The impact of prolonged frozen storage on the preparation quality of bird skins and skeletons in zoological collections

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Abstract

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

Keywords: frozen storage, museum collections, skins, skeletons, vertebrates, zoological preparation.

Declarations

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Conflicts of interest

The authors declare that they have no conflict of interest.

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Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

Code availability

Not applicable.

Authors' contributions

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

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Introduction

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

Most of the specimens housed in natural history collections must be prepared before they become part of these repositories. Zoological collections house animal specimens prepared, for instance, as skins and skeletons (Cato and Jones 1991; Beck 2018). How a specimen is prepared may greatly affect not only its value for research but also its long-term conservation (Simmons and Voss 2009, Carrillo-Ortiz et al. 2021). A controversial issue in research based on natural history collections is whether or not museum specimens provide information that is comparable to the information that can be gathered from specimens in the field (i.e. live specimens). Some studies have shown that some properties including the colour of fur or plumage (Doucet and Hill 2009; Kennedy 2010; Sandoval Salinas et al. 2018) and the length of limbs (Knox 1980; Kuczyński et al. 2002) may change over time after the specimen's death; likewise, the use of certain products during preparation (e.g. arsenic as a tanning agent and insecticide for skins) may affect the integrity and attributes of a specimen (Marte et al. 2006; Pohland and Mullen 2006; Töpfer et al. 2011). This suggests that the methods and protocols used for the preparation of specimens destined for natural history collections could affect the reliability of the results obtained from the study of such specimens. Therefore, the end-users of these collections should be aware of how the details and the preparation of these specimens could affect their results.

Many natural history museum and university collections still use preparation techniques that have barely changed for over a century (Simmons and Snider 2012). The continued use of traditional preparation methods confirms that in general they are both valid and effective. Understanding how these techniques affect specimens and researching systematically how preparation protocols can improve the long-term conservation of collections without causing damage are two issues that are worthy of inquiry. Surprisingly though, zoological preparation as a technical discipline has not traditionally been subject to rigorous systematic research. In recent years, there have been calls to improve protocols in conservation and how material from zoological collections is consulted by applying more objective quantitative approaches. Hence, it is now urgent to conduct hypothesis-based experiments to understand how preparation protocols affect the integrity and feasibility of using natural history collections (Simmons and Snider 2012). To date, studies have assessed the quality of museum specimens subject to different preparation processes (Williams and Hawks 1987; Horie 1990; Kite and Thomson 2006) or explored the optimal conditions for storing and exhibiting specimens after preparation (Staniforth 1984; Mathias 1994; Viscardi et al. 2006). However, the quality and condition of museum specimens will also greatly depend on how these specimens were preserved before being prepared, a further challenge that must be faced as soon as the animal dies. Therefore, what occurs between the death and the preparation of a specimen is also relevant despite often being ignored (Winker 2000).

Skins and skeletons are amongst the most frequently studied elements in vertebrate scientific collections (Csuti 1980). In taxidermy (i.e. animals mounted in lifelike poses), skins are ideally prepared as soon as possible after animal death to avoid any deterioration and ensure the preservation of all its properties (Péquignot et al. 2006). When zoological preparation is not feasible or necessary on death, it is important to check for tissue decay, preserve specimens until preparation, and prevent potential infestations by placing museum specimens in quarantine in a freezer (Bergh et al. 2006; White and Dusek 2015; Windsor et al. 2015; Campbell and Baars 2019). Typically, due to backlogs in admissions in natural history museums, specimens are often not prepared immediately after quarantine and in some cases they remain stored in

freezers for years before preparation. It has been remarked that freezing only temporarily preserves museum specimens since they deteriorate even when frozen (Winker 2000). Despite this, how frozen storage affects the quality of zoological preparation is still poorly known; unfortunately, most knowledge of this subject is not based on objective criteria such as experimental results and so it is not usually publishable.

