

# Persistent organic pollutant burden, experimental POP exposure and tissue properties affect metabolic profiles of blubber from grey seal pups

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1 **Title:**

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4

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22 **Keywords:**

23 Dioxin-like PCBs; glucose uptake; lipolysis; lactate production; blubber depth;  
24 energetic state; environmental contamination; fasting; feeding

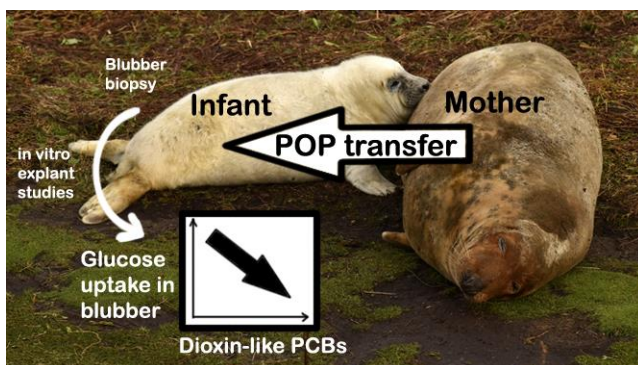
25

26 **Abstract**

27 Persistent organic pollutants (POPs) are toxic, ubiquitous, resist breakdown,  
28 bioaccumulate in living tissue and biomagnify in food webs. POPs can also alter energy  
29 balance in humans and wildlife. Marine mammals experience high POP concentrations,  
30 but consequences for their tissue metabolic characteristics are unknown. We used  
31 blubber explants from wild, grey seal (*Halichoerus grypus*) pups to examine impacts of  
32 intrinsic tissue POP burden and acute experimental POP exposure on adipose metabolic  
33 characteristics. Glucose use, lactate production and lipolytic rate differed between  
34 matched inner and outer blubber explants from the same individuals and between  
35 feeding and natural fasting. Glucose use decreased with blubber dioxin-like PCBs (DL-  
36 PCB) and increased with acute experimental POP exposure. Lactate production  
37 increased with DL-PCBs during feeding, but decreased with DL-PCBs during fasting.  
38 Lipolytic rate increased with blubber dichlorodiphenyltrichloroethane (DDT) and its  
39 metabolites (DDX) in fasting animals, but declined with DDX when animals were  
40 feeding. Our data show that POP burdens are high enough in seal pups to alter adipose  
41 function early in life, when fat deposition and mobilisation are vital. Such POP-induced  
42 alterations to adipose glucose use may significantly alter energy balance regulation in  
43 marine top predators with the potential for long term impacts on fitness and survival.

44

45 **TOC/Abstract Art**



46

47 **Introduction**

48           The impact of environmental and organismal accumulation of man-made  
49 chemicals is a growing global challenge<sup>1,2</sup>. Persistent organic pollutants (POPs) are  
50 toxic<sup>3</sup>, accumulate in living tissues<sup>4</sup>, and resist biological and chemical degradation<sup>5</sup>.  
51 Widespread bans on their production initially lowered environmental POP levels<sup>6-9</sup>, but  
52 reductions have slowed or ceased more recently<sup>10-13</sup>. Legacy POPs are thus ubiquitous  
53 in the marine environment and remain a serious concern for top predators, in which  
54 biomagnification produces the highest burdens<sup>4,11,14-16</sup>.

55           At high exposure levels POPs, such as polychlorinated biphenyls (PCBs),  
56 polybrominated diphenyl ethers (PBDEs) and organochlorine pesticides (OCPs),  
57 including dichlorodiphenyltrichloroethane (DDT), impair fertility, reproductive function,  
58 skeletal and neurological development, immunity and thyroid function in humans and  
59 wildlife<sup>17-24</sup>. Recently, several POPs have also been implicated as lipid disruptors in  
60 humans and rodents<sup>25,26</sup>. In fish and zooplankton, POPs can increase fat deposition and  
61 lipogenic gene expression<sup>27-31</sup>. Conversely, exposure to POPs such as dioxins, lindane  
62 and OCPs can cause rapid energy reserve depletion, termed ‘wasting syndrome’<sup>32-35</sup>.  
63 These apparently conflicting effects of different POPs on a variety of energetic  
64 processes highlight the need for further investigation of pollutant mixtures, doses and  
65 exposure routes, and their physiological consequences for organisms in natural  
66 environments.

67           POP-induced alterations to energy balance may have significant individual and  
68 population level consequences because fatness is often positively associated with  
69 reproductive fitness and survival<sup>36</sup>. This is particularly true in marine mammals<sup>37-39</sup>,  
70 which rely heavily on subcutaneous fat reserves for insulation<sup>40-41</sup> and as a metabolic  
71 fuel when food is scarce. Many marine mammal species also undergo natural periods of

72 fasting during moulting, breeding, migration, or the postweaning fast that occurs in  
73 many phocid pups<sup>37,42-46</sup>.

74 Marine mammals typically experience exceptionally high POP burdens due to  
75 their high fat content and top trophic level, and are regarded as sentinels of marine  
76 pollution<sup>14,16</sup>. POPs are transferred to suckling young as they undergo rapid fat  
77 accretion<sup>47</sup>. In fasting individuals, less hydrophobic POPs are released into the blood  
78 stream as lipid is mobilised and more recalcitrant POPs concentrate in the remaining  
79 blubber<sup>47-54</sup>. Phocid pups thus experience high POP exposure during both feeding and  
80 fasting, two fundamentally important periods for adipogenesis, fat mobilisation and  
81 maturation of energy balance pathways. Their reliance on adipose tissue to fuel  
82 metabolism during fasting or while searching for food in changing environments<sup>55</sup> place  
83 these top predators at high risk from adverse effects of POP exposure as a result of  
84 impaired fat cell function.

85 Marine mammals show structural changes in fatty acid (FA) content and thermal  
86 properties with blubber depth<sup>54,56-61</sup>. Effects of blubber depth and nutritional state on  
87 metabolic function and sensitivity to POP exposure have not been explored. High POP  
88 levels are associated with altered adipose gene expression in seals<sup>62-64</sup>, polar bears  
89 (*Ursus maritimus*)<sup>55</sup>, and killer whales (*Orca orcinus*)<sup>65</sup>. POP cocktails from polar bear  
90 fat can induce adipogenesis in mouse (*Mus musculus*) pre-adipocyte cell lines and  
91 primary polar bear pre-adipocytes<sup>66</sup>. However, the consequences of POP-induced gene  
92 expression changes for fat tissue function are unknown.

