

1 **DISEASE IN WILDLIFE OR EXOTIC SPECIES**

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3 **Intrasarcoplasmic Polyglucosan Inclusions in Heart and Skeletal Muscles of Long-**  
4 **Finned Pilot Whales (*Globicephala melas*) May be Age-Related**

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18

## Summary

19 Polysaccharide storage myopathies have been described in several animal species and are  
20 characterised by periodic Acid–Schiff (PAS)-positive, diastase-resistant intrasarcoplasmic  
21 inclusions in myocytes. Skeletal and cardiac muscle samples from a subset of a single pod of  
22 stranded long-finned pilot whales (*Globicephala melas*) were evaluated by light and  
23 transmission electron microscopy. Twelve individuals demonstrated sporadic basophilic  
24 packets of PAS-positive, diastase-resistant complex polysaccharide material, either centrally  
25 or peripherally in skeletal and cardiac myocytes. Few microscopic myopathic changes were  
26 found but included focal inflammation and internalized nuclei. Ultrastructurally, the  
27 inclusions consisted of loosely arranged, tangled filaments and were not membrane-bound,  
28 which is consistent with polyglucosan bodies. Within skeletal muscle, the number of  
29 inclusions had a marginal statistically significant ( $p = 0.0536$ ) correlation with length, as a  
30 proxy for age, suggesting that such inclusions in skeletal muscles may be age-related,  
31 although the cause remains unclear.

## Introduction

32  
33 Polyglucosan bodies are periodic-Acid-Schiff (PAS)-positive and diastase-resistant  
34 intracytoplasmic inclusions composed of poorly branched, long strands of abnormal glycogen  
35 with a characteristic fibrillar ultrastructure (Cheville, 2009). In humans, they occur as  
36 incidental, age-related changes, such as in corpora amylacea in neural tissue or basophilic  
37 degeneration of the myocardium, and also as pathological accumulations as a key feature of  
38 the wide ranging polyglucosan body diseases (Cavanaugh, 1999). They occasionally occur in  
39 skeletal or cardiac muscle in conditions such as Lafora body disease, or as a primary lesion  
40 sites of myopathies, including glycogen-storage disorders types IV, VII and XV, and AMP-  
41 activated protein kinase deficiency (Cavanaugh, 1999; Hedberg-Oldfors and Oldfors, 2015).  
42 In animals, polyglucosan body accumulation in muscle has been most thoroughly  
43 characterized in equine polysaccharide storage myopathy (EPSM) (Valentine *et al*, 1997).

44 Specific carbohydrate metabolism pathway dysfunctions have been identified for  
45 most of these disorders, including Type 1 EPSM (Hedberg-Oldfors and Oldfors, 2015;  
46 McCue *et al*, 2008). Clinical signs referable to muscle, vary with disease subtype in people,  
47 but generally include muscle weakness and atrophy, and exercise intolerance (Hedberg-  
48 Oldfors and Oldfors, 2015). Similarly, clinical signs in EPSM depend on chronicity, with  
49 muscle fasciculations and stiffness observed in the acute presentation and muscle atrophy and  
50 gait abnormalities in the chronic form (Valberg *et al*, 2011).

51 Similar intramyofibre PAS-positive, diastase-resistant aggregates and granules have  
52 been described within the skeletal muscles of 11 cetacean species, including short-finned  
53 pilot whales (*Globicephala macrorhynchus*) (Sierra *et al*, 2012) and within the myocardium  
54 of pilot whales (Scotti, 1962; Cowan, 1966). Inclusions in skeletal muscle were accompanied  
55 by chronic myopathic changes, muscle atrophy and necrosis (Sierra *et al*, 2012), whereas  
56 those in heart were considered akin to age-related “basophilic degeneration” in humans

57 (Cowan, 1966). However, the pathogenesis and clinical significance of polyglucosan  
58 inclusions in cetaceans remain undetermined.

59 Long-finned pilot whales (*Globicephala melas*) are large pelagic odontocetes, the  
60 Northern and Southern subspecies of which, inhabit boreal and subarctic parts of the Atlantic  
61 Ocean and circumpolar Antarctic Ocean, respectively. Pod size is typically 10 to 20 closely  
62 related members, thought to be formed around adult females and their offspring (Reeves *et al*,  
63 2002). Pilot whales are commonly involved in both mass and single strandings with 254  
64 strandings of individual animals in Scotland alone in the six-year period of 2005–2019  
65 (Scottish Marine Animal Stranding Scheme, 2020).

