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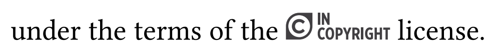

1 **THE IMPACT OF PROLONGED FROZEN STORAGE ON THE PREPARATION**

2 **QUALITY OF BIRD SKINS AND SKELETONS IN ZOOLOGICAL**

3 **COLLECTIONS**

4

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15 **Abstract**

16 Specimens from zoological collections play a pivotal role in improving scientific
17 knowledge in many natural science disciplines. To guarantee an optimum state of
18 conservation and ensure their usefulness, the preparation process employed is crucial.
19 Skins and skeletons are key elements in vertebrate scientific collections and, ideally, are
20 prepared from recently deceased animals; however, specimens are often stored in a
21 frozen state for a long time (years) prior to preparation. Whether the duration of this
22 frozen state has a deleterious effect on preparation quality has rarely been studied. The
23 main objective of this study was thus to contribute towards research into zoological
24 preparation by testing to see whether prolonged frozen storage hinders the preparation
25 of bird skins and skeletons. We used the common buzzard (*Buteo buteo*) and the barn
26 owl (*Tyto alba*) as biological models. Our results showed that long-term frozen storage
27 led to weight loss, bone marrow acidification and solidification, and hampered skin
28 preparation. The necropsy affected weight loss and decreased the skin tear resistance,
29 probably due to tissue dehydration. Thus, prolonged frozen storage appears to have a
30 harmful effect on the preparation quality of vertebrate specimens. Since frozen storage
31 could ultimately have an impact on the conservation and scientific use of museum
32 specimens, practices should be implemented to minimise the amount of time
33 specimens are frozen or to mitigate any detrimental effects. More importance should be
34 attached to research on zoological preparation since it is fundamental for optimising the
35 quality, conservation status, and value of museum collections.

36

37

38 **Keywords**

39 Frozen storage - Museum collections - Skeletons - Skins - Vertebrates - Zoological
40 preparation

41

42 Introduction

43 Natural history collections are a natural legacy handed down by our predecessors and
44 have long been essential sources for research (Suarez and Tsutsui 2004; Winker 2004;
45 MacDonald and Ashby 2011). Traditionally, biological scientific collections have formed
46 the basis of natural science disciplines such as taxonomy and phylogeny (Ruane and
47 Austin 2017; Miralles et al. 2020), paleozoology (Lyman 2010), and ecology and
48 evolution (Holmes et al. 2016; Nattier 2018; Lamichhaney et al. 2019). These collections
49 also assist in the monitoring of environmental contaminants (Ratcliffe 1967; Miller et al.
50 1972; Hayes et al. 2002), global climate change (Parmesan et al. 1999; Hellberg et al.
51 2001), and biological invasions (Fonseca et al. 2001; Suarez et al. 2001); contribute to
52 public health and safety (Leirs et al. 1999; Tsangaras and Greenwood 2012; Tiee et al.
53 2018); and are widely used in forensic science (Murmann et al. 2006; Corrieri and
54 Márquez-Grant 2015).

55 Most of the specimens housed in natural history collections must be prepared before
56 they become part of these repositories. Zoological collections house animal specimens
57 prepared, for instance, as skins and skeletons (Cato and Jones 1991; Beck 2018). How a
58 specimen is prepared may greatly affect not only its value for research but also its
59 longterm conservation (Simmons and Voss 2009; Carrillo-Ortiz et al. 2021). A
60 controversial issue in research based on natural history collections is whether or not

61 museum specimens provide information that is comparable to the information that can
62 be gathered from specimens in the field (i.e. live specimens). Some studies have shown
63 that some properties including the colour of fur or plumage (Doucet and Hill 2009;
64 Kennedy 2010; Sandoval Salinas et al. 2018) and the length of limbs (Knox 1980;
65 Kuczyński et al. 2002) may change over time after the specimen's death; likewise, the
66 use of certain products during preparation (e.g. arsenic as a tanning agent and
67 insecticide for skins) may affect the integrity and attributes of a specimen (Marte et al.
68 2006; Pohland and Mullen 2006; Töpfer et al. 2011). This suggests that the methods and
69 protocols used for the preparation of specimens destined for natural history collections
70 could affect the reliability of the results obtained from the study of such specimens.
71 Therefore, the end-users of these collections should be aware of how the details and the
72 preparation of these specimens could affect their results.

73 Many natural history museum and university collections still use preparation techniques
74 that have barely changed for over a century (Simmons and Snider 2012). The continued
75 use of traditional preparation methods confirms that, in general, they are both valid and
76 effective. Understanding how these techniques affect specimens and researching
77 systematically how preparation protocols can improve the long-term conservation of
78 collections without causing damage are two issues that are worthy of inquiry.
79 Surprisingly though, zoological preparation as a technical discipline has not traditionally
80 been subject to rigorous systematic research. In recent years, there have been calls to
81 improve protocols in conservation and how material from zoological collections is
82 consulted by applying more objective quantitative approaches. Hence, it is now urgent
83 to conduct hypothesis-based experiments to understand how preparation protocols
84 affect the integrity and feasibility of using natural history collections (Simmons and

85 Snider 2012). To date, studies have assessed the quality of museum specimens subject
86 to different preparation processes (Williams and Hawks 1987; Horie 1990; Kite and
87 Thomson 2006) or explored the optimal conditions for storing and exhibiting specimens
88 after preparation (Staniforth 1984; Mathias 1994; Viscardi et al. 2006). However, the
89 quality and condition of museum specimens will also greatly depend on how these
90 specimens were preserved before being prepared, a further challenge that must be
91 faced as soon as the animal dies. Therefore, what occurs between the death and the
92 preparation of a specimen is also relevant despite often being ignored (Winker 2000).

93 Skins and skeletons are amongst the most frequently studied elements in vertebrate
94 scientific collections (Csuti 1980). In taxidermy (i.e. animals mounted in lifelike poses),
95 skins are ideally prepared as soon as possible after animal death to avoid any
96 deterioration and ensure the preservation of all its properties (Péquignot et al. 2006).
97 When zoological preparation is not feasible or necessary on death, it is important to
98 check for tissue decay, preserve specimens until preparation, and prevent potential
99 infestations by placing museum specimens in quarantine in a freezer (Bergh et al. 2006;
100 White and Dusek 2015; Windsor et al. 2015; Campbell and Baars 2019). Typically, due to
101 backlogs in admissions in natural history museums, specimens are often not prepared
102 immediately after quarantine, and in some cases, they remain stored in freezers for
103 years before preparation. It has been remarked that freezing only temporarily preserves
104 museum specimens since they deteriorate even when frozen (Winker 2000). Despite
105 this, how frozen storage affects the quality of zoological preparation is still poorly
106 known; unfortunately, most knowledge of this subject is not based on objective criteria
107 such as experimental results and so it is not usually publishable.

108 The main aim of the present study was thus to contribute towards research on
109 zoological preparation and help establish or optimise suitable preservation strategies
110 prior to preparation in order to enhance preparation quality and therefore improve the
111 scientific utility and long-term conservation of zoological collections. We thus assessed
112 the impact of prolonged frozen storage on the quality of preparation of birds' skins and
113 skeletons intended to form part of a scientific zoological collection by analysing a series
114 of variables. We hypothesised that long-term frozen storage (1) hinders sex
115 determination via the examination of gonads; (2) leads to a gradual decrease in body
116 weight over time as a result of dehydration; (3) decreases skin tear resistance; (4) alters
117 the physicochemical properties of bone marrow; and, overall, (5) hampers the
118 preparation of skins and skeletons of vertebrate specimens, thereby negatively affecting
119 the quality of these elements.