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

Materials and methods

Sample

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

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

Variables and data collection

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

Sexing

Barn owls can be sexed by plumage given that they have a degree of sexual dimorphism (Ravindran et al. 2018). However, many other species are not sexually dimorphic, so they can only be sexed by examining gonads during preparation. In order to assess the potential effect of long-term frozen storage on internal organs, barn owl specimens were scored as sexable or not sexable by gonad inspection. Since barn owls can breed during their first year of life (Marti 1994), we used specimens with mature gonads, that is, birds hatched the previous calendar year

183 and currently in their second calendar year, i.e. age EURING 5/AHY (Gustafson et al. 1997;
184 EURING 2010) or older.
185
186 ***Body weight***
187 Specimens were weighed to determine whether or not variation in weight occurred during
188 frozen storage. Necropsy was taken into account for this and some subsequent analyses since it
189 entails the opening up of the abdominal cavity, which is likely to influence how birds are
190 exposed to the freezing drying conditions. Here, we used the common buzzard as the biological
191 model because we knew that some specimens had been necropsied; out of the 117 buzzard
192 specimens, 26 had been necropsied and 79 had not; for the 12 remaining birds no data indicated
193 whether they had been necropsied or not. Specimens were weighed with a precision scale
194 (Salter®, HoMedics Group Ltd, max 2kg, d=1g).
195
196 ***Skin tear resistance***
197 Skins' resistance to tearing was also analysed in common buzzard specimens to infer whether or
198 not long-term frozen storage and necropsy had an effect. A fresh piece of skin measuring 2 x 5
199 cm was taken from the apterium (i.e. featherless) chest of each specimen, at a certain distance
200 from the incision in the case of necropsied specimens. The variable defining tear resistance was
201 the weight that these pieces of skin could bear. A structure was designed in which a fragment of
202 skin was fixed to a bucket into which water was added at a constant speed. The volume of water
203 (in litres) was calculated by means of graduation marks (±0.1 litres) on the container. The
204 weight (calculated from the volume of water) that the piece of skin was able to bear before
205 breaking was used as an indicator of tear resistance.
206
207 ***Difficulty of skin preparation***
208 The difficulty in preparing the skins of each barn owl specimen was assessed and scored on a
209 scale ranging from 1 to 4 by skin preparers on the basis of a series of criteria (i.e. whether it was
210 necessary to moisten the specimen regularly, whether the skin was tough, or whether the skin

broke easily): *1* = the skin was very easy to prepare; *2* = some of these difficulties were encountered during preparation and the skin was generally easy to prepare; *3* = at least two of these difficulties were encountered; or, despite their severity, all problems could be resolved; and *4* = so many difficulties were encountered and could not be overcome that the preparer recommended that the specimen be used for the preparation of other elements instead of the skin (e.g. piece of skin, tissues, skeleton).

Bone marrow pH and consistency

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

Data analysis

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

When sexing using the barn owls' internal anatomy, the feasibility or impossibility of sexing a specimen was scored as 'sexed' and 'non-sexed', respectively. A General Linear Model (GLM) with binomial distribution, tested for goodness of fit with the Hosmer-Lemeshow (HL) test, was carried out with the binomial dependent variable *sexing practicability* (*sexed*/*non-*

239 *sexed*) in relation to the independent variable *years of frozen storage*. *Body weight* was
240 introduced as an offset variable in order to minimise heteroscedasticity from the time of freezing
241 onwards. After maximising the likelihood function, a Wald test was conducted.
242 The effect of length of time in the freezer and the performance (or otherwise) of a necropsy
243 on specimens' weights was evaluated. In this case, a GLM with normal distribution was applied,
244 using the independent variables or factors *years of frozen storage* and *necropsy* as well as their
245 interaction (*years of frozen storage* x *necropsy*), and the dependent variable *body weight*. A
246 Wald test was used for this assessment.
247 To analyse how skin tear resistance is modulated by the duration of frozen storage and
248 necropsy, a GLM with normal distribution of the dependent variable *weight* in relation to the
249 independent variables *years of frozen storage* and *necropsy* was performed. The *years of frozen*
250 *storage x necropsy* interaction was not used because previous analyses had shown that the
251 gradients between *weight* and *years of frozen storage* were similar for necropsied and non-
252 necropsied specimens, probably because the pieces of skin were obtained at a distance from the
253 necropsy incision. A Wald test was subsequently performed.
254 Next, to assess whether the difficulty of skin preparation varied depending on the amount of
255 time that the skins were frozen, a GLM with normal distribution was performed in which *years*
256 *of frozen storage* was the independent variable and *difficulty of skin preparation* was the
257 dependent variable. After using the maximum likelihood function, a Wald test was performed.
258 To assess the effect of frozen storage and necropsy on bone marrow pH, a GLM with normal
259 distribution was implemented with *years of frozen storage* and *necropsy* as independent
260 variables for common buzzards, and just *years of frozen storage* as the independent variable for
261 barn owls, and *bone marrow pH* as the dependent variable for both species. Evaluation was
262 conducted with a Wald test.
263 For bone marrow consistency, a multinomial GLM was applied, with *years of frozen storage*
264 and *necropsy* as independent variables for common buzzards, just *years of frozen storage* as the
265 independent variable for barn owls, and *bone marrow consistency* as the multinomial dependent
266 variable in both cases. A Wald test was used for this assessment.