93 The major functions of fat tissue are to store lipid and release it for use as  
94 metabolic fuel. The balance between lipogenesis and lipolysis depends on the  
95 nutritional state of the organism and the hormonal milieu to which the tissue is exposed,  
96 and may be influenced by POPs. Glucose uptake, lactate production and glycerol release

97 are important metabolic properties of adipose tissue that contribute to whole animal  
98 glucose homeostasis and energy balance. Adipose tissue synthesises triacylglycerol  
99 (TAG) from glucose<sup>67,68</sup>, such that glucose uptake rates can be used to investigate  
100 metabolic function of adipose tissue. Glucose uptake is altered by POPs at tissue and the  
101 whole animal level in other animals<sup>69-87</sup>. Of glucose taken up by adipose, 20% is stored  
102 as TAG, and 70% is used to synthesise lactate<sup>67</sup>. Lactate production is thus an important  
103 route of glucose disposal and may be influenced by POP exposure. Glycerol is produced  
104 during lipolysis<sup>88</sup>, and as a by-product of glycolysis<sup>68</sup>, and is thus a major secretory  
105 product of adipose tissue. Unlike FA, glycerol cannot be re-esterified by adipose *in*  
106 *vitro*<sup>89,90</sup>, making its production a useful marker of lipolytic<sup>88</sup>, and, to some extent,  
107 glycolytic<sup>68</sup> activity.

108         We measured glucose uptake, lactate production and glycerol release as indices  
109 of metabolic activity in blubber by employing an explant approach we have developed  
110 using blubber biopsies from wild grey seals (*Halichoerus grypus*)<sup>91</sup>. Our approach  
111 allows experimental work to be performed *in vitro* in remote field locations to  
112 investigate fat tissue function<sup>91</sup>, which, in marine mammals, was previously limited to  
113 measurement of enzyme activities<sup>92</sup>. Here, we used blubber explants from wild grey seal  
114 pups, which remain on land during both feeding (during suckling) and fasting (during  
115 the post-weaning fast). We investigated whether metabolic properties of blubber differ  
116 by tissue depth, nutritional state and intrinsic POP burden. We also investigated acute  
117 changes in blubber metabolic characteristics in response to exogenous experimental  
118 treatments with high, but biologically relevant, POP concentrations.

119  
120  
121

122 **Methods**

123

124 *Study site and animals*

125 Field work was conducted on the Isle of May, Scotland (56°11'N, 02°33'W)  
126 from 7<sup>th</sup> November to 18<sup>th</sup> December 2016 under permit from Scottish Natural Heritage  
127 (SNH). Grey seal females that were part of a long-term study were observed from when  
128 they came ashore, and the date of birth of their pups was recorded. Pups were included  
129 if they were at an appropriate predicted mass for their age (number of days post-partum)  
130 late in the suckling period and if they did not have any obvious indications of ill health.  
131 We collected blubber biopsies for POP analysis and explant culture from 27 pups late in  
132 the suckling period(day 15). Pups were then left to wean naturally from their mothers,  
133 which occurred at approximately 18 days old. We then attempted to resample the same  
134 pups once during the postweaning fast (day 12 after weaning). As pups were still  
135 feeding for several days between the two sampling events, additional mass gain and  
136 increases in blubber lipid percentage were possible between the feeding/fasting  
137 sampling points. Four pups were removed from the study because they developed an  
138 unrelated infection. This gave a total of 27 pups (9 female and 18 male) sampled while  
139 feeding and 23 (7 female and 16 male) of the same animals sampled again during  
140 fasting.

141 Pups were defined as weaned based on daily observation to determine maternal  
142 departure date<sup>43</sup>. Pups were then transferred to a large (~600m<sup>2</sup>) fenced off area of the  
143 island, away from the main colony, with access to a pool of fresh water, in accordance  
144 with UK Home Office guidelines and based on prior studies<sup>43,93</sup>.

145 The sex of all pups was determined at early lactation (~ day 5 of suckling). Pups  
146 were weighed at early and late lactation; when they were initially transferred into the

147 pen (within 2 days after weaning); and every 5 days in the pen. Pups less than 30 kg at  
148 weaning were excluded from the study. To ensure pups were of a viable departure mass,  
149 early release criteria were set at loss of 25% of weaning mass or reaching 30kg,  
150 whichever was the higher mass<sup>43</sup>. It was not necessary to release any of the study  
151 animals early. All pups were released into the colony with ready access to the sea.

152 All animal procedures were performed under the UK Home Office project  
153 licence #70/7806 and conformed to the UK Animals (Scientific Procedures) Act, 1986.  
154 All research received prior ethical approval from Abertay University and the University  
155 of St Andrews Animal Welfare and Ethics Committee.

156

#### 157 *Biopsy sampling*

158 Biopsy sampling, transport, explant creation and culture protocols were adapted  
159 from a previous study<sup>91</sup>. Prior to sampling, pups were given a mass-specific intravenous  
160 dose of Zoletil<sup>TM</sup> and subcutaneous injections of Lignol<sup>TM</sup> at the biopsy sites. Three full  
161 depth blubber biopsies, one 6 mm and two 10 mm, were taken<sup>94</sup>. The 6 mm biopsy was  
162 immediately wrapped in foil and frozen at -20 °C for POP analysis<sup>47,50</sup>. The two 10 mm  
163 biopsies were cut immediately, using sterile surgical scissors, into ‘inner’ (blubber  
164 closest to muscle layer) and ‘outer’ (blubber closest to the skin) tissue, and placed into  
165 separate 15 ml centrifuge tubes filled with warm (37 °C) sterile Krebs Ringer solution  
166 (pH 7.4; NaCl; 7.89 g/l; KCl; 0.373 g/l; MgSO<sub>4</sub>; 0.12 g/l; K<sub>2</sub>HPO<sub>4</sub>; 0.07 g/l; Glucose;  
167 0.99 g/l; HEPES; 4.77 g/l; CaCl<sub>2</sub>; 0.11 g/l<sup>95</sup> with 1% Antibiotic Antimycotic Solution  
168 (all chemicals supplied by Sigma- Aldrich) for transport back to the field laboratory. A  
169 single hidden suture (Ethicon Vicryl Suture (W9114) Violet, 3-0, 20mm, 75cm with  
170 VICRYL Suture 1/2 circle ‘Taper Point Plus’ Needle) was used to close 10 mm biopsy  
171 sites , either vertically or horizontally through the skin<sup>96</sup>. Pups were observed remotely



172 on a daily basis, and biopsy sites checked at each reweighing to document healing. The  
173 second sample was not taken until all the previous biopsy sites had healed.