66 This aim of this study was to describe and characterize intrasarcoplasmic inclusions in  
67 skeletal and cardiac muscles from 12 long-finned pilot whales from a single pod involved in a  
68 mass stranding event (MSE) to determine if they were pathological, age-related or a  
69 combination of both.

70

## 71 **Materials and Methods**

### 72 *Animals and Samples*

73 Thirty-nine pilot whales from a single pod of approximately 70 animals, presumed to be  
74 genetically-related, stranded on July 22, 2011 at the Kyle of Durness, Scotland, UK  
75 (58°32'1.2516" N, -4°48'9.252" W) and 19 are known to have died following a re-flotation  
76 attempt. Sixteen pilot whales (8 females and 8 males) ranging in length from 2.83–5.55 m were  
77 recovered and necropsied on site. Where possible, the age of individual animals (Table 1) was  
78 estimated retrospectively on the basis of tooth analysis (Luque *et al*, 2009).

79 Necropsies were performed according to a standard protocol (Kuiken and Baker, 1991) and a  
80 wide range of tissue samples from each animal was collected for histological evaluation.

81 Skeletal muscle samples were taken from the *longissimus dorsi* muscle immediately

82 craniolateral to the leading edge of the dorsal fin after the blubber had been removed.  
83 Ventricular myocardium was also sampled. Tissues for histology were fixed in 10% neutral  
84 buffered formalin, processed routinely through graded alcohols, embedded in paraffin wax,  
85 sectioned (4 $\mu$ m) and mounted on glass slides. Initial diagnostic histology on haematoxylin  
86 and eosin (HE)-stained sections allowed selection of 12 individuals (seven females and five  
87 males) based on histological assessment of post-mortem tissue preservation sufficient to  
88 permit sensible interpretation. Serial sections were cut from skeletal and cardiac muscle  
89 samples and stained with HE, PAS or PAS-dia­stase (PAS-D) (Bancroft and Cook, 1994).  
90 Additional heart and skeletal muscle serial sections were taken from two representative  
91 individuals and stained with Grocott–Gomori’s methenamine silver (GGMS), toluidine blue  
92 or von Kossa (Bancroft and Cook, 1994) to assess the presence of carbohydrate, acidic  
93 residues and mineralization, respectively. All sections were evaluated for the presence or  
94 absence of the following changes: cytoplasmic vacuolation, inflammation, small group  
95 atrophy, myocyte regeneration, myocyte degeneration, haemorrhage, fibrosis and parasitism.

96

#### 97 *Quantification and Statistical Analyses*

98 For skeletal muscle only, the numbers of angular fibres and internalized nuclei were counted  
99 in 10 random microscopic fields at a final magnification of  $\times 200$ . The total number of  
100 polyglucosan inclusion-containing myocytes was counted in each section stained with HE,  
101 PAS or PAS-D. To standardise the inclusion counts across muscle types and sections, the  
102 area of each tissue section was measured using AnalySIS (Soft Imaging System software,  
103 Olympus, Tokyo, Japan). The inclusion density was calculated by dividing the total inclusion  
104 count on the slide by the total area of that tissue section.

105 To establish a mean cell diameter for each sample, the diameters of two cells devoid of  
106 inclusions were measured in PAS-stained sections from each of five random microscopic

107 fields at  $\times 200$  magnification. For every inclusion, the diameter of the inclusion and the  
108 diameter of the cell containing it, were measured using AnalySIS. The proportion of the cell  
109 occupied by the inclusion was estimated by dividing the area of the inclusion by the area of  
110 the host cell, and multiplying by 100, to give a relative percentage. Means were calculated for  
111 inclusion diameter, the diameter of cells containing inclusions and the diameter of cells  
112 devoid of inclusions.

113 As the diameter of skeletal muscle cells was highly correlated with the length of the  
114 whale ( $p < 0.001$ ), the inclusion density, based on standardized number of myocytes, was used  
115 for analysis. This was calculated by multiplying the inclusion density by the average myocyte  
116 cross-sectional area from the sample.

