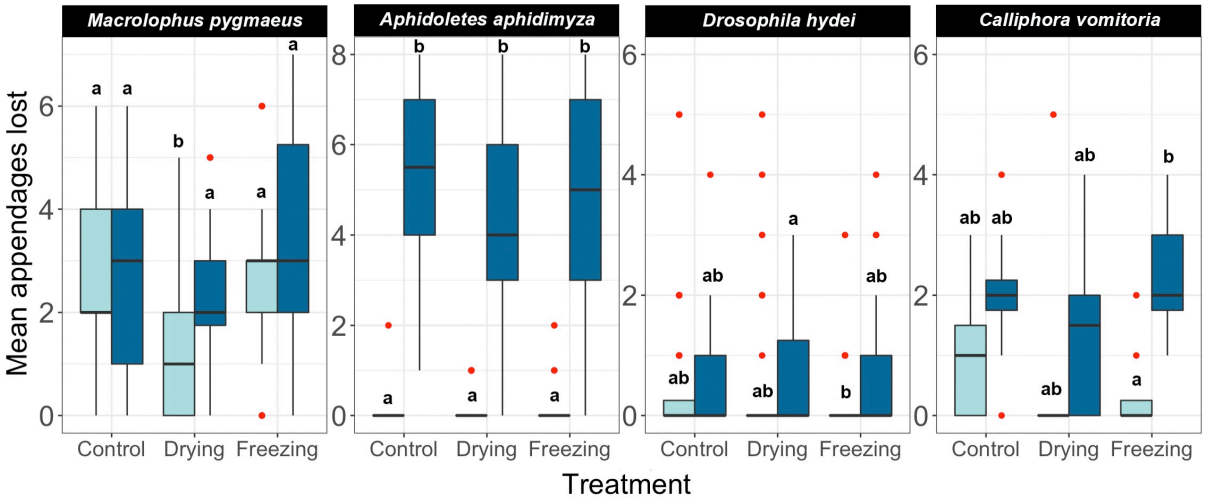

Figure 4 Effect of different pre-treatments on the number of appendages lost by each species at two different concentrations of ethanol. All samples were shaken under a Gentle regime. Light blue boxes represent samples kept in 70 % ethanol and dark blue boxes represent samples kept in 95 % ethanol. Red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

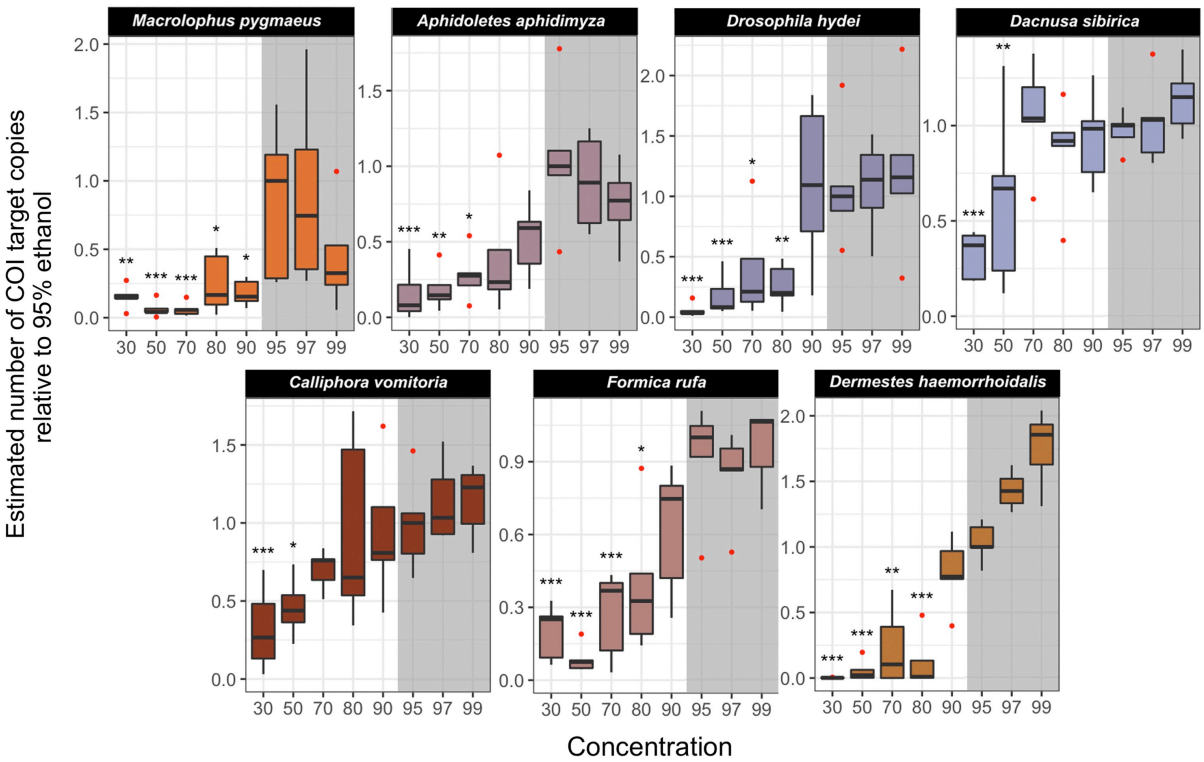

Figure 5 The estimated number of COI target copies in samples of 7 insect species after one year of storage at room-temperature at eight ethanol concentrations. The numbers were standardized relative to 95 % ethanol concentration median. Barplots were based on 5 or 4 (in four cases) replicates. The concentrations in which the number of COI copies was statistically different from the 95 % ethanol concentration treatment are indicated with asterisks (*** p-value < 0.001, ** p < 0.01, * p < 0.05). The shadowed area corresponds to the ethanol concentrations in which DNA is optimally preserved according to literature.