174

#### 175 *Explant culture and POP exposure*

176 All biopsies were returned to the field laboratory, processed and placed in the  
177 incubator within 30 minutes of collection, which is within timeframes used in prior  
178 explant studies on livestock<sup>97,98</sup> and wild seals<sup>91</sup>. Upon arrival, tissue was washed with 1  
179 ml sterile, warm Krebs Ringer buffer. Visible hair, muscle or blood contaminated tissue  
180 was removed. Tissue was minced using a sterile scalpel into 5-10 mg pieces, and 100  
181 mg portions of tissue were weighed out in a sterile Petri dish. The 100 mg explants were  
182 dispensed into pre-prepared 12 well plates warmed to 37°C in a sterile, non-ducted PCR  
183 hood . In total, 1500µl of either complete cell culture media (medium 199, Hanks'  
184 Balanced Salts with 1% Antibiotic Antimycotic Solution, 1% FA supplement and 5%  
185 charcoal stripped foetal bovine serum) or complete media containing 150ng/ml POP  
186 mix (PCB Aroclor standard mix 1 (Sigma-Aldrich), containing Aroclor 1016; 500  
187 µg/mL, Aroclor 1260; 500 µg/mL, Decachlorobiphenyl (PCB 209); 50 µg/mL and  
188 2,4,5,6-Tetrachloro-m-xylene (an OCP); 50 µg/mL in acetone:methanol solution (2:3))  
189 was added per well to generate control and +POP experiments. Plates were placed in a  
190 humidified incubator maintained at 37°C and 5% CO<sub>2</sub> (Thermo Scientific, Midi 40 CO<sub>2</sub>  
191 Incubator, model: 3404) for 24 h. All media was drawn off after 24 h and frozen at -20  
192 °C. Explants were snap frozen in liquid nitrogen and stored at -80 °C.

193

#### 194 *Metabolite measurement*

195 Glucose, lactate and glycerol concentrations were measured in media from  
196 explant experiments using Randox (County Antrim, UK) kits (glucose: GL364, lactate:

197 LC2389, glycerol: GY105) and standards in an RX Monza (Randox) Clinical Chemistry  
198 analyser (Model: 328-14-0914) as described previously<sup>91</sup>. Internal quality control  
199 measurements lay within  $< \pm 15\%$ . Intra and inter assay variability for sample  
200 analysis was  $< 5\%$ . Mean variation between replicates from the same individual and of  
201 the same nutritional state and tissue type for the three metabolites analysed were as  
202 follows; glucose 26.6% (range 0.6-144%), lactate 46.9% (range 3-164%) and glycerol  
203 19.8% (range 0.5-106%). Rates of glucose removal and accumulation of lactate (indices  
204 of glycolysis) and glycerol (indices of lipolysis) were calculated per 100 mg tissue<sup>-1</sup>  
205 hour<sup>-1</sup>.

206

#### 207 *POP analysis*

208       Extraction and detection of POPs took place at the Center for Analytical  
209 Research and Technology (CART), University of Liège, Belgium, using standard  
210 methods (supporting information SI 1). The 6 mm biopsies yielded  $385 \pm 7$  (s.e.) mg  
211 blubber tissue per sample. Six non Dioxin-Like PCBs (NDL-PCBs) (28, 58, 101, 138,  
212 153, 180), eight Dioxin-Like PCBs (DL-PCBs) (105, 114, 118, 123, 156, 157, 167, 189)  
213 and nine PBDEs (28, 47, 66, 85, 99, 100, 153, 154, 183) were analysed at CART in the  
214 Department of Chemistry. Four OCPs (o,p'-DDT, p,p'-DDT,  
215 dichlorodiphenyldichloroethane (DDD), dichlorodiphenyldichloroethylene (DDE)) were  
216 analysed at CART in the Laboratory of Animal Ecology and Ecotoxicology (LEAE).

217       POP concentrations were measured in ng g<sup>-1</sup> lipid, summed into  $\sum$ NDL-PCBs,  
218  $\sum$ DL-PCBs,  $\sum$ PBDEs and  $\sum$ DDXs (o,p'-DDT, p,p'-DDT, DDD, DDE) and log  
219 transformed prior to statistical analysis.

220

221

222 *Statistical analysis*

223           Analyses were performed using the statistical package R 3.4.1<sup>99</sup>. T-tests were  
224 used to compare log transformed POP concentrations between feeding and fasting  
225 samples. Pearson's correlation coefficient was calculated between all four  $\Sigma$ POP types  
226 (log transformed  $\Sigma$ NDL-PCBs,  $\Sigma$ DL-PCBs,  $\Sigma$ PBDEs and  $\Sigma$ DDXs). As all POP classes  
227 were highly significantly correlated, we explored the effects of each POP class  
228 separately. Generalised additive mixed models (GAMM)<sup>100</sup> were used to identify the  
229 variables that best explained glucose uptake, lactate production and glycerol production.  
230 Explanatory variables explored in these models included log transformed  $\Sigma$ NDL-PCBs,  
231  $\Sigma$ DL-PCBs,  $\Sigma$ PBDEs and  $\Sigma$ DDXs in the blubber at time of sampling (each POP class  
232 tested separately in each model), the mass of the pup, the tissue depth (inner or outer),  
233 the nutritional state at sampling (feeding or fasting), the experimental conditions  
234 (control or +POP) and the pup's sex. Interaction terms between nutritional state, tissue  
235 type, experimental conditions and POP concentrations were also investigated (see SI  
236 Table 1). The model was fitted using the multiple generalized cross validation library  
237 *mgcv*<sup>101</sup>. The identities of individual pups were fitted as random effect smooths<sup>102</sup> to  
238 account for the same individual generating multiple explants and any variation between  
239 replicates. The smoothing parameters were set by maximum likelihood to reduce the  
240 risk of overfitting<sup>103</sup>. The models were fitted with a Gaussian error distribution. Glucose  
241 data were log transformed and glycerol data were square root transformed. No  
242 transformation was needed for lactate data. Model selection was performed by  
243 backwards stepwise elimination through examination of  $R^2$  values, Akaike's  
244 information criterion (AIC) values,  $\Delta$ AIC, Akaike's weights, QQ and residual plots  
245  
246

247 **Results and Discussion**

248

249 *POP concentrations*

250 Mean concentrations and ranges of the summed POPs in grey seal pup blubber  
 251 are given in Table 1.

252

253 Table 1. Means and ranges for study pup masses and fat content of 6mm biopsies, with  
 254 median concentrations and ranges of POPs in grey seal pup blubber in feeding and  
 255 fasting states.