117 Statistical analyses were performed using R software (version 2.15.1, R Foundation  
118 for Statistical Computing, Vienna, Austria). Analysis of variance was used to compare  
119 categorical variables (sex, muscle type, and presence of inflammation and degeneration) and  
120 linear models were used to compare continuous variables (length and density of inclusions,  
121 and percentage of cell occupied by inclusions). Approximation to normality was judged by  
122 means of the Shapiro-Wilks W test. The results indicate that  $\log(x-1)$  gave the best  
123 transformation. For all analyses,  $p \leq 0.05$  was considered significant.

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### 125 *Electron Microscopy*

126 One  $\text{mm}^3$  samples of skeletal muscle were taken from 4 pilot whales  
127 (SW2011/303.01, SW2011/303.09, SW2011/303.13 and SW2011/303.04) that were suitably  
128 well-preserved and had relatively higher numbers of inclusions, as detected by light  
129 microscopy, post-fixed in osmium tetroxide, dehydrated and embedded in Epon resin 812  
130 (Hexion, Columbus, Ohio, USA) for electron microscopy. Myocardial tissue was extracted  
131 from a paraffin wax block (SW2011/303.01) and prepared for electron microscopy as above.

132 Sections (1  $\mu\text{m}$ ) from all resin-embedded samples were stained with toluidine blue.  
133 Intrasarcolemmic inclusions were identified in the skeletal muscle of whale SW2011/303.09  
134 and myocardium of whale SW2011/303.01. Sections from these two blocks were serially  
135 sectioned at 60 nm, stained with uranyl acetate and lead citrate (Ellis, 2007) and examined  
136 with a Joel 1200EX transmission electron microscope (Joel, Tokyo, Japan).

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## Results

### *Histopathology*

139 Ten of the 12 cases demonstrated minimal (0 to 2 per  $\times 200$  field) intrasarcolemmic  
140 inclusions in both cardiac and skeletal muscle samples in HE-, PAS- and PAS-D-stained  
141 sections. The remaining two cases (SW2011/303.02, SW2011/303.11) contained only a  
142 single intrasarcolemmic inclusion in either cardiac or skeletal myocytes, which were only  
143 observed in PAS- and PAS-D-stained sections.  
144

145 The cardiac and skeletal muscle inclusions were morphologically identical, had  
146 sharply demarcated borders and frequently appeared as discrete or aggregated packets (Fig.  
147 1), although sometimes they appeared as uniform amorphous aggregates. In HE-stained  
148 sections, the inclusions varied from pale to deeply basophilic, occasionally with a darker  
149 staining centre, and were located both peripherally and centrally within the myocyte  
150 sarcoplasm. The myocytes that contained inclusions were frequently perifascicular or on the  
151 edge of perimysium and often appeared to displace myofibrils and, in cardiac muscle, also  
152 displace nuclei. The inclusions were positive in PAS-stained serial sections, staining bright  
153 pink, and were resistant to diastase digestion (Fig. 2), positive for carbohydrate with GGMS  
154 (Fig. 3a), metachromatic with toluidine blue, indicative of acidic residues (Fig. 3b) and were  
155 devoid of mineralization with von Kossa staining.

156 In a few sections, small inflammatory foci were centred on inclusion-containing  
157 myocytes and composed of macrophages, predominantly, with fewer lymphocytes and  
158 neutrophils (Fig. 4a). Some macrophages contained PAS-positive, diastase-resistant material  
159 (Fig. 4b). The affected myocytes were degenerate as indicated by loss of cross-striations and  
160 a markedly fragmented sarcolemma resulting in a “moth-eaten” appearance. A moderate  
161 number of internalised myocyte nuclei were present. None of the samples examined showed  
162 vacuolation, small group atrophy, regeneration, haemorrhage, fibrosis, fat infiltration, or  
163 protozoal or metazoan parasites.