120

121 **Materials and methods**

122 **Sample**

123 The sample used in this study consisted of 117 common buzzards *Buteo buteo*
124 (Linnaeus, 1758) and 139 barn owls *Tyto alba* (Scopoli, 1769) (see Online Resource 1).
125 These specimens were donated to the Natural Science Museum of Barcelona in 1995–
126 2010 (common buzzards) and 1995–2012 (barn owls) by different wildlife recovery
127 centres in Catalonia. Upon their arrival in the museum, these specimens were kept
128 frozen at – 20 °C in a freezing chamber (length × width × height = 2.5 × 2.0 × 2.3 m,
129 AGEFRED®) inside plastic boxes for a number of years. Throughout 2012 and 2013, these
130 specimens were thawed and subjected to a series of observations and a battery of

131 experimental protocols in the Zoological Preparation Laboratory of the Natural Science
132 Museum of Barcelona before being prepared for the ornithological collection. These
133 experimental protocols involved procedures relating to the preparation of skins and
134 skeletons. Skin preparation involves the skinning, i.e. the removal of the skin, and then
135 the cleaning; defatting; tanning; and, if necessary, mounting of the skin (Hendry 1999;
136 Quevedo et al. 2005; Péquignot 2006). Skeleton preparation entails the elimination of all
137 soft tissues surrounding the bones and the removal of bone marrow, which typically
138 seeps out and gradually impregnates bones (Hildebrand 1968; McDonald 2006). The
139 protocols followed are those used by the Zoological Preparation Laboratory of the
140 Natural Science Museum of Barcelona (Orta and Roqué 2011; Orta et al. 2011).

141

142 **Variables and data collection**

143 In order to assess the effects of prolonged frozen storage, a set of variables were
144 measured when the specimens were thawed. Whenever possible, we worked with
145 average annual values for the variables chosen. Some of the background details of the
146 specimens were unknown to us (e.g. physical conditions at the moment of recollection,
147 and how long and under what conditions they had been stored in freezers before arriving
148 at the museum). In addition, sometimes, only one of the two model species was used to
149 analyse certain variables. This was due to operational requirements, since the priority
150 was to prepare the specimens for the museum collection and to the fact that
151 information on whether necropsy had been performed before donation was available
152 only for some specimens.

153

154 Sexing

155 Barn owls can be sexed by plumage given that they have a degree of sexual dimorphism
156 (Ravindran et al. 2018). However, many other species are not sexually dimorphic, so they
157 can only be sexed by examining gonads during preparation. In order to assess the
158 potential effect of long-term frozen storage on internal organs, barn owl specimens were
159 scored as sexable or not sexable by gonad inspection. Since barn owls can breed during
160 their first year of life (Marti 1994), we used specimens with mature gonads, that is, birds
161 hatched the previous calendar year and currently in their second calendar year, i.e. age
162 EURING 5/AHY (Gustafson et al. 1997; EURING – The European Union for Bird Ringing
163 2010) or older.

164

165 Body weight

166 Specimens were weighed to determine whether or not variation in weight occurred
167 during frozen storage. Necropsy was taken into account for this and some subsequent
168 analyses since it entails the opening up of the abdominal cavity, which is likely to
169 influence how birds are exposed to the freezing-drying conditions. Here, we used the
170 common buzzard as the biological model because we knew that some specimens had
171 been necropsied; out of the 117 buzzard specimens, 26 had been necropsied and 79
172 had not; for the 12 remaining birds, no data indicated whether they had been necropsied
173 or not. Specimens were weighed with a precision scale (Salter®, HoMedics Group Ltd.,
174 max 2 kg, d = 1 g).

175

176 Skin tear resistance

177 Skins' resistance to tearing was also analysed in common buzzard specimens to infer
178 whether or not longterm frozen storage and necropsy had an effect. A fresh piece of skin
179 measuring 2 × 5 cm was taken from the apterium (i.e. featherless) chest of each
180 specimen, at a certain distance from the incision in the case of necropsied specimens.
181 The variable defining tear resistance was the weight that these pieces of skin could bear.
182 A structure was designed in which a fragment of skin was fixed to a bucket into which
183 water was added at a constant speed. The volume of water (in litres) was calculated by
184 means of graduation marks (± 0.1 L) on the container. The weight (calculated from the
185 volume of water) that the piece of skin was able to bear before breaking was used as an
186 indicator of tear resistance.

187

188 Difficulty of skin preparation

189 The difficulty in preparing the skins of each barn owl specimen was assessed and
190 scored on a scale ranging from 1 to 4 by skin preparers on the basis of a series of criteria
191 (i.e. whether it was necessary to moisten the specimen regularly, whether the skin was
192 tough, or whether the skin broke easily): 1 = the skin was very easy to prepare; 2 = some
193 of these difficulties were encountered during preparation and the skin was generally
194 easy to prepare; 3 = at least two of these difficulties were encountered; or, despite their
195 severity, all problems could be resolved; and 4 = so many difficulties were encountered
196 and could not be overcome that the preparer recommended that the specimen be used
197 for the preparation of other elements instead of the skin (e.g. piece of skin, tissues,
198 skeleton).

199

200 Bone marrow pH and consistency

201 The impact of long-term frozen storage on the physicochemical properties of bone
202 marrow was assessed. The pH and the consistency of bone marrow were analysed at
203 the same temperature in each specimen of both species so that the effect of necropsy
204 could also be evaluated. The bone marrow samples were extracted from the proximal
205 end of the ulna of each specimen. This bone was chosen because it is easily accessible
206 and generally contains a lot of marrow since it is not pneumatised in birds (McLelland
207 1992). The pH of bone marrow was analysed with a pocket-sized pH metre (Hanna
208 instruments®). Bone marrow in freshly prepared specimens is a semiliquid tissue that
209 consists of a solid and a fluid portion. However, it is often observed that marrow in
210 frozen specimens is more solidified, possibly due to dehydration. For this reason, the
211 semiliquid marrow consistency was taken as a reference of consistency in fresh
212 specimens and bone marrow samples were qualitatively and comparatively categorised
213 into three categories in terms of their degree of dryness: semiliquid, semisolid, or solid.

214

215 **Data analysis**

216 Analyses were performed with annual averages whenever possible and were conducted
217 using Statistica 12 (STATSOFT, Inc. 2014).

218 When sexing using the barn owls' internal anatomy, the feasibility or impossibility of
219 sexing a specimen was scored as 'sexed' and 'non-sexed', respectively. A general linear
220 model (GLM) with binomial distribution, tested for goodness of fit with the Hosmer–

221 Lemeshow (HL) test, was carried out with the binomial-dependent variable *sexing*
222 *practicability* (*sexed*/*non-sexed*) in relation to the independent variable *years of frozen*
223 *storage*. *Body weight* was introduced as an offset variable in order to minimise
224 heteroscedasticity from the time of freezing onwards. After maximising the likelihood
225 function, a Wald test was conducted.

