



# Predicting consequences of POP-induced disruption of blubber glucose uptake, mass gain rate and thyroid hormone levels for weaning mass in grey seal pups

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## ABSTRACT

Persistent organic pollutants (POPs) are endocrine disruptors that alter adipose tissue development, regulation and function. Top marine predators are particularly vulnerable because they possess large fat stores that accumulate POPs. However, links between endocrine or adipose tissue function disruption and whole animal energetics have rarely been investigated. We predicted the impact of alterations to blubber metabolic characteristics and circulating thyroid hormone (TH) levels associated with polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), and organochlorine pesticides (OCPs) on suckling mass gain and weaning mass in wild grey seal pups. Glucose uptake by inner blubber was a strong predictor of whole animal mass gain rate, which in turn, resulted in heavier weaning mass. Weaning mass was predicted to increase by  $3.7 \pm 1.59$  (sem) %, through increased mass gain rate, in the absence of the previously reported suppressive effect of dioxin-like PCB (DL-PCBs) on blubber glucose uptake. PBDEs were, conversely, associated with faster mass gain. Alleviation of this effect was predicted to reduce weaning mass by  $6.02 \pm 1.86\%$  (sem). To better predict POPs effects on energy balance, it is crucial to determine if and how PBDEs promote mass gain in grey seal pups. Weaning mass was negatively related to total T3 (TT3) levels. A 20% (range = 9.3–31.7%) reduction in TT3 by DL-PCBs partially overcame the effect of DL-PCB-mediated reduction in blubber glucose uptake. Overall, DL-PCBs were thus predicted to reduce weaning mass by  $1.86 \pm 1.60\%$ . Organohalogen impacts on whole-animal energy balance in grey seal pups appear to partially offset each other through opposing effects on different mechanisms. POP effects were generally minor, but the largest POP-induced reductions in weaning mass were predicted to occur in pups that were already small. Since weaning mass is positively related to first-year survival, POPs may disproportionately affect smaller individuals, and could continue to have population-level impacts even when levels are relatively low compared to historical values. Our findings show how *in vitro* experiments combined with measurements *in vivo* can help elucidate mechanisms that underpin energy balance regulation and help to quantify the magnitude of disruptive effects by contaminants and other stressors in wildlife.

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## 1. Introduction

Legacy contaminants, such as persistent organic pollutants (POPs), including organohalogenated compounds, are toxic, lipophilic, accumulate in living tissue and biomagnify up food chains (Goerke et al 2004; Kannan et al 2000). Post-ban POP reductions have had positive effects on reproductive success and population dynamics in many species (AMAP 2018; Houde et al 2017; Rig  t et al 2019; Roos et al 2012). However, POPs remain a threat to top predators with large fat stores, such as marine mammals (Desfor  ges et al 2018; Houde et al 2017; Jepson and Law 2016; Jepson et al 2016; Rig  t et al 2016; Tartu et al 2017).

Research efforts to identify biological effects of POPs in marine mammals have largely focussed on their immune (Hammond et al 2005; Penin et al 2018), carcinogenic (Martineau et al 2002), reproductive (Helle et al 1976 a & b; Murphy et al 2010), thyroid hormone (TH) and estrogen disrupting effects (Brouwer et al 1989; Tabuchi et al 2006; Villanger et al 2011; 2013). However, lipid disrupting effects of many POPs have been identified at all trophic levels, altering pathways that control accumulation or mobilisation of fat reserves (Arsenescu et al., 2008; Lee et al 2016; Robinson et al 2018; Speranza et al 2016; Swanepoel et al 1999; Wen et al 2019; Yadetie et al 2014; 2017). Indeed, altered expression of genes involved in energy balance in liver and fat of ringed (*Pusa hispida*) and harbour seal (*Phoca