164

#### 165 *Electron Microscopy*

166 By electron microscopy, the skeletal muscle myocytes, recognized by light microscopy as  
167 containing diastase-resistant inclusions, were seen to contain several large aggregates of non-  
168 membrane bound filamentous material in both sarcoplasmic and subsarcolemmal locations  
169 (Fig. 5a). This material was frequently interspersed with, and displaced, the myofibrils. The  
170 inclusions consisted of irregularly arranged filaments, often with more electron-dense cores  
171 in which individual filaments could not be discerned, while other inclusions appeared less  
172 well aggregated and were composed of loosely arranged, short, tangled and randomly-  
173 oriented filaments (Fig. 5b). Consistently, inclusions which contained an electron-dense core  
174 also appeared more compact and electron-dense with fewer visible filaments.

175 In the cardiac myocytes, inclusions were present in central or peripheral locations and  
176 distributed between myofibrils, often displacing them (Fig. 6a). These inclusions were  
177 morphologically very similar to those in the skeletal muscle, consisting of moderately  
178 electron-dense, short, non-membrane bound filaments that were randomly oriented, loosely  
179 amassed and frequently contained a homogeneous more electron-dense core (Fig. 6b). Both  
180 inclusion types were deemed consistent with polyglucosan bodies.



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182 ***Quantitative and Statistical Analyses***

183 The mean densities of inclusions in PAS-stained skeletal and cardiac muscle sections were  
184 2.40/cm<sup>2</sup> and 5.39/cm<sup>2</sup>, respectively. In the same sections, where present, the mean  
185 proportion of cell occupied by inclusions was 64.1% and 72.5% in skeletal and cardiac  
186 muscle, respectively. There was a marginally significant correlation (p =0.0536) between the  
187 inclusion density, based on standardized number of myofibres and whale length in muscle  
188 samples, based on examination of PAS-stained sections. No correlation was found between  
189 the number of inclusions per standardized number of myofibres and whale length in  
190 myocardium. There were no correlations between the number of inclusions per standardised  
191 number of myofibres with sex or presence of inflammation, degeneration or angular fibres,  
192 nor between the percentage of myocyte cell diameter occupied by inclusions with sex or  
193 length.

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**Discussion**

196 The basophilic inclusions documented in this study appear similar to those reported in the  
197 skeletal muscles of other cetacean species (Sierra *et al*, 2012). However, we characterized the  
198 changes using different parameters, including frequency, density, proportion of cell occupied,  
199 and presence in myocardium, which was more appropriate to our goal of determining if the  
200 inclusions were age-related rather than disease-related. Furthermore, ultrastructural  
201 examination, in addition to the histological and histochemical analyses, revealed that the  
202 inclusions were consistent with polyglucosan bodies.

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Electron microscopy demonstrated that, like those found in EPSM and human polyglucosan body diseases, the inclusions in this study were not membrane-bound, which suggests they originated intracellularly. Most, but not all, contained more electron dense

206 cores, which could represent progressive consolidation of material (Valentine *et al*, 1997;  
207 Cavanaugh, 1999; McCue *et al*, 2009). The displacement of cardiac and skeletal myofibrils  
208 was also reported in EPSM, in which it was proposed to play a role in the pathogenesis of  
209 muscle dysfunction (Naylor *et al*, 2012). Glycogen accumulation is proposed to be one of the  
210 initial steps in the formation of diastase-resistant complex polysaccharide in EPSM  
211 (Valentine and Cooper, 2006). Glycogen granules or aggregates were not identified  
212 ultrastructurally or histologically in this study, as diastase-sensitive material could have been  
213 depleted during the live-stranding process or lost through routine processing of the tissues.

214         The identical staining characteristics and ultrastructure of the inclusions in cardiac  
215 and skeletal muscle and simultaneous presence in both tissues in most cases (83%), are  
216 suggestive of a shared pathogenesis, likely involving a defect in carbohydrate metabolism. In  
217 addition to cardiac and skeletal muscle, polysaccharide inclusions have been found in various  
218 smooth muscles of horses with EPSM, including urinary bladder, ureter, penis and arrector  
219 pili muscles (Larcher *et al*, 2008). However, there were no inclusions in smooth muscle in the  
220 sections examined from the pilot whales in the present study (Brownlow *et al*, 2015). The  
221 foci of inflammation, centred on inclusion-containing myocytes, suggests that a pathological  
222 process leads to its accumulation, and the presence of a moderate number of internalised  
223 nuclei in some skeletal muscle sections is indicative of chronic myopathy. However, there  
224 was no associated atrophy or acute necrosis as described in other cetaceans (Sierra *et al*,  
225 2012). Ubiquitin, which targets abnormal proteins, has been reported in polysaccharide  
226 inclusion-containing myocytes in cetaceans (Sierra *et al*, 2012) and horses (Valentine *et al*,  
227 2006), further supporting a pathological origin. Ubiquitination of glycogen aggregates is  
228 proposed to play a role in the development of diastase resistance (Valentine and Cooper,  
229 2006), potentially in response to abnormal folding of the glycogenin protein component of