226 The effect of length of time in the freezer and the performance (or otherwise) of a
227 necropsy on specimens' weights was evaluated. In this case, a GLM with normal
228 distribution was applied, using the independent variables or factors *years of frozen*
229 *storage* and *necropsy* as well as their interaction (*years of frozen storage* × *necropsy*),
230 and the dependent variable *body weight*. A Wald test was used for this assessment.

231 To analyse how skin tear resistance is modulated by the duration of frozen storage and
232 necropsy, a GLM with normal distribution of the dependent variable *weight* in relation to
233 the independent variables *years of frozen storage* and *necropsy* was performed. The
234 *years of frozen storage* × *necropsy* interaction was not used because previous analyses
235 had shown that the gradients between *weight* and *years of frozen storage* were similar
236 for necropsied and non-necropsied specimens, probably because the pieces of skin
237 were obtained at a distance from the necropsy incision. A Wald test was subsequently
238 performed.

239 Next, to assess whether the difficulty of skin preparation varied depending on the
240 amount of time that the skins were frozen, a GLM with normal distribution was
241 performed in which *years of frozen storage* was the independent variable and *difficulty of*
242 *skin preparation* was the dependent variable. After using the maximum likelihood
243 function, a Wald test was performed.

244 To assess the effect of frozen storage and necropsy on bone marrow pH, a GLM with
245 normal distribution was implemented with *years of frozen storage* and *necropsy* as
246 independent variables for common buzzards, and just *years of frozen storage* as the
247 independent variable for barn owls, and *bone marrow pH* as the dependent variable for
248 both species. Evaluation was conducted with a Wald test.

249 For bone marrow consistency, a multinomial GLM was applied, with *years of frozen*
250 *storage* and *necropsy* as independent variables for common buzzards, just *years of*
251 *frozen storage* as the independent variable for barn owls, and *bone marrow consistency*
252 as the multinomial dependent variable in both cases. A Wald test was used for this
253 assessment.

254

255 **Results**

256 **Sexing based on gonadal inspection**

257 Of the 139 barn owl specimens, only 81 were sexually mature individuals; 56 could be
258 sexed (69.14%) and 25 could not (30.86%). The model showed a good fit (goodness of fit
259 HL = 2.321, $P = 0.69$) indicating that the results did not support the hypothesis that the
260 time spent in the freezer significantly affected the practicability of sexing the birds
261 ($W(1,81) = 1.015$, $P = 0.314$).

262

263 **Effect of frozen storage on body weight**

264 Significant effects of both necropsy and the duration of frozen storage were detected on
265 the mean weight per amount of years of frozen storage (Table 1). However, a marginal

266 effect of the interaction term was detected, meaning that the effects of the two
267 independent variables were not interdependent (Table 1). In general terms, the
268 specimens that had undergone necropsy weighed less ($\bar{x}$ = 500.57 g, SE = 33.95) than
269 the whole specimens ($\bar{x}$ = 622.60 g, SE = 48.32) (Fig. 1a). Specimens that had been
270 frozen for longer weighed less than those frozen for a shorter period, especially in the
271 case of whole specimens (Fig. 1b). Necropsied specimens weighed less during the first
272 few years of frozen storage and lost less weight when frozen. Conversely, specimens
273 that had not been necropsied weighed more at the beginning of frozen storage and lost
274 weight more quickly over the years (Fig. 1b).

275

276 **Effect of frozen storage on skin condition**

277 The performance of necropsy had a significant effect on the amount of weight that the
278 pieces of skin could bear; by contrast, the amount of time that the specimens were in
279 the freezer did not have a significant effect on skin tear resistance (Table 2). The skin of
280 necropsied specimens was less resistant and bore less weight before breaking ($\bar{x}$ = 3.63
281 kg, SE = 0.35) than the skin of whole specimens ($\bar{x}$ = 5.57 kg, SE = 0.24) (Fig. 2).

282 The length of frozen storage was found to have a clear effect on the difficulty of skin
283 preparation (intercept: $W(1,12) = 0.650$, $P = 0.420$; frozen storage(1,12): $W = 30.68$, $P <$
284 0.001) and, in particular, specimens frozen for longer were more difficult to prepare (Fig.
285 3). Body regions with less flesh (e.g. skull, distal parts of wings, and legs) were the most
286 difficult parts of the specimens to process.

287

288 **Effect of frozen storage on bone marrow properties**

289 Bone marrow pH was not significantly affected by the performance of necropsy in
290 common buzzards (Table 3). In addition, in this species, the duration of frozen storage
291 had a marginal effect on bone marrow pH as there was a trend towards bone marrow
292 acidification with greater time of frozen storage (Table 3, Fig. 4a). This marginal effect
293 was mainly due to the results from specimens frozen since 1995, which had abnormally
294 high (i.e. basic) pH values. When these individuals were removed, the model was highly
295 significant ($P < 0.01$). Given that the necropsy effect was not significant, we performed
296 the same analysis on barn owls. With this species, the duration of frozen storage
297 significantly affected bone marrow pH (intercept: $W_{(1,13)} = 1526.302$, $P < 0.001$; frozen
298 storage$_{(1,13)}$: $W = 5.11$, $P = 0.024$) as bone marrow acidified with time of frozen storage
299 (Fig. 4b). The decrease in pH during the storage period was around 1.5 points in both
300 species, and pH values reached around 5.5 in common buzzards and around 6.0 in barn
301 owls.

302 Unlike necropsy, the duration of frozen storage significantly affected the consistency of
303 bone marrow in common buzzards (Table 4) and in barn owls ($W(4,130) = 10.44$, $P =$
304 0.005). The comparison of semiliquid bone marrow consistency with the two other
305 consistency categories at the same temperature in each species revealed that the
306 duration of frozen storage significantly explained the differences between them (Tables 5
307 and 6). The increasing solidification of bone marrow with longer frozen storage time
308 seemed to occur in both species, although the transition between the three consistency
309 categories was more evident in barn owls (Fig. 5).

310

311 **Discussion**

312 Freezing specimens immediately after death while awaiting preparation in museums is a
313 common strategy that reduces any risk of imminent decay caused by bacterial
314 proliferation or enzymatic activity (Winker 2000; Herren 2012; Tortora et al. 2019).
315 However, certain results in this study suggest that long-term frozen storage as a practice
316 significantly affects preparation procedures and alters the state of vertebrate skins and
317 skeletons held in natural history collections. Specifically, specimens stored for longer in
318 a freezer lose more weight, their skin is more difficult to prepare, and their bone marrow
319 becomes more acidic and solid. In addition, necropsy aggravates some of the negative
320 effects of frozen storage and also exacerbates certain features that are not directly
321 influenced by the duration of frozen storage.