	Feeding mean	Feeding range	Fasting mean	Fasting range
Pup mass (kg)	41.9	31.4 – 52.6	40	32.8 – 50.4
Biopsy fat content (% lipid)	78.5	67.8 – 85.5	82.9	56.3 – 88.9
	Feeding median	Feeding range	Fasting median	Fasting range
$\Sigma$ NDL-PCB (ng g <sup>-1</sup> lipid)	354.29	194.2 – 790.5	541.73	302.5 – 1253.8
$\Sigma$ DL-PCBs (ng g <sup>-1</sup> lipid)	10.78	5.5 – 45.6	19.78	9.6 – 151.2
$\Sigma$ PBDE (ng g <sup>-1</sup> lipid)	13.81	6.4 – 57.9	20.2	10.2 – 70.1
$\Sigma$ DDX (ng g <sup>-1</sup> lipid)	169.09	98.5 – 420.8	209.56	128.4 – 345.7

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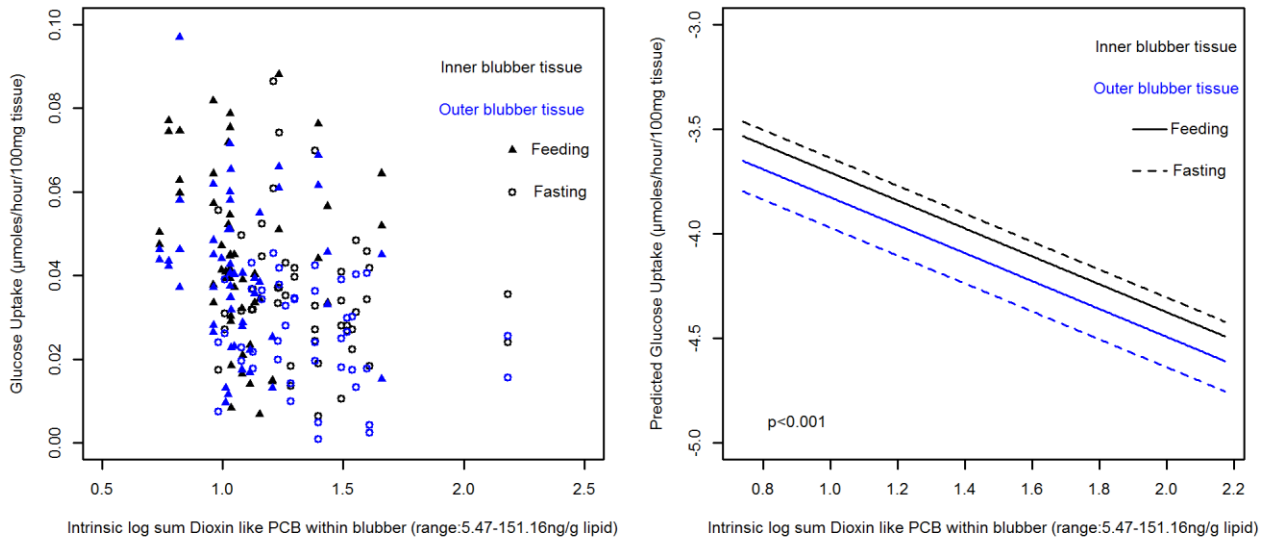
257 Higher POP levels when pups were fasting (Welch's two sample t-test,  $p < 0.001$   
258 for all log transformed POP classes) are consistent with previous work showing that  
259 lipid mobilisation during fasting concentrates more lipophilic POPs in remaining fat  
260 tissue<sup>50,51,53</sup>. Blubber may thus be more vulnerable to disruptive effects of contaminants  
261 during fasting if the POPs can interact with cellular machinery to exert negative effects.

262

### 263 *Glucose uptake*

264  $\Sigma$ DL-PCBs, experimental conditions, tissue depth, and nutritional state best  
265 explained variation in glucose uptake, along with an interaction between tissue depth  
266 and nutritional state (GAMM:  $R^2 = 0.36$ , SI Table 2).  $\Sigma$ NDL-PCBs,  $\Sigma$ PBDEs and  
267  $\Sigma$ DDX had no significant impact on glucose uptake (SI Table 1). Uptake was higher in  
268 inner compared to outer tissue ( $p < 0.001$ ). Glucose uptake was lower during fasting  
269 than feeding in outer blubber ( $p = 0.035$ ), but did not differ between nutritional states in  
270 inner tissue (Figure 1). In both inner and outer tissue, glucose uptake was inversely  
271 related to  $\Sigma$ DL-PCBs ( $p < 0.001$ ) (Figure 1) and the slope of the relationship did not  
272 depend on nutritional state. Acute exogenous POP exposure increased glucose uptake  
273 compared to controls, (GAMM:  $R^2 = 0.36$ ,  $p < 0.001$ , SI Table 2, Figure 2), and the  
274 increase was not affected by nutritional state or tissue depth. Doses of each PCB congener  
275 given in the explant POP exposure conditions, with the estimated percentage change for  
276 each PCB type measured in blubber are given in SI Table 3. A boxplot of glucose  
277 uptake data during different nutritional states and tissue depths is provided in SI Figure  
278 1.