230 glycogen (Valentine *et al*, 2006). Unfortunately, immunolabelling for ubiquitin was not  
231 performed in our study due to limited funds.

232         Although only marginally statistically significant, most likely due to the small sample  
233 size of this pod, there was a correlation between the inclusion density by standardized  
234 number of myofibres and whale length in skeletal muscle samples. Length being used as a  
235 proxy for age, this finding suggests that intrasarcoplasmic polyglucosan inclusions in skeletal  
236 muscles may be age-related in this species. This finding is supported by previous reports, in  
237 which, with the exception of a single juvenile bottlenose dolphin, polysaccharide inclusions  
238 in skeletal muscle of other cetacean species were only found in adult or adult–senile  
239 specimens (Sierra *et al*, 2012). Accumulation of PAS-positive inclusions in cardiac muscle of  
240 pilot whales has also been reported to be age-related (Cowan, 1966), although such an  
241 association was not found in myocardium in this population. The overall prevalence of  
242 polysaccharide inclusions was highest in the current study of long-finned pilot whales  
243 (100%), compared with that reported in other cetacean species (22.6%) or short finned pilot  
244 whales (16.6%, 2/12 animals examined) (Sierra *et al*, 2012; Sierra *et al*, 2017). Cowan (1966)  
245 observed a prevalence (60%) of basophilic degeneration in long-finned pilot whales, which is  
246 closer to that in the present study. All of the animals in this study originated from the same  
247 pod, and so were highly likely to have been genetically related. Thus, it is unclear to what  
248 extent polysaccharide accumulation in long-finned pilot whales is a species-related  
249 phenomenon or whether there could have also been a familial component in this pod.

250         The most likely cause of stranding in this pod was an underwater explosion and the  
251 causes of death were attributable to the effects of live-stranding (Brownlow *et al*, 2015).  
252 Therefore, the physiological or pathophysiological origin and clinical significance of these  
253 inclusions remain elusive. The relative lack of associated histopathological features contrasts  
254 with the findings of acute necrosis and atrophy by Sierra *et al* (2012) despite chronic

255 myopathic and inflammatory changes being identified. As muscle weakness and dysfunction  
256 are a hallmark of polyglucosan body myopathies, it is possible that these inclusions could  
257 contribute to cetacean strandings but, in this species, are more likely to represent an  
258 incidental degenerative, age-related change unrelated to muscle dysfunction.

259         Screening future cases of stranded and non-stranded cetaceans for this lesion,  
260 including other populations of long-finned pilot whales unrelated to those examined here,  
261 may help determine the significance of this finding. Additionally, comparing long and deep-  
262 diving with short and non-deep diving cetacean species, may determine if longer episodes of  
263 hypoxia could contribute to this condition.

264

265

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269 Moredun Research Institute, the Royal Zoological Society of Scotland and Marine Scotland.

270 **Figure Legends**

271 Fig. 1. Long-finned pilot whale. Intracytoplasmic inclusions comprising pale amphophilic to  
272 basophilic material in (a) skeletal and (b) cardiac myocytes. HE. Bar, 50  $\mu\text{m}$ .

273

274 Fig. 2. Long-finned pilot whale, skeletal muscle. Periodic Acid-Schiff-positive, diastase-  
275 resistant inclusions, consistent with complex polysaccharide, at periphery of a skeletal  
276 myocyte. PAS-D. Bar, 100  $\mu\text{m}$ .