322

323 **Impact of prolonged frozen storage on general condition**

324 During freezing, about 80% of the water content of specimens is expected to solidify into
325 pure ice crystals (Cano-Muñoz 1991). However, freezing also causes tissues to
326 dehydrate, that is, water sublimates through biological membranes when a specimen is
327 exposed to an atmosphere under a different water pressure (relative humidity). This
328 phenomenon, known as freeze-drying, leads to the recondensation of water outside the
329 tissues and is the most common problem with frozen specimens (Winker 2000;
330 Campañone et al. 2005; Zaritzky 2008). If freezing is slow, water migrates out of the cells
331 until freezing is complete (i.e. when the centre of the frozen object has a temperature of
332 – 12 °C or less), which typically leads to the formation of large ice crystals on the outside
333 of the cells (Cano-Muñoz 1991; Zaritzky 2008; Herren 2012). Ice crystals, even when

334 small and formed within cells, can also cause cells to burst by perforating cellular
335 membranes. This histological damage, added to moisture loss, provokes more water
336 release during thawing and therefore dehydrates specimens even further (Cano-Muñoz
337 1991; Zaritzky 2008). Ultimately, these processes result in weight loss in frozen
338 specimens (Compagno 2001; Edwards et al. 2002; Campañone et al. 2005). The weight
339 variations in our study could be explained by these phenomena and, furthermore, point
340 to accumulative dehydration over time since weight loss was found to increase as the
341 period of frozen storage increased, especially in whole specimens. Specimens were as a
342 rule not physically insulated very well and fluctuations in freezer temperatures could
343 have occurred during long storage periods, two factors that could have contributed to
344 progressive weight loss.

345 Dehydration caused by frozen storage is expected to have an impact on the whole body
346 of frozen specimens, including their internal organs. Indeed, dehydration often
347 complicates the examination of the gonads and can hinder sex determination or even
348 make it impossible (Edwards et al. 2002). In species with no evident sexual dimorphism,
349 the inability to sex a specimen after scrutiny of the gonads may lessen its scientific
350 usefulness, thereby undermining the quality of the scientific collection it belongs to.
351 Even though we were unable to detect any significant impact of dehydration on sex
352 determination, we might have encountered greater difficulties when sexing specimens if
353 a smaller bird species—i.e. with smaller gonads and likely to suffer more quickly and
354 severely from dehydration—had been chosen. Another reason why we did not come
355 across any major impediments when sexing specimens might have been thanks to the
356 expertise of preparers in sexing specimens by gonad inspection, since expert preparers
357 usually make very few mistakes when compared to DNA sexing (< 5%, JQ unpublished

358 data). This expertise may play an important role in their ability to sex an animal that is
359 decomposed or dehydrated, as was the case in some of the specimens in this study.

360 The epidermis of avian skin is a barrier with a facultative capacity for waterproofing that
361 prevents excessive water loss (Horie 1990; Elias and Menon 1991; Menon et al. 1996).
362 Given that the necropsies of vertebrate specimens typically entail opening the
363 abdomen, this protective skin layer is disrupted in this region in necropsied specimens.
364 Consequently, internal organs and cavities are more exposed in these specimens, which
365 make them more susceptible to dehydration than whole specimens during frozen
366 storage. According to our results, necropsied animals were initially lighter than those
367 that had not been necropsied (Fig. 1a), and the rate of weight loss in relation to the years
368 elapsed since the onset of frozen storage differed between necropsied and whole
369 specimens (Fig. 1b). Taken together, these results suggest that there could be two
370 processes operating simultaneously in weight loss in relation to necropsy. On the one
371 hand, the fact that necropsy usually entails the evisceration of specimens could
372 contribute to some extent to explaining the weight difference between entire and
373 necropsied specimens at the beginning of the storage period. On the other hand, the
374 fact that necropsied specimens are more exposed to dehydration and might have less
375 tissue from which to lose water could lead to an immediate water loss from the
376 remaining tissues in the first few years of frozen storage that, consequently, would
377 explain the lack of any relationship in necropsied specimens between weight loss and
378 the duration of frozen storage. By contrast, non-necropsied specimens could steadily
379 lose weight over time due to the dehydration of all their tissues and organs, which
380 supports the hypothesis that weight loss results from constant or regular dehydration
381 over years of frozen storage.

382

383 **Impact of prolonged frozen storage on skin properties**

384 Contrary to our initial hypothesis, the duration of frozen storage was not found to affect
385 the tear resistance of the skin. As for the effect of necropsy, necropsied specimens were
386 found to have less tear resistance since their pieces of skin were able to bear
387 significantly less weight than those from whole specimens. Even though pieces of skin
388 were obtained at a certain distance from the incision, the significant effect of necropsy
389 on tear resistance suggests that skin desiccation could increase in necropsied
390 specimens; consequently, skin in the less dehydrated whole specimens is more elastic
391 and resistant in comparison. Vertebrate skin is often considered a non-linear-elastic
392 material with low strain-rate sensitivity (Lanir and Fung 1974; Fung 1981). Its mechanical
393 properties are dictated by the principal constituents of the dermis, i.e. elastin and
394 especially type 1 collagen (Yang et al. 2015). Experiments testing the tear resistance of
395 skin are usually conducted on hydrated specimens in order to reflect reality. However,
396 the comparison of stress–strain curves of skins with different levels of hydration shows
397 that the mechanical response of skin is significantly altered when its water content is
398 abnormally low (Yang et al. 2015). Specifically, the most severely dehydrated pieces of
399 skin have the greatest loss of tear resistance, which is explained by the fact that slipping
400 between collagen fibrils is severely limited when there is a lack of water molecules (Yang
401 et al. 2015). This alteration in the skin's mechanical response could explain the lower
402 skin tear resistance detected in necropsied specimens in the present study.

403 In terms of the degree of difficulty in preparing skins, prolonged frozen storage seriously
404 hampers skin manipulation and processing. Our results support the idea that the

405 duration of frozen storage has a cumulative adverse impact on the feasibility of proper
406 skin preparation. Additionally, the skin of the body regions with less soft tissue
407 underneath (i.e. the skull and the distal part of the limbs) was more difficult to remove
408 properly (CO and LR, pers. obs.), probably due to the fact that these regions were the
409 most freeze-dried. Hence, it is to be expected that the less dehydrated a specimen is,
410 the more manoeuvrable and the less fragile its skin will be. Skin preparation in frozen
411 specimens that have been freeze-dried can be aided by soaking the affected parts or
412 even the whole specimen in water in a refrigerator for 1–2 days (Winker 2000). This
413 strategy reflects the need to moisten more regularly specimens frozen for longer periods
414 of time when preparing their skins, evidence for the impact of dehydration due to frozen
415 storage.

416

417 **Impact of prolonged frozen storage on the properties of bone marrow**

418 The distal limb elements of birds are not commonly pneumatised and usually contain
419 non-haematopoietic bone marrow adipose tissue in their medullary cavity (Gurevitch et
420 al. 2007; Canoville et al. 2019). Lipids in biological systems, including marrow adipose
421 tissue, are vulnerable to oxidation through oxidative and hydrolytic rancidity (Laitinen et
422 al. 2006; Wazir et al. 2019). Although the speed of lipid hydrolysis and oxidation (as well
423 as the resulting changes in marrow fatty acid composition) are greater at higher
424 temperatures, these processes also take place even below 0 °C (Laitinen et al. 2006;
425 Zhou et al. 2018; Blasco et al. 2019). In fact, changes in the biochemical nature of lipids
426 have been reported from bone specimens frozen at – 20 °C due to hydrolysis (Mularchuk
427 and Boskey 1990), and risk of a high lipid oxidation rate in fresh-frozen bone allografts

428 exists even at – 30 °C (Laitinen et al. 2006). As a result of the hydrolysis of lipids such as
429 triacylglycerol and polar lipids, fatty acids are released and, since some of them are
430 acidic, they can lead to a decrease in pH (Deeth and Fitz-Gerald 2006; Zhou et al. 2018).
431 In addition, a drop in pH during cold storage can result from the decomposition of
432 adenosine triphosphate (ATP) into acidic substances such as phosphoric acid (Ozawa et
433 al. 1990). Although the acid–base status of bone marrow has been little studied
434 (Nikolaeva 2018), the abovementioned phenomena may help explain the higher bone
435 marrow acidification detected in the specimens of our study that had been frozen for a
436 longer time.