279

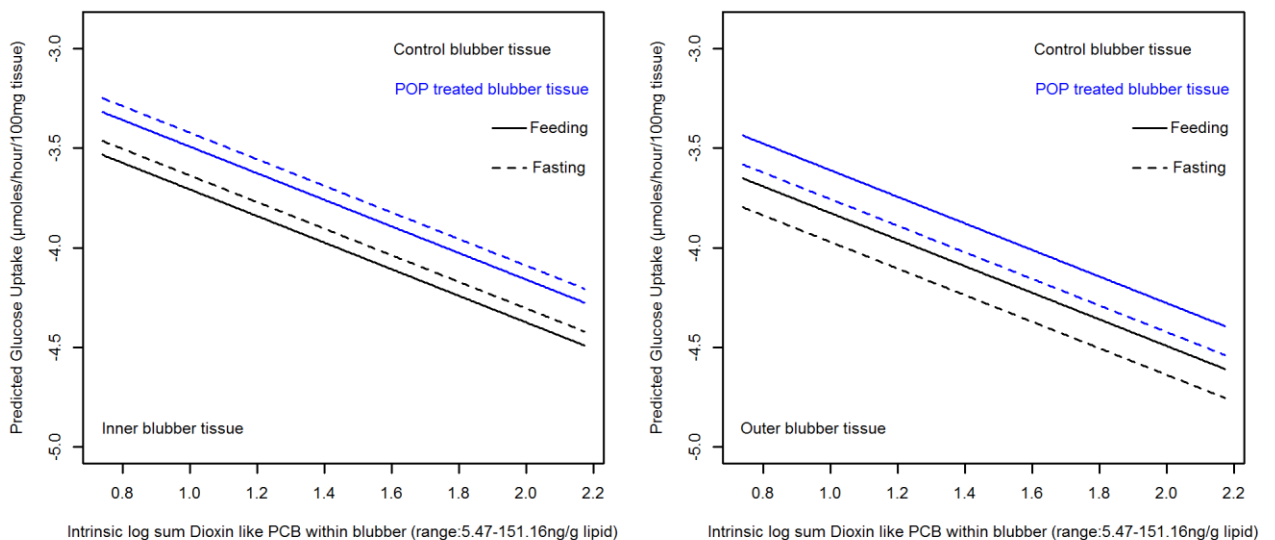


280

281 Figure 1. Scatter plot (left panel) and prediction line plot (right panel) showing the  
 282 impact of intrinsic  $\Sigma$ DL-PCBs on glucose uptake in blubber from grey seal pups,  
 283 comparing inner (black) /outer (blue) tissue and feeding (filled triangles or solid lines)  
 284 /fasting (open circles or dashed lines) states (n = 27 feeding and n = 23 fasting pups).

285 Individual was included as a random effect in the model.

286



287

288 Figure 2. Prediction line plots showing the impact of exposure to acute exogenous POPs  
 289 on glucose uptake in blubber from grey seal pups, comparing inner (left panel) and

290 outer (right panel) tissue and feeding/fasting states across the range of intrinsic  $\Sigma$ DL-  
291 PCBs in the blubber. Individual was included as a random effect in the model.

292

293         These data show for the first time that metabolic activity in phocid blubber is  
294 altered by the POPs accumulated *in utero* and during suckling. POPs have been  
295 postulated to be ‘obesogens’ from epidemiological data in humans<sup>24</sup>. Experimental  
296 studies have demonstrated that POPs can alter adipocyte function in rodents and  
297 human cell lines<sup>25,104</sup>. POPs are associated with altered adipose and liver expression of  
298 metabolic pathway genes in adult polar bears<sup>55,66</sup> and ringed seals (*Pusa hispida*)<sup>63,64</sup>,  
299 but the functional consequences of such changes for fat tissue have not been identified.  
300 Recent experimental studies that replicate environmental or physiological POP  
301 concentrations and congener mixtures provide causal evidence of their  
302 immunological<sup>105</sup> and adipogenic effects<sup>91</sup>. Our work builds on these findings by  
303 showing that existing POP concentrations in blubber tissue, generated *in utero* and  
304 through lactational transfer, can alter metabolic properties of adipose tissue in young,  
305 developing seals.

306         Our data are consistent with experimental studies showing PCB 77, 153 and  
307 dioxin reduce glucose and insulin tolerance in mice, guinea pigs (*Cavia porcellus*),  
308 rabbits (*Oryctolagus cuniculus*) and in human cell lines<sup>69-77</sup>. PCBs are also associated  
309 with dysfunctional glucose homeostasis in humans<sup>78-81</sup>. Here, only  $\Sigma$ DL-PCBs altered  
310 glucose uptake in blubber explants, consistent with an aryl hydrocarbon receptor (AhR)-  
311 dependent pathway in seals, as seen in other species<sup>71,78</sup>. POP-induced reduced glucose  
312 use is accompanied by a fall in glucose transporter, GLUT4, mRNA expression and  
313 GLUT1 protein levels in other animals<sup>75</sup>, and may also occur in seals. The opposite  
314 effects of an exogenous POP exposure to the intrinsic  $\Sigma$ DL-PCB levels on glucose

315 uptake may reflect the difference between short (overnight) *in vitro* and long-term (*in*  
316 *utero* and over 15 days of suckling) exposure, which may produce opposing effects on  
317 membrane fluidity and transport properties<sup>106</sup>. Alternatively, either the DL-PCB dose in  
318 the acute exposure experiments may have been too low to affect glucose uptake (SI  
319 Table 3) or the exogenous POP cocktail produces different effects from the *in vivo*  
320 contaminant mixture. We cannot distinguish between these possibilities from our data.

321 Lower glucose uptake caused by DL-PCBs is likely to reduce fat deposition rate,  
322 which may be problematic for pups laying down fat while suckling. However, fat  
323 deposition may increase if acute exogenous POP exposure increases glucose uptake.  
324 Indeed, short-term exposure to a POP cocktail from polar bear blubber induced  
325 adipogenesis in a mouse pre-adipocyte cell line<sup>66</sup>. The timecourse of POPs on both  
326 adipogenesis and lipogenesis needs to be explored.

327 NDL-PCBs, DDX and PBDEs in blubber did not affect glucose uptake, despite  
328 evidence of their impacts on glucose homeostasis in other animals<sup>82-87, 107</sup>. Our data  
329 suggest that the negative relationship between PBDEs and first year survival of grey  
330 seal pups<sup>104</sup> is not driven by direct alterations to adipose metabolic properties. PBDEs  
331 may alter adipose function indirectly through endocrine disrupting effects, for example,  
332 by altering thyroid or insulin function<sup>86, 87, 108, 109</sup>, highlighting the need for improved  
333 understanding of energy balance regulation in seals, and its disruption by POPs.

334 Glucose uptake was higher in inner tissue under all conditions, suggesting  
335 deeper blubber has greater metabolic activity than superficial tissue. This finding is  
336 consistent with regional stratification in structure; the theorised role of superficial  
337 blubber in thermoregulation; the possibility of smaller, more numerous adipocytes that  
338 are more metabolically active<sup>92</sup> in deeper tissue; and the observation that deeper blubber



339 contains higher glycolytic enzyme levels compared to superficial blubber in fin whales  
340 (*Balaenoptera physalis*<sup>92</sup>).