Results

Sexing based on gonadal inspection

Of the 139 barn owl specimens, only 81 were sexually mature individuals; 56 could be sexed (69.14 %) and 25 could not (30.86%). The model showed a good fit (goodness of fit HL=2.321, P=0.679) indicating that the results did not support the hypothesis that the time spent in the freezer significantly affected the practicability of sexing the birds ($W_{(1,81)}$=1.015, P=0.314).

Effect of frozen storage on body weight

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

Effect of frozen storage on skin condition

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

294 The length of frozen storage was found to have a clear effect on the difficulty of skin
295 preparation (Intercept: $W_{(1,12)}$=0.650, P=0.420; Frozen storage$_{(1,12)}$: W=30.68, P<0.001) and, in
296 particular, specimens frozen for longer were more difficult to prepare (Fig. 3). Body regions
297 with less flesh (e.g. skull, distal parts of wings and legs) were the most difficult parts of the
298 specimens to process.
299
300 **Effect of frozen storage on bone marrow properties**
301 Bone marrow pH was not significantly affected by the performance of necropsy in common
302 buzzards (Table 3). In addition, in this species the duration of frozen storage had a marginal
303 effect on bone marrow pH as there was a trend towards bone marrow acidification with greater
304 time of frozen storage (Table 3, Fig. 4A). This marginal effect was mainly due to the results
305 from specimens frozen since 1995, which had abnormally high (i.e. basic) pH values. When
306 these individuals were removed, the model was highly significant (P<0.01). Given that the
307 necropsy effect was not significant, we performed the same analysis on barn owls. With this
308 species, the duration of frozen storage significantly affected bone marrow pH (Intercept:
309 $W_{(1,13)}$=1526.302, P<0.001; Frozen storage$_{(1,13)}$: W=5.11, P=0.024) as bone marrow acidified
310 with time of frozen storage (Fig. 4b). The decrease in pH during the storage period was around
311 1.5 points in both species and pH values reached around 5.5 in common buzzards and around
312 6.0 in barn owls.
313 Unlike necropsy, the duration of frozen storage significantly affected the consistency of bone
314 marrow in common buzzards (Table 4) and in barn owls ($W_{(4,130)}$=10.44, P=0.005). The
315 comparison of semiliquid bone marrow consistency with the two other consistency categories at
316 the same temperature in each species revealed that the duration of frozen storage significantly
317 explained the differences between them (Tables 5 and 6). The increasing solidification of bone
318 marrow with longer frozen storage time seemed to occur in both species, although the transition
319 between the three consistency categories was more evident in barn owls (Fig. 5).
320
321 **Discussion**

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

Impact of prolonged frozen storage on general condition

During freezing, about 80% of the water content of specimens is expected to solidify into pure ice crystals (Cano-Muñoz 1991). However, freezing also causes tissues to dehydrate, that is, water sublimates through biological membranes when a specimen is exposed to an atmosphere under a different water pressure (relative humidity). This phenomenon, known as freeze-drying, leads to the recondensation of water outside the tissues and is the most common problem with frozen specimens (Winker 2000; Campañone et al. 2005; Zaritzky 2008). If freezing is slow, water migrates out of the cells until freezing is complete (i.e. when the centre of the frozen object has a temperature of -12ºC or less), which typically leads to the formation of large ice crystals on the outside of the cells (Cano-Muñoz 1991; Zaritzky 2008; Herren 2012). Ice crystals, even when small and formed within cells, can also cause cells to burst by perforating cellular membranes. This histological damage, added to moisture loss, provokes more water release during thawing and therefore dehydrates specimens even further (Cano-Muñoz 1991; Zaritzky 2008). Ultimately, these processes result in weight loss in frozen specimens (Compagno 2001; Edwards et al. 2002; Campañone et al. 2005). The weight variations in our study could be explained by these phenomena and, furthermore, point to accumulative dehydration over time since weight loss was found to increase as the period of frozen storage increased, especially in whole specimens. Specimens were as a rule not physically insulated

very well and fluctuations in freezer temperatures could have occurred during long storage periods, two factors that could have contributed to progressive weight loss.