437 Dehydration causes structural and mechanical changes in bones by provoking a
438 decrease in the spacing between collagen fibrils (Lees et al. 1984; Lievers et al. 2010).
439 Principally, dehydration results in less strength and fracture strain and hence less
440 toughness in the cortical bone (Nyman et al. 2006). Although these parameters were not
441 analysed in this study, during skeleton preparation, it is often noticed that bones of
442 specimens frozen for a long time are more fragile and that some even break easily in the
443 hand, which would be nearly impossible in a fresh specimen (LR, pers. obs.). Therefore,
444 these parameters could be incorporated into future studies since such fragility could
445 negatively affect the quality of bone preparation and so of osteological collections.
446 Since bones can be affected by dehydration, it is likely that a fraction of water
447 associated with bone marrow could be displaced during frozen storage. This loss of
448 water would lead to a change in bone marrow consistency over time, making it more
449 solid, as detected in this study. The protocols for skeleton preparation frequently entail
450 drilling a hole at each end of long bones and then using hot water and a pressurised
451 water gun to remove the bone marrow from the inside of the bones (McDonald 2006;

452 Orta et al. 2011). However, the solidification of bone marrow could obstruct its removal
453 and some marrow may be left inside the bones. Acidification is a major concern in long-
454 term conservation in museums and heritage institutions and, in the case of biological
455 collections, it is well known that residual fat left in skins acidifies over time and speeds
456 up skin deterioration (Winker 2000; Kite and Thomson 2006). Given that lipid
457 acidification occurs to a greater extent at higher temperatures, room temperature at
458 museums is regarded as a serious threat for the stability of bone marrow residues in
459 bones and, consequently, for the welfare of prepared skeletons. As well, poorly
460 prepared osteological collections are very vulnerable to infestation, since pests are
461 primarily attracted to the fat in bones (McDonald 2006). Therefore, the solidification of
462 bone marrow as a result of prolonged frozen storage may not just increase the difficulty
463 and lessen the quality of bone preparation but could also impede long-term bone
464 conservation by triggering detrimental biochemical reactions and inciting pest
465 infestations.

466

467 **Guidelines on frozen storage for improving preparation protocols**

468 Several results from this study point towards long-term frozen storage as being
469 potentially counterproductive for zoological preparation; additionally, they suggest that
470 frozen storage leads to progressive dehydration and fails to halt the biochemical
471 processes that modify the physicochemical properties of skin and bone marrow over
472 time. As a result, prolonged frozen storage hinders the proper preparation and integrity
473 of vertebrate specimens that are to be stored as skins and skeletons in scientific

474 collections. However, by taking into account these considerations and seeking
475 solutions, the preparation quality of vertebrate specimens could be greatly improved.

476 Probably, the most logic way of avoiding the harmful effects of prolonged frozen storage
477 would be to minimise the time that specimens are stored in freezers before preparation
478 (Winker 2000). This would require greater coordination in the input of specimens into the
479 museums and their capacity for zoological preparation, which unfortunately is not
480 always the case. Nowadays, many natural history museums—especially in Europe—do
481 not collect animals in the wild but, instead, rely on specimens donated by scientific or
482 administrative institutions to increase their collections. As a result, these museums
483 cannot control the flow (unless they reject donations) and the growth of their collections
484 becomes opportunistic. For as long as the capability of preparation is limited, priority
485 should be given to the preparation of necropsied, wounded, and small specimens (e.g.
486 passerines and micromammals), especially if they are to be kept as skins or skeletons in
487 scientific collections. The greater exposure of internal cavities in necropsied and
488 wounded specimens, and the larger surface-area-to-volume ratios in smaller animals,
489 mean that these type of specimens suffer disproportionately more severely from the
490 harmful effects of long-term frozen storage.

491 Another measure that would help mitigate the drawbacks of prolonged frozen storage in
492 zoological preparation is the complete isolating of the specimens while frozen through
493 the use of materials that are stable at low temperatures and resistant to water vapour
494 and oxygen (i.e. to keep oxygen out and moisture in) (Pham and Mawson 1997; Zaritzky
495 2008; Herren 2012). Isolation could mean simply wrapping specimens with plastic film
496 and placing them in plastic bags with as much air as possible removed, or, best of all,

497 vacuum-packing them in plastic bags and then storing them inside isolating boxes until
498 preparation (Cano-Muñoz 1991; Winker 2000). There is a wide range of available plastic
499 packaging materials (e.g. polyethylene terephthalate, polypropylene, etc.); however,
500 some are not considered to be suitable for long-term conservation storage (Tétreault
501 2017). Once again, isolation measures are particularly important for necropsied,
502 wounded, or small specimens. According to Winker (2000), it is possible to prepare
503 small specimens that have been frozen for 3 years if they have been properly isolated
504 and stored. Given that this author considers 3 years to be an exceptionally long period of
505 time, even greater isolation measures should be adopted when longer frozen periods are
506 foreseeable. This also sheds light on another interesting consideration: the size of
507 specimens could influence the deleterious effects of frozen storage or the aptness of
508 the measures used in this work. We used relatively large species as model species but
509 the effects of long-term frozen storage on smaller species are still unknown, and would
510 be an interesting future field of study.

511 An additional recommendation for optimising the preservation of specimens with a view
512 to improving their preparation is to use a freezing system that minimises dehydration;
513 this could, however, imply investment in renewing existing infrastructure, something that
514 may be beyond the capacity of many modestly budgeted museums. In this regard,
515 dehydration could be minimised by combining short freezing times and good
516 aerodynamics. The faster the temperature of the subject is dropped, the less
517 dehydration takes place (Herren 2012). Proper regulation of aerodynamic parameters in
518 freezers (i.e. air velocity, air pressure, and relative humidity) could stop snow from
519 forming due to the humidity lost from the specimens, which prompts further
520 dehydration. Frost-free freezers should be avoided because they undergo dramatic

521 temperature fluctuations that could mobilise water from specimens (Winker 2000).
522 Specimens should be maintained at or below – 20 °C and at a very high relative humidity
523 (95–98%) in order to prevent dehydration (Cano-Muñoz 1991; Winker 2000). Blast
524 freezers are recommendable since their use of high-velocity air and temperatures up to
525 – 40 °C allows for quick freezing (Cano-Muñoz 1991; Herren 2012).

526 Thawing is another critical phase in frozen storage because it involves a change from ice
527 crystals to melted water, which can then be reabsorbed by the animal tissue (Cano-
528 Muñoz 1991; Pham and Mawson 1997; Kennedy 2000). Slow-thawing specimens at
529 around 5 °C guarantees an efficient reabsorption of melted water (Calvelo 1981; Cano-
530 Muñoz 1991). During thawing, the level of air circulation should be kept low, and relative
531 humidity should be kept low at the beginning (70%)—to prevent frost from forming on
532 the surface—but should be increased towards the end of the thawing period (90–95%)
533 (Cano-Muñoz 1991).