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278 Fig. 3. Long-finned pilot whale, skeletal muscle. (a) Intramyocytic inclusions stain black,  
279 consistent with carbohydrate. Grocott–Gomori’s methenamine silver. Bar, 100  $\mu\text{m}$ . (b)  
280 Inclusions are metachromatic indicating presence of acidic residues. Toluidine blue. Bar, 100  
281  $\mu\text{m}$ .

282

283 Fig. 4. Long-finned pilot whale, skeletal muscle. Mixed inflammatory response including  
284 macrophages and a few lymphocytes and neutrophils associated with inclusion-containing  
285 myocytes. (a) HE. Bar, 50  $\mu\text{m}$ . (b) PAS-D. Bar, 50  $\mu\text{m}$ .

286

287 Fig. 5a. Long-finned pilot whale, skeletal muscle. Non-membrane bound inclusion material  
288 (arrows) displaces myofibrils (M). Z-lines (Z). TEM. Bar, 2  $\mu\text{m}$ .

289

290 Fig. 5b. Long-finned pilot whale, skeletal muscle. Higher magnification of short, loosely  
291 packed, randomly oriented and tangled filaments in inclusion. TEM. Bar, 1  $\mu\text{m}$ .

292

293 Fig. 6a. Long-finned pilot whale, cardiac muscle. Distinct, non-membrane bound,  
294 intramyocytic inclusion mass (arrows) with central electron-dense core (C). Normal

295 myofibrils (M) and sarcomeric bands at periphery of cardiomyocyte. Erythrocyte (E).

296 Basement lamina (BM). TEM. Bar, 2  $\mu\text{m}$ .

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298 Fig. 6b. Long-finned pilot whale, cardiac muscle. Variegated, aggregated short fibrils form an

299 electron-dense core in which individual fibrils are not easily discernible. TEM. Bar, 0.2  $\mu\text{m}$ .

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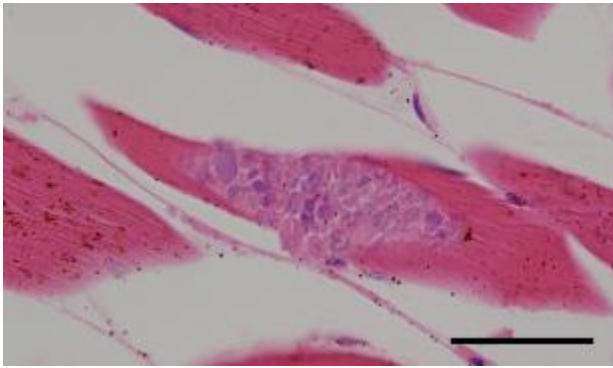
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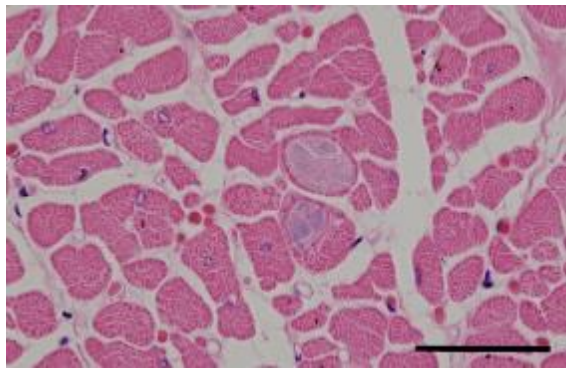
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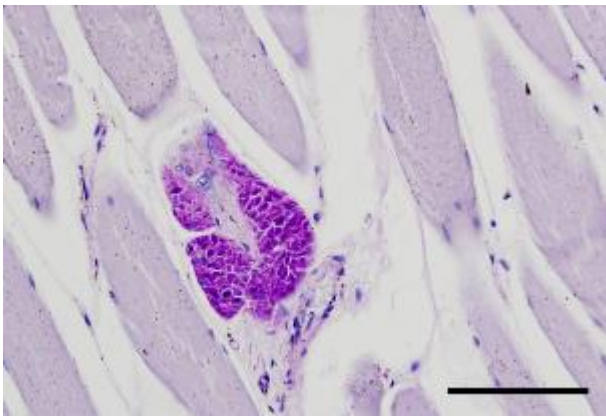
Figure 1a