Dehydration caused by frozen storage is expected to have an impact on the whole body of frozen specimens, including their internal organs. Indeed, dehydration often complicates the examination of the gonads and can hinder sex determination or even make it impossible (Edwards et al. 2002). In species with no evident sexual dimorphism, the inability to sex a specimen after scrutiny of the gonads may lessen its scientific usefulness, thereby undermining the quality of the scientific collection it belongs to. Even though we were unable to detect any significant impact of dehydration on sex determination, we might have encountered greater difficulties when sexing specimens if a smaller bird species – i.e. with smaller gonads and likely to suffer more quickly and severely from dehydration – had been chosen. Another reason why we did not come across any major impediments when sexing specimens might have been thanks to the expertise of preparers in sexing specimens by gonad inspection, since expert preparers usually make very few mistakes when compared to DNA sexing (<5%, JQ unpublished data). This expertise may play an important role in their ability to sex an animal that is decomposed or dehydrated, as was the case in some of the specimens in this study.

The epidermis of avian skin is a barrier with a facultative capacity for waterproofing that prevents excessive water loss (Horie 1990; Elias and Menon 1991; Menon et al. 1996). Given that the necropsies of vertebrate specimens typically entail opening the abdomen, this protective skin layer is disrupted in this region in necropsied specimens. Consequently, internal organs and cavities are more exposed in these specimens, which make them more susceptible to dehydration than whole specimens during frozen storage. According to our results, necropsied animals were initially lighter than those that had not been necropsied (Fig. 1a), and the rate of weight loss in relation to the years elapsed since the onset of frozen storage differed between necropsied and whole specimens (Fig. 1b). Taken together, these results suggest that there could be two processes operating simultaneously in weight loss in relation to necropsy. On the one hand, the fact that necropsy usually entails the evisceration of specimens could contribute to some extent to explaining the weight difference between entire and necropsied specimens at the

beginning of the storage period. On the other hand, the fact that necropsied specimens are more exposed to dehydration and might have less tissue from which to lose water could lead to an immediate water loss from the remaining tissues in the first few years of frozen storage that, consequently, would explain the lack of any relationship in necropsied specimens between weight loss and the duration of frozen storage. By contrast, non-necropsied specimens could steadily lose weight over time due to the dehydration of all their tissues and organs, which supports the hypothesis that weight loss results from constant or regular dehydration over years of frozen storage.

Impact of prolonged frozen storage on skin properties

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

406 In terms of the degree of difficulty in preparing skins, prolonged frozen storage seriously
407 hampers skin manipulation and processing. Our results support the idea that the duration of
408 frozen storage has a cumulative adverse impact on the feasibility of proper skin preparation.
409 Additionally, the skin of the body regions with less soft tissue underneath (i.e. the skull and the
410 distal part of the limbs) was more difficult to remove properly (CO and LR, pers. obs.),
411 probably due to the fact that these regions were the most freeze-dried. Hence, it is to be
412 expected that the less dehydrated a specimen is, the more manoeuvrable and the less fragile its
413 skin will be. Skin preparation in frozen specimens that have been freeze-dried can be aided by
414 soaking the affected parts or even the whole specimen in water in a refrigerator for 1–2 days
415 (Winker 2000). This strategy reflects the need to moisten more regularly specimens frozen for
416 longer periods of time when preparing their skins, evidence for the impact of dehydration due to
417 frozen storage.
418
419 **Impact of prolonged frozen storage on the properties of bone marrow**
420 The distal limb elements of birds are not commonly pneumatised and usually contain non-
421 hematopoietic bone marrow adipose tissue in their medullary cavity (Gurevitch et al. 2007;
422 Canoville et al. 2019). Lipids in biological systems, including marrow adipose tissue, are
423 vulnerable to oxidation through oxidative and hydrolytic rancidity (Laitinen et al. 2006; Wazir
424 et al. 2019). Although the speed of lipid hydrolysis and oxidation (as well as the resulting
425 changes in marrow fatty acid composition) are greater at higher temperatures, these processes
426 also take place even below 0ºC (Laitinen et al. 2006; Zhou et al. 2018; Blasco et al. 2019). In
427 fact, changes in the biochemical nature of lipids have been reported from bone specimens frozen
428 at -20ºC due to hydrolysis (Mularchuk and Boskey 1990) and risk of a high lipid oxidation rate
429 in fresh-frozen bone allografts exists even at -30°C (Laitinen et al. 2006). As a result of the
430 hydrolysis of lipids such as triacylglycerol and polar lipids, fatty acids are released and, since
431 some of them are acidic, they can lead to a decrease in pH (Deeth and Fitz-Gerald 2006; Zhou et
432 al. 2018). In addition, a drop in pH during cold storage can result from the decomposition of
433 adenosine triphosphate (ATP) into acidic substances such as phosphoric acid (Ozawa et al.