534 If it is too late to apply these type of preventive measures but it is necessary to prepare
535 specimens that have been frozen for a long time, the best way to optimise the time taken
536 and preparation quality is to prepare elements other than skins or skeletons (e.g. piece
537 of skin, muscle, liver) from these specimens. In this case, the preparation of only fresh
538 enough specimens as skins and skeletons is not only quicker but also gives better
539 results and improves the quality of the scientific collections. Nevertheless, the
540 measures finally adopted to mitigate the impact of long-term frozen storage and improve
541 the preparation quality of vertebrate skins and skeletons will depend in the ultimate
542 instance on the particular conditions and circumstances of each natural history
543 museum.

544

545 **Relevance of research on zoological preparation**

546 The protocols followed to prepare museum specimens have a crucial impact on the
547 appropriateness and usefulness of these specimens as sources of biological
548 information. For instance, small changes in specimens' posture during preparation may
549 improve the scientific value of an ornithological collection by facilitating the taking of
550 relevant information such as biometric measurements or moult data (Carrillo-Ortiz et al.
551 2021). The present work, which used as a case study an assessment of the impact of
552 prolonged frozen storage on the preparation quality of bird skins and skeletons,
553 highlights the suitability and worth of conducting research on zoological preparation as
554 a means of optimising the current quality of zoological preparation for scientific
555 collections. Given that long-term frozen storage leads to progressive dehydration and
556 does not stop the biochemical processes that provoke skin ageing and acidification of
557 bone marrow, this procedure could potentially affect future studies based on zoological
558 collections. This highlights the need to understand how zoological preparation
559 establishes or optimises preservation techniques and therefore improves the scientific
560 worth of zoological collections. Here, we have shown that systematic questions and
561 experimental designs focused on zoological preparation can help to improve knowledge
562 of how certain practices affect the quality of the final result, and how they can
563 contribute towards improving preservation and preparation protocols that will make
564 scientific collections more durable, usable, and valuable. Hence, this study not only
565 highlights how important it is to study zoological preparation but also aims to encourage
566 fresh studies to be conducted of other collection elements and species to broaden

567 current knowledge and further optimise zoological preparation. Research into zoological
568 preparation aims to optimise the conservation, utility, and value of natural history
569 collections for the benefit of the scientific community and society as a whole, and so
570 any contribution towards the improvement of this technical discipline is of great
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572

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578

579 Author contribution

580 Study conception and design: JQ, LR, CO; development of methodology: JQ, CO, IdC,
581 LR, JC-O; material preparation and data collection: LR; formal analysis of the study data:
582 JQ, JM-V, JC-O; drafting of the manuscript: JM-V, JC-O, IdC, JQ; critical revision of the
583 manuscript: JM-V, JQ; research supervision and project administration: JQ. All authors
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590

591 Data availability

592 Data sharing is not applicable to this article as no datasets were generated or analysed
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594

595 Code availability

596 Not applicable.

597

598 **Declarations**

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603

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849 **Tables**

850 **Table 1.** Wald test assessing the effects of necropsy and time of frozen storage on body weight in common buzzards

851

852		Level of effect	Estimate parameter	SE parameter	Wald statistic	P-value
853	Intercept		6.545	0.099	4413.222	0.000
854	Necropsy	No	0.261	0.099	7.018	*0.008*
855	Years of frozen storage		−0.023	0.009	6.628	*0.010*
856	Necropsy × years of	1	−0.016	0.009	3.516	0.061
857	frozen storage					

858

859 Values in italics stand for statistically significant factors

860

861 **Table 2.** Wald test assessing the effects of necropsy and time of frozen storage on skin tear resistance in common buzzards

862

863		Level of effect	Estimate parameter	SE parameter	Wald statistic	P-value
864	Intercept		1.425	0.095	0.00	0.000
865	Necropsy	No	0.219	0.048	0.00	*0.008*
866	Years of frozen		0.008	0.008	0.008	0.330
867	storage					

868

869 Values in italics stand for statistically significant factors

870

871 **Table 3.** Wald test assessing the effects of necropsy, duration of frozen storage, and their interaction on bone marrow pH in common

872 buzzards

873

874		Level of effect	Estimate parameter	SE parameter	Wald statistic	P-value
875	Intercept		1.969	0.068	838.917	0.000
876	Necropsy	No	−0.039	0.068	0.334	0.564
877	Years of frozen storage		−0.011	0.006	3.558	0.059
878	Necropsy × years of	1	0.000	0.006	0.001	0.976
879	frozen storage					

880

881 **Table 4.** Likelihood test (type III) of necropsy, duration of frozen storage, and their interaction on bone marrow consistency in common

882 buzzards

883		Degrees of freedom	Wald statistic	*P*-value
884	Intercept	2	3.740	0.154
885	Necropsy	2	1.568	0.457
886	Years of frozen storage	2	8.750	*0.013*
887	Necropsy × years offrozen storage	2	1.133	0.567

888

889 Values in italics stand for statistically significant factors

890

891 **Table 5**. Estimated variance–covariance matrix test assessing differences in bone marrow consistency in terms of years of frozen storage

892 in common buzzards

893		Level of response	Estimate parameter	SE parameter	Wald statistic	*P*-value
894	Intercept 1	Semiliquid vs. semisolid	−2.544	1.411	3.251	0.071
895	Necropsy	Semiliquid vs. semisolid	−0.919	1.411	0.424	0.515
896	Years of frozen storage	Semiliquid vs. semisolid	0.295	0.113	6.774	*0.009*
897	Necropsy × years of frozen storage	Semiliquid vs. semisolid	0.068	0.113	0.365	0.546
898	Intercept 2	Semiliquid vs. solid	−1.021	0.815	1.568	0.210
899	Necropsy	Semiliquid vs. solid	0.649	0.815	0.634	0.426
900	Years of frozen storage	Semiliquid vs. solid	0.182	0.075	5.904	*0.015*
901	Necropsy × years of frozen storage	Semiliquid vs. solid	−0.038	0.075	0.260	0.610

902

903 Values in italics stand for statistically significant factors

904 **Table 6.** Estimated variance–covariance matrix test assessing differences in bone marrow consistency in terms of years of frozen storage
905 in barn owls

906		Level of response	Estimate parameter	SE parameter	Wald statistic	*P*-value
907	Intercept 1	Semiliquid vs. semisolid	−1.462	1.021	2.052	0.152
908	Years of frozen storage	Semiliquid vs. semisolid	0.171	0.072	5.737	*0.017*
909	Intercept 2	Semiliquid vs. solid	−4.234	1.648	6.603	0.010
910	Years of frozen storage	Semiliquid vs. solid	0.327	0.110	8.924	*0.003*

911

912 Values in italics stand for statistically significant factors

913

914 **Figure captions**

915 **Fig. 1. a** Comparison of body weight ranges in whole and necropsied common buzzard
916 specimens. **b** Regression trends of body weight over years of frozen storage in whole and
917 necropsied common buzzard specimens

918

919 **Fig. 2.** Weight ranges borne by the skin of whole and necropsied common buzzard
920 specimens