341           Glucose uptake fell as the animals transitioned from feeding to fasting in outer  
342 blubber, but not in inner blubber. This likely reflects high levels of fat deposition  
343 throughout the blubber during feeding, followed by cessation of fat accumulation during  
344 fasting. Metabolic rate falls in fasting pups<sup>42,110</sup>, and this is likely reflected in reduced  
345 demands by all tissues, including blubber. Lower sensitivity to residual insulin present  
346 in stripped FBS may explain lower glucose uptake and greater decrease in outer tissue  
347 from fasting animals, a state in which many animals become less sensitive to the effect  
348 on insulin of glucose uptake<sup>111-113</sup>. The lack of decreased glucose uptake in inner  
349 blubber in fasting animals is surprising. It is however plausible that cell membrane  
350 expression of GLUT4 and/or insulin -independent GLUT1, are reduced to a greater  
351 extent in outer compared to inner blubber during fasting. Indeed, fasting northern  
352 elephant seals (*Mirounga angustirostris*) maintain expression of GLUT4 in adipose cell  
353 membranes<sup>114</sup>.

354           Previous studies of metabolic effects of POPs on marine mammals have  
355 focussed on adults<sup>55,63,64,66</sup>. Concentrations of POPs in adult seals are an order of  
356 magnitude greater than those found in pups, with adult seal blubber containing several  
357 thousand  $\Sigma$ PCBs (ng/g)<sup>115</sup> compared to the levels detected in the pups here, which were  
358 in the hundreds of ng/g range (Table 1). Here, we have shown that pups have already  
359 accumulated high enough POP levels in their blubber to impact on adipose function at  
360 less than three weeks of age, when the ability to generate and regulate blubber is a key  
361 factor for survival<sup>116</sup>. Growing and dividing adipocytes are more vulnerable to  
362 disruption by DL-PCBs because they have higher AhR levels than mature,  
363 differentiated adipocytes<sup>117,118</sup>. The sensitivity to POP-induced alterations to adipose

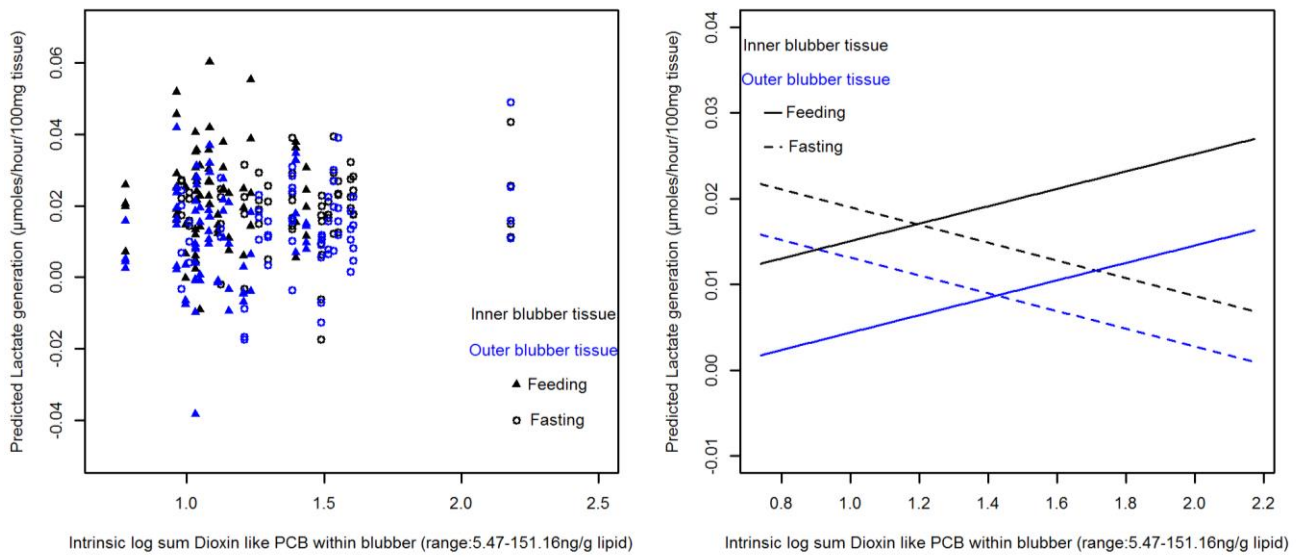
364 tissue metabolism is therefore likely to be greatest in young marine mammals, which  
365 are exposed to large quantities of POPs from their mothers<sup>47,50</sup> and are likely to have a  
366 high proportion of proliferating pre-adipocytes as a result of rapid fat deposition<sup>119</sup>. The  
367 energy balance and survival consequences of reduced glucose uptake in young pups  
368 now needs to be explored.

369

### 370 *Lactate production*

371  $\Sigma$ DL-PCBs, tissue depth, nutritional state, pup sex and interactions between  
372  $\Sigma$ DL-PCBs and nutritional state, and tissue depth and nutritional state were retained in  
373 the model that best explained lactate production (SI Table 1 and 2).  $\Sigma$ NDL-PCBs,  
374  $\Sigma$ PBDEs and  $\Sigma$ DDX had no significant impact on lactate production (SI Table 1).  
375 Lactate production increased with DL-PCBs in feeding pups, but decreased with DL-  
376 PCBs when pups were fasting (GAMM:  $R^2 = 0.29$ ,  $p = 0.003$ ; SI Table 2, Figure 3).  
377 Lactate generation was higher in males than in females ( $p = 0.05$ ) and higher in inner  
378 tissue than outer tissue for any given DL-PCB load in any given nutritional state ( $p =$   
379  $0.001$ ) (Figure 3). The difference between inner and outer tissue tended to be greater  
380 during feeding than during fasting ( $p = 0.055$ ). Lactate generation was not impacted by  
381 acute exogenous experimental POP exposure. A boxplot of lactate generation/uptake  
382 data during different nutritional states and tissue depths is provided in SI Figure 2.

383



384

385 Figure 3. Scatter plot (left panel) and prediction line plot (right panel) showing the  
 386 impact of intrinsic  $\Sigma$ DL-PCBs on lactate generation/uptake in blubber from male grey  
 387 seal pups (n=34), comparing inner (black) /outer (blue) tissue and feeding (filled  
 388 triangles or solid lines) /fasting (open circles or dashed lines) states. Individual was  
 389 included as a random effect in the model.