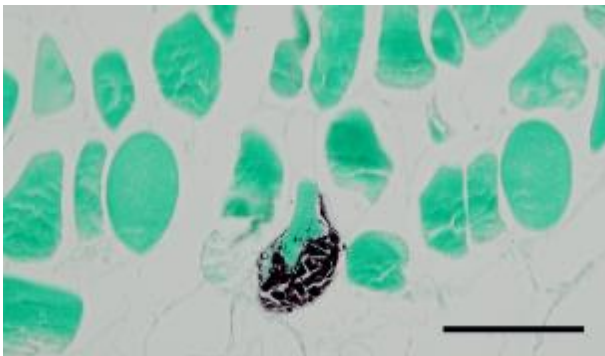
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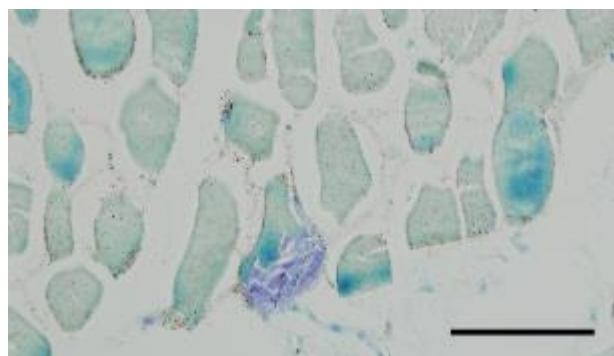
2



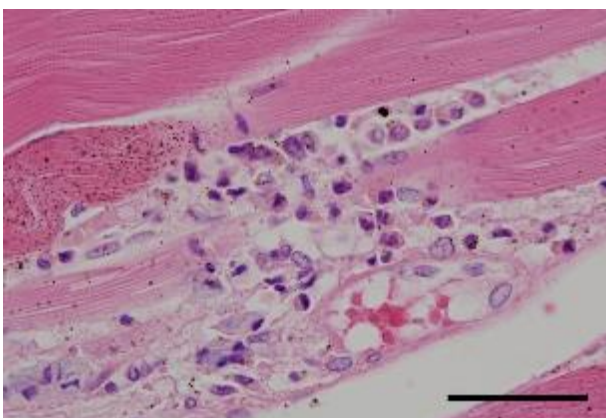
3a



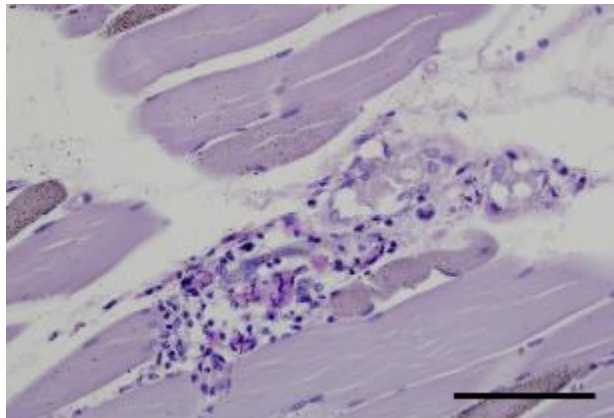
3b



4a



4b

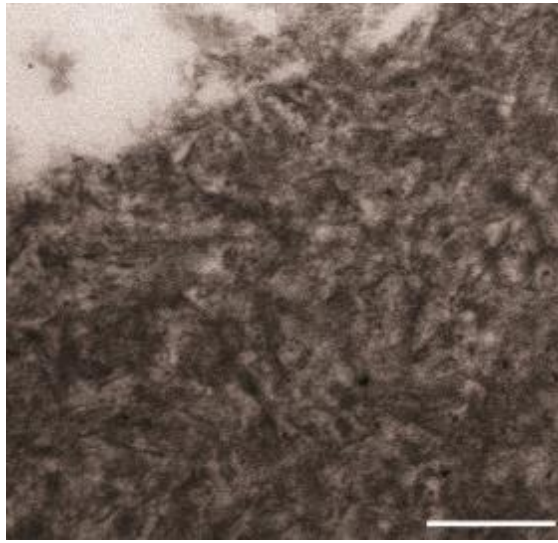


5a

5b



Figure 5a.tif



6a

6b