921

922 **Fig. 3.** Trend of the scores rating the difficulty of skin preparation in barn owl specimens
923 in relation to years of frozen storage

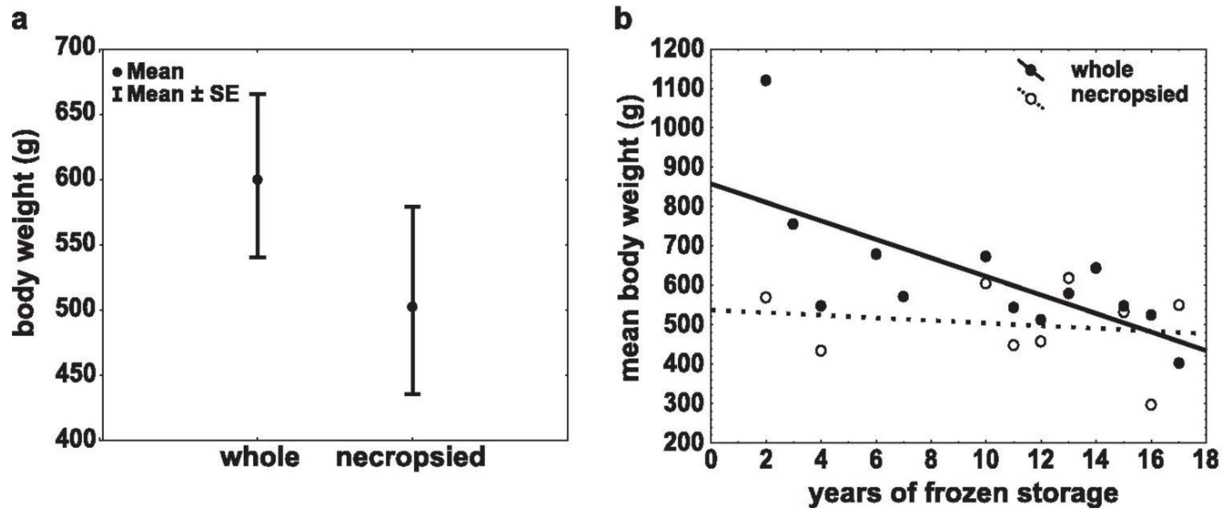
924

925 **Fig. 4.** Regression trends of bone marrow pH in common buzzards (**a**) and barn owls (**b**)
926 as the period of frozen storage increases

927

928 **Fig. 5.** Bone marrow consistencies in common buzzards (**a**) and barn owls (**b**) in terms of
929 the years of frozen storage. The graphs show the range of years of frozen storage (*X*-axis,
930 quantitative variable) for which the bone marrow was observed to exist in each of the
931 three categories of consistency (*Y*-axis, categorical variable)

932 Fig. 1



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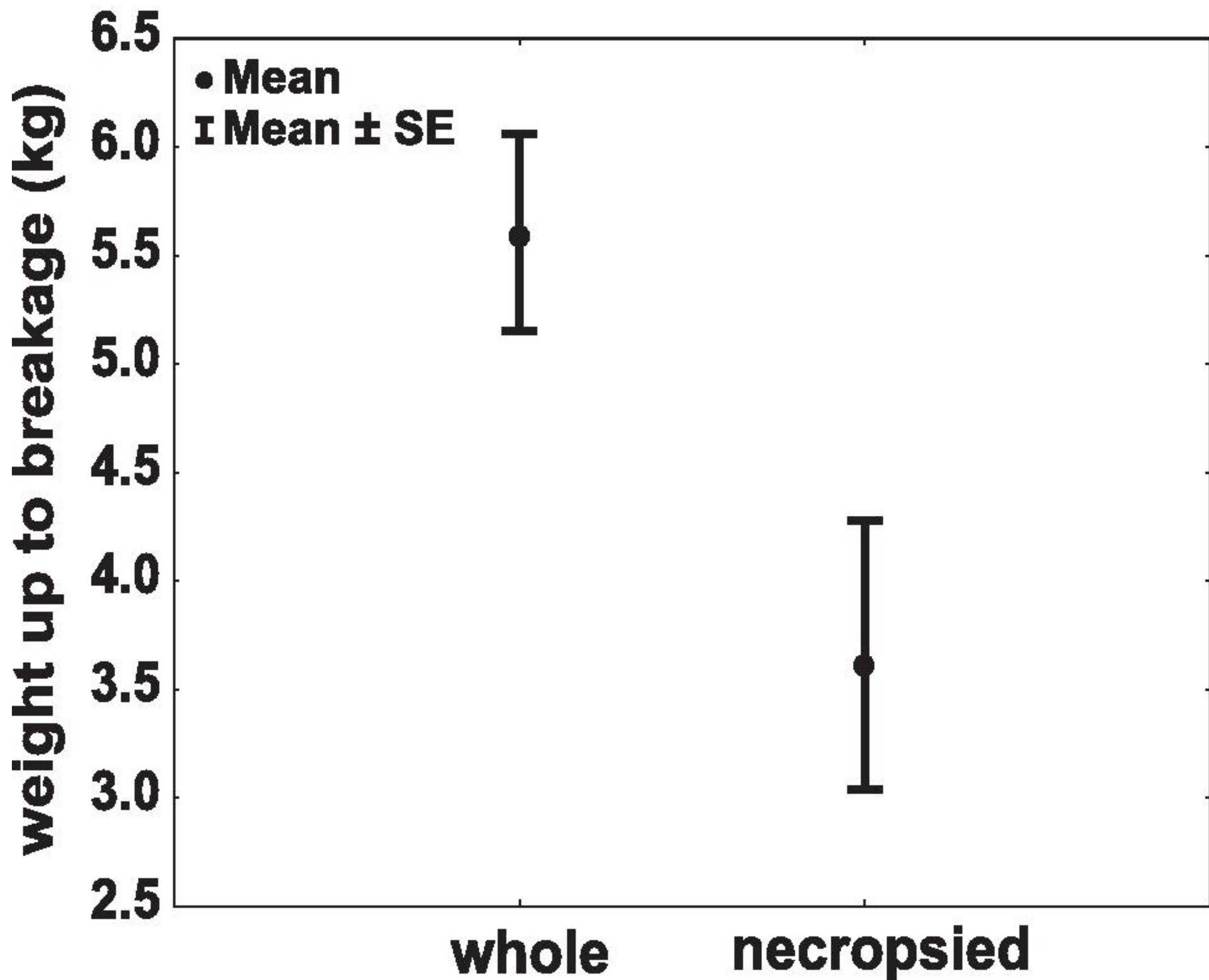
935 Fig. 2