1990). Although the acid-base status of bone marrow has been little studied (Nikolaeva 2018), the abovementioned phenomena may help explain the higher bone marrow acidification detected in the specimens of our study that had been frozen for a longer time.

Dehydration causes structural and mechanical changes in bones by provoking a decrease in the spacing between collagen fibrils (Lees et al. 1984; Lievers et al. 2010). Principally, dehydration results in less strength and fracture strain and hence less toughness in the cortical bone (Nyman et al. 2006). Although these parameters were not analysed in this study, during skeleton preparation it is often noticed that bones of specimens frozen for a long time are more fragile and that some even break easily in the hand, which would be nearly impossible in a fresh specimen (LR, pers. obs.). Therefore, these parameters could be incorporated into future studies since such fragility could negatively affect the quality of bone preparation and so of osteological collections. Since bones can be affected by dehydration, it is likely that a fraction of water associated with bone marrow could be displaced during frozen storage. This loss of water would lead to a change in bone marrow consistency over time, making it more solid, as detected in this study. The protocols for skeleton preparation frequently entail drilling a hole at each end of long bones and then using hot water and a pressurised water gun to remove the bone marrow from the inside of the bones (McDonald 2006; Orta et al. 2011). However, the solidification of bone marrow could obstruct its removal and some marrow may be left inside the bones. Acidification is a major concern in long-term conservation in museums and heritage institutions and, in the case of biological collections, it is well known that residual fat left in skins acidifies over time and speeds up skin deterioration (Winker 2000; Kite and Thomson 2006). Given that lipid acidification occurs to a greater extent at higher temperatures, room temperature at museums is regarded as a serious threat for the stability of bone marrow residues in bones and, consequently, for the welfare of prepared skeletons. As well, poorly prepared osteological collections are very vulnerable to infestation, since pests are primarily attracted to the fat in bones (McDonald 2006). Therefore, the solidification of bone marrow as a result of prolonged frozen storage may not just increase the difficulty and lessen the quality of bone preparation but

could also impede long-term bone conservation by triggering detrimental biochemical reactions and inciting pest infestations.

Guidelines on frozen storage for improving preparation protocols

Several results from this study point toward long-term frozen storage as being potentially counterproductive for zoological preparation; additionally, they suggest that frozen storage leads to progressive dehydration and fails to halt the biochemical processes that modify the physicochemical properties of skin and bone marrow over time. As a result, prolonged frozen storage hinders the proper preparation and integrity of vertebrate specimens that are to be stored as skins and skeletons in scientific collections. However, by taking into account these considerations and seeking solutions, the preparation quality of vertebrate specimens could be greatly improved.

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

Another measure that would help mitigate the drawbacks of prolonged frozen storage in zoological preparation is the complete isolating of the specimens while frozen through the use

of materials that are stable at low temperatures and resistant to water vapour and oxygen (i.e. to keep oxygen out and moisture in) (Pham and Mawson 1997; Zaritzky 2008; Herren 2012). Isolation could mean simply wrapping specimens with plastic film and placing them in plastic bags with as much air as possible removed, or, best of all, vacuum-packing them in plastic bags and then storing them inside isolating boxes until preparation (Cano-Muñoz 1991; Winker 2000). There is a wide range of available plastic packaging materials (e.g. polyethylene terephthalate, polypropylene, etc.); however, some are not considered to be suitable for long-term conservation storage (Tétreault 2017). Once again, isolation measures are particularly important for necropsied, wounded or small specimens. According to Winker (2000), it is possible to prepare small specimens that have been frozen for three years if they have been properly isolated and stored. Given that this author considers three years to be an exceptionally long period of time, even greater isolation measures should be adopted when longer frozen periods are foreseeable. This also sheds light on another interesting consideration: the size of specimens could influence the deleterious effects of frozen storage or the aptness of the measures used in this work. We used relatively large species as model species but the effects of long-term frozen storage on smaller species are still unknown, and would be an interesting future field of study.