390

391 Lactate production is a major route of glucose disposal in fat tissue<sup>67</sup>. However,  
 392 the inhibitory effect of DL-PCBs on glucose uptake is only consistent with lactate  
 393 production during fasting. During feeding, lactate production increased with increased  
 394 DL-PCBs in the face of reduced glucose uptake. One explanation is that the electron  
 395 transfer chain experiences increased push force during suckling, in combination with  
 396 inhibition by DL-PCBs<sup>120,121</sup>, which could elevate lactate production through increased  
 397 reliance on glycolysis. Suckling pups with higher DL-PCB burdens could thus  
 398 experience greater risk of metabolic acidosis from higher adipose lactate production,  
 399 with attendant risk of adipose dysfunction, greater demand on liver gluconeogenesis by

400 to process the lactate, and thus heightened energetic costs that could impact on  
401 efficiency of mass transfer and fat accretion during suckling.

402 Adipose lactate production is a direct consequence of lactate dehydrogenase  
403 (LDH) activity<sup>122</sup>. LDH activity can fall during fasting<sup>123</sup>, and LDH gene expression  
404 and enzyme activity also differ substantially between adipose depots in rats<sup>122</sup>. LDH  
405 activity can also be modulated by PCB exposure in rat and dog liver, and the effect is  
406 dose dependent<sup>124,125</sup>. Shifts in LDH expression and activity may thus contribute to the  
407 differences in lactate production between depths and nutritional states seen here in  
408 response to DL-PCBs. Higher lactate generation in inner tissue and the greater  
409 difference between regions during feeding may be a result of greater glucose uptake and  
410 metabolic activity in inner blubber during suckling. Regional differences in metabolic  
411 properties may also relate to adipocyte size<sup>126</sup>.

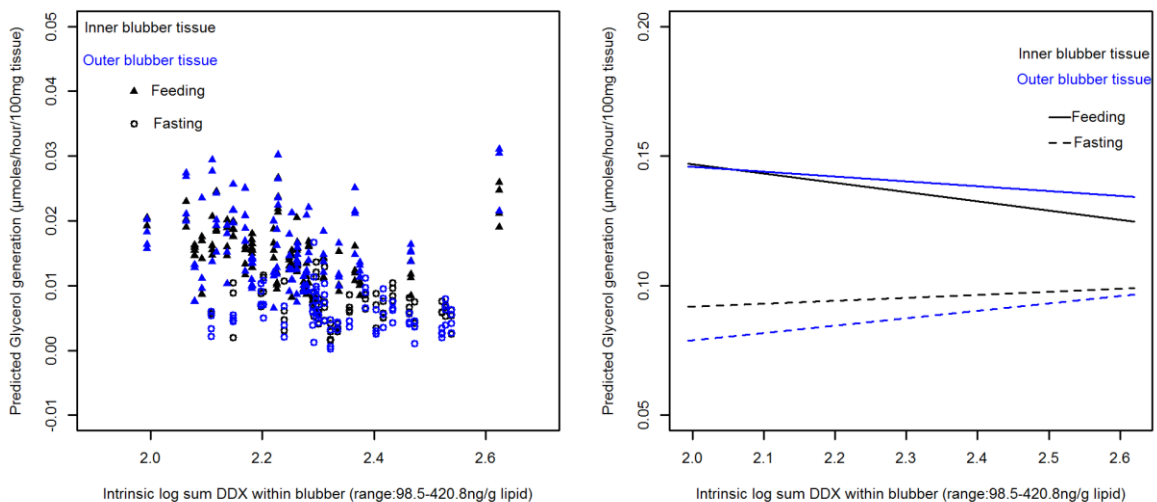
412 Higher lactate production by adipose from males than females has not been  
413 reported, either in adult seals<sup>91</sup> or in humans and rodents<sup>122</sup> and may result from the  
414 small number of females here. However, since adult females accumulate fat faster than  
415 adult males<sup>127</sup> and female juvenile northern elephant seals spare fat more effectively  
416 than males<sup>128</sup>, it is possible that some metabolic differences are present in pups' blubber  
417 prior to overt size dimorphism. For example, females may use more lactate to synthesise  
418 FA<sup>129</sup> than males, releasing less net lactate.

419

#### 420 *Glycerol production*

421 Nutritional state, an interaction between tissue depth and nutritional state, and an  
422 interaction between nutritional state and intrinsic blubber DDX were significant terms  
423 in the best model to explain glycerol production (SI Table 1 and 2, Figure 4). In  
424 addition, extrinsic POP treatment, mass and sex were retained as non significant

425 variables. Unexpectedly, glycerol production, was higher during feeding than fasting  
 426 (GAMM:  $R^2 = 0.74$ ,  $p < 0.001$  Figure 4). In addition, lipolysis was higher in outer than  
 427 in inner tissue during feeding, whereas inner tissue had a higher lipolytic rate than outer  
 428 tissue in the fasted state ( $p = 0.0003$ ; SI Table 2). Finally, lipolytic rate increased with  
 429 intrinsic blubber DDX in fasting animals, whereas the opposite relationship between  
 430 DDX and lipolytic rate occurred in feeding pups ( $p < 0.0001$ , Figure 4). A boxplot of  
 431 glycerol generation data from different nutritional states and tissue depths is provided in  
 432 SI Figure 3.  
 433



434  
 435 Figure 4. Scatter plot (left panel) and prediction line plot (right panel) showing the  
 436 impact of intrinsic  $\Sigma$ DDX on glycerol generation in blubber from grey seal pups,  
 437 comparing inner (black) /outer (blue) tissue and feeding (filled triangles or solid lines)  
 438 /fasting (open circles or dashed lines) states ( $n = 27$  feeding and  $n = 23$  fasting pups).  
 439 Individual was included as a random effect in the model.

440  
 441 POPs, such as dioxin and related compounds, and OCPs typically produce  
 442 ‘wasting syndrome’ at high doses<sup>32-35</sup>, likely due to increased WAT lipolysis<sup>130</sup>. In  
 443 contrast, some studies on rat adipose or rodent cell lines show no effect of DDT or DDE

444 on lipolysis at lower doses<sup>131,132</sup>. Our data show that lipolysis is increased in blubber of  
445 fasting grey seal pups in response to intrinsic tissue levels of DDT and its metabolites,  
446 but decreased in feeding pups. These data suggest, that, as in bats, seal pups in negative  
447 energy balance may be most vulnerable to the presence of DDX in their tissues<sup>34</sup>.  
448 Interestingly, the increase in lipolysis is greater in outer tissue, which is proposed to be  
449 important for insulation<sup>56-61</sup>, such that DDT may negatively affect both  
450 thermoregulatory and metabolic capability of blubber during fasting. A reduction in  
451 lipolytic rate with DDX in feeding animals could reflect inhibition of tissue turnover of  
452 FFA, increased lipogenesis or a shift towards lactate production rather than glycerol  
453 generation during glycolysis<sup>68</sup>.