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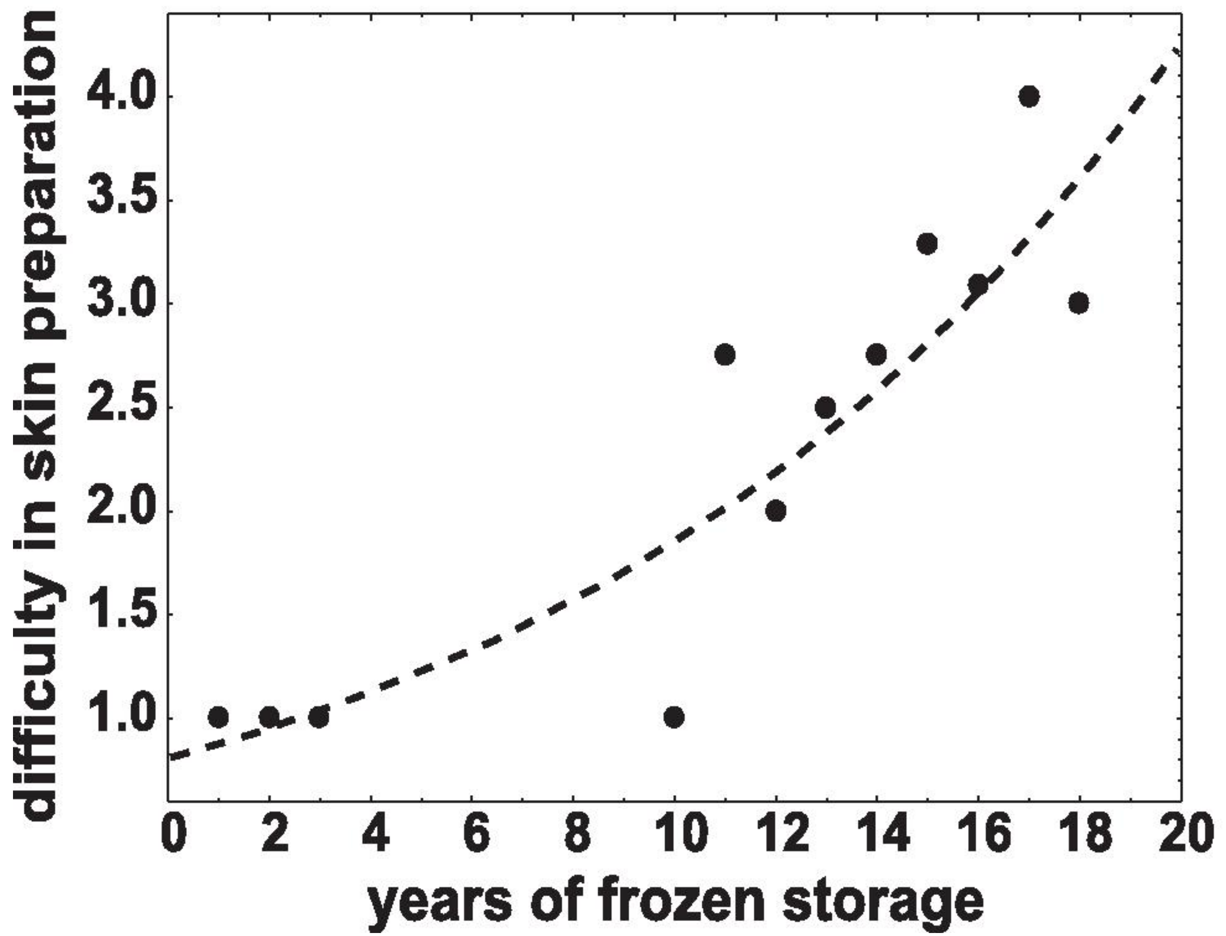
937 Fig. 3



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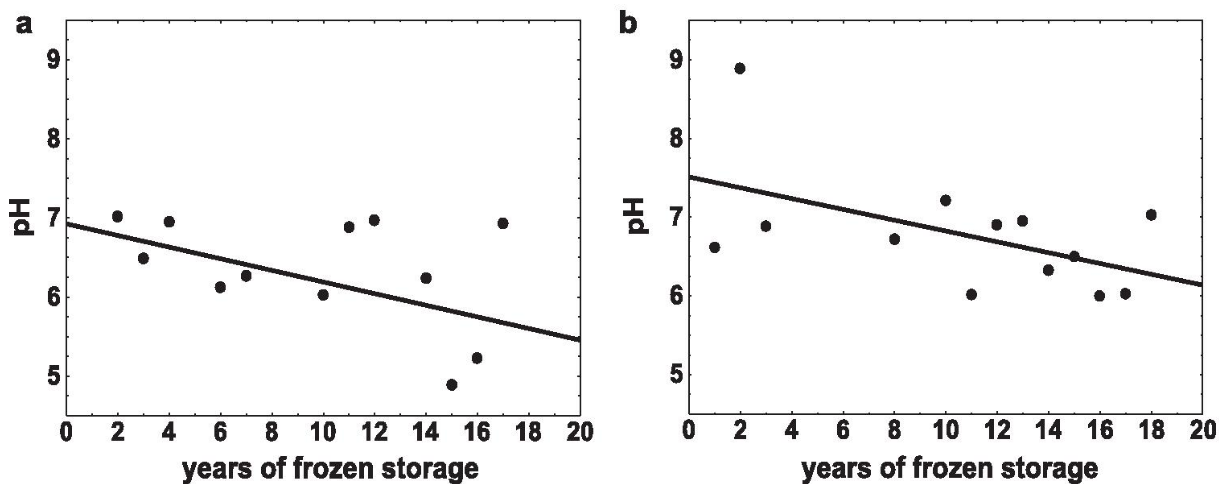
940 Fig. 4



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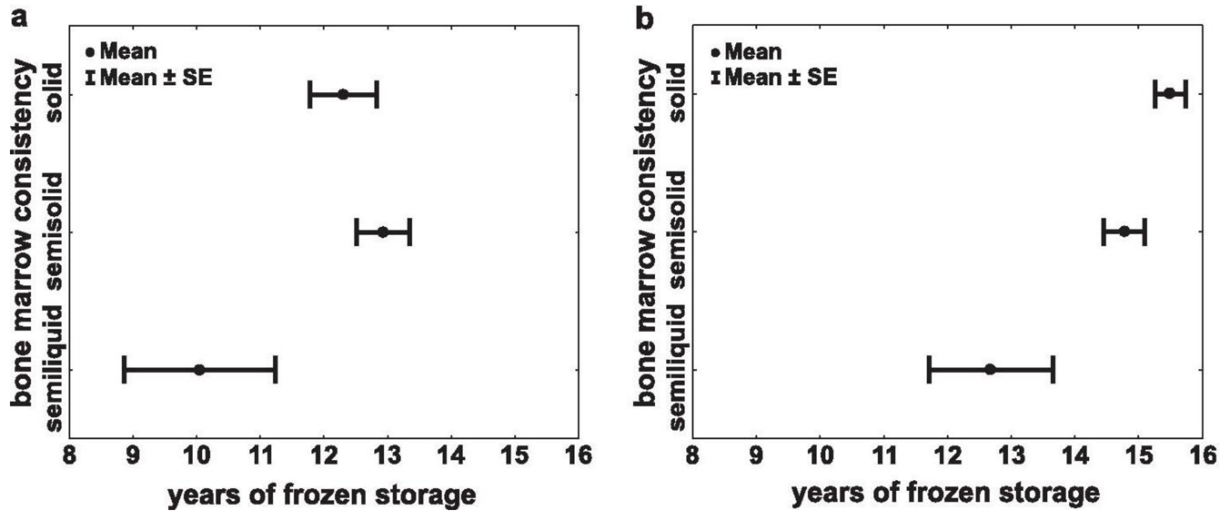
942 Fig. 5



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