An additional recommendation for optimizing the preservation of specimens with a view to improving their preparation is to use a freezing system that minimises dehydration; this could, however, imply investment in renewing existing infrastructure, something that may be beyond the capacity of many modestly budgeted museums. In this regard, dehydration could be minimised by combining short freezing times and good aerodynamics. The faster the temperature of the subject is dropped, the less dehydration takes place (Herren 2012). Proper regulation of aerodynamic parameters in freezers (i.e. air velocity, air pressure and relative humidity) could stop snow from forming due to the humidity lost from the specimens, which prompts further dehydration. Frost-free freezers should be avoided because they undergo dramatic temperature fluctuations that could mobilise water from specimens (Winker 2000). Specimens should be maintained at or below -20ºC and at a very high relative humidity (95–

98%) in order to prevent dehydration (Cano-Muñoz 1991; Winker 2000). Blast freezers are recommendable since their use of high-velocity air and temperatures up to -40ºC allows for quick freezing (Cano-Muñoz 1991; Herren 2012).

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

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

Relevance of research on zoological preparation

The protocols followed to prepare museum specimens have a crucial impact on the appropriateness and usefulness of these specimens as sources of biological information. For instance, small changes in specimens' posture during preparation may improve the scientific value of an ornithological collection by facilitating the taking of relevant information such as biometric measurements or moult data (Carrillo-Ortiz et al. 2021. The present work, which used as a case study of assessment of the impact of prolonged frozen storage on the preparation quality of bird skins and skeletons, highlights the suitability and worth of conducting research

on zoological preparation as a means of optimizing the current quality of zoological preparation for scientific collections. Given that long-term frozen storage leads to progressive dehydration and does not stop the biochemical processes that provoke skin aging and acidification of bone marrow, this procedure could potentially affect future studies based on zoological collections. This highlights the need to understand how zoological preparation establishes or optimises preservation techniques and therefore improves the scientific worth of zoological collections. Here, we have shown that systematic questions and experimental designs focused on zoological preparation can help to improve knowledge of how certain practices affect the quality of the final result, and how they can contribute towards improving preservation and preparation protocols that will make scientific collections more durable, usable and valuable. Hence, this study not only highlights how important it is to study zoological preparation but also aims to encourage fresh studies to be conducted of other collection elements and species to broaden current knowledge and further optimise zoological preparation. Research into zoological preparation aims to optimise the conservation, utility and value of natural history collections for the benefit of the scientific community and society as a whole, and so any contribution towards the improvement of this technical discipline is of great interest.

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Tables

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

	Level of effect	Estimate parameter	SE parameter	Wald statistic	P-value
Intercept		6.545	0.099	4413.222	0.000
Necropsy	NO	0.261	0.099	7.018	**0.008**
Years of frozen storage		-0.023	0.009	6.628	**0.010**
Necropsy × Years of frozen storage	1	-0.016	0.009	3.516	0.061

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

	Level of effect	Estimate parameter	SE parameter	Wald statistic	P-value
Intercept		1.425	0.095	0.00	0.000
Necropsy	NO	0.219	0.048	0.00	**0.008**
Years of frozen storage		0.008	0.008	0.008	0.330

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

	Level of effect	Estimate parameter	SE parameter	Wald statistic	P-value
Intercept		1.969	0.068	838.917	0.000
Necropsy	NO	-0.039	0.068	0.334	0.564
Years of frozen storage		-0.011	0.006	3.558	0.059
Necropsy × Years of frozen storage	1	0.000	0.006	0.001	0.976

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

	Degrees of freedom	Wald statistic	P-value
Intercept	2	3.740	0.154
Necropsy	2	1.568	0.457
Years of frozen storage	2	8.750	**0.013**
Necropsy × Years of frozen storage	2	1.133	0.567

Table 5 Estimated variance-covariance matrix test assessing differences in bone marrow consistency in terms of years of frozen storage in common buzzards.

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

836 **Table 6** Estimated variance-covariance matrix test assessing differences in bone marrow

837 consistency in terms of years of frozen storage in barn owls.

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

838

839

840

Figure captions

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

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

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

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

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