454 Lower glycerol production during fasting compared to feeding contrasts starkly  
455 with the typical fasting increase in lipolysis<sup>133,134</sup>. Regional and nutritional differences  
456 may result from depth changes in metabolic activity and adipocyte size<sup>135</sup>. Lower  
457 glycerol production in fasting pups could also arise from a reduction in aldolase  
458 activity<sup>136</sup>. In addition, higher lipolytic rates in feeding animals may reflect their higher  
459 metabolic rate<sup>42,110</sup>, faster rates of triglyceride turnover and/ or greater production of 3  
460 carbon (3C) intermediates to protect tissues against high fuel availability and provide  
461 fuel for actively growing tissue elsewhere<sup>68,122</sup>. High rates of glycerol production in  
462 feeding animals could protect adipocytes from metabolic acidosis, minimise  
463 overproduction of oxidising equivalents and maximise energy efficiency<sup>68,137</sup>.

464

465 *Value and wider application of the explant method.*

466 Wildlife species are facing increasing anthropogenic threats that can limit their  
467 ability to find food and store fat as an energy reserve for lean times. Constrained,  
468 fragmented or reduced foraging ranges<sup>138</sup> and decreased prey availability<sup>139,140</sup> limit

469 opportunities for fat accumulation, and force individuals to rely on adipose stores more  
470 frequently to sustain them until the next feeding opportunity. Understanding the impact  
471 of natural and anthropogenic stressors on energy balance requires experimental  
472 interventions that are practically and ethically challenging to accomplish in marine  
473 mammals and other wildlife species. *In vitro* methods are increasingly being explored as  
474 alternatives to whole animal work and lethal sampling. Prior efforts have generated  
475 viable skin tissue samples for culture via dermal punches and biopsy darts in both  
476 marine<sup>141,142</sup> and terrestrial<sup>143</sup> wildlife species. Given the importance of fat tissue to the  
477 health and survival of wildlife in rapidly changing environments, there is a critical need  
478 for methods to better understand their adipose physiology. We previously used blubber  
479 explants from wild seals, which were returned to tissue culture facilities in a mainland  
480 laboratory<sup>91</sup>. Here, we collected and maintained viable blubber explants for 24 h in a  
481 remote field laboratory, with only core tissue culture equipment. The methodology  
482 outlined here could therefore be adapted for use in other wildlife species in remote field  
483 locations or where alternative *in vitro* methods are not available.

484

#### 485 *Outlook*

486 Our explant method clearly shows promise in better understanding adipose  
487 tissue responses to contaminants and can be expanded beyond the parameters measured  
488 here. Although establishment of cell lines would permit a wider range of exposures,  
489 including time courses, dose responses and mixtures over longer duration experiments,  
490 such approaches have been hampered by the absence of typical proliferation and  
491 differentiation responses in primary cells from non-model species<sup>53</sup>. A focus on  
492 understanding the requirements of such cell types and generation of cell lines would  
493 greatly facilitate progress in toxicological studies on adipose from wildlife species.

494 In summary, we have shown that dioxin-like PCBs disrupt adipose glucose  
495 uptake and lactate production, and DDX disrupt lipolysis in grey seal pups, using an  
496 experimental explant approach. Our results are particularly important because they  
497 demonstrate POP-induced alterations to adipose tissue function in young animals in the  
498 first weeks of life, for which rapid fat accumulation during suckling and mobilisation  
499 during fasting are vital for survival. They also highlight that different POPs may impact  
500 different metabolic characteristics, and that their effects are modified by nutritional state  
501 and tissue depth, producing complex effects on tissue function. Wider screening of  
502 metabolic properties of cells and tissue from non-model organisms is key to identify  
503 energy-balance disrupting effects of legacy and emerging contaminants. Our methods  
504 and findings also have wider application in understanding drivers of juvenile survival of  
505 other top marine predators, particularly in species that simultaneously experience high  
506 POP loads and rapid alterations in prey availability and habitat.

507

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524

## 525 **Supporting Information**

526

527 SI 1. Methodological details for POP detection.

528

529 SI Table 1. Candidate GAMM models exploring different summed POP types, potential  
530 interactions and the top models in the selection process.

531

532 SI Table 2. Model output from all final GAMMs analysing metabolic characteristics in  
533 explant experiments with their standard errors, estimates and p values. If predictor  
534 variables have parentheses, this indicates the tested group.

535

536 SI Table 3. Doses of each PCB type given in the explant PCB culture conditions, with  
537 the estimated percentage change for each PCB type measured in blubber tissue.

538  $\sum$ DL-PCB within total dose: 1.6ng.  $\sum$ NDL-PCB within total dose: 202.6ng.

539

540 SI Figure 1. Glucose uptake in inner and outer blubber tissue from feeding and fasting  
541 grey seal pups (n = 27 feeding and n = 23 fasting pups) with median, upper and lower  
542 quartiles, 1.5 x interquartile range and outliers shown. Significant differences between

543 groups are detailed in the main text, as not all significant parameters and interactions  
544 influencing glucose uptake are plotted. Individual was included as a random effect in  
545 the model.

546

547 SI Figure 2. Lactate production in inner and outer blubber tissue from feeding and  
548 fasting grey seal pups (n = 27 feeding and n = 23 fasting pups) with median, upper and  
549 lower quartiles, 1.5x interquartile range and outliers shown. Significant differences  
550 between groups are detailed in the main text, as not all significant parameters and  
551 interactions influencing lactate generation/uptake are plotted. Individual was included as  
552 a random effect in the model.

553

554 SI Figure 3. Glycerol generation in inner and outer blubber tissue from feeding and  
555 fasting grey seal pups (n = 27 feeding and n = 23 fasting pups) with median, upper and  
556 lower quartiles, 1.5x interquartile range and outliers shown. Significant differences  
557 between groups are detailed in the main text, as not all significant parameters and  
558 interactions influencing glycerol generation are plotted. Individual was included as a  
559 random effect in the model.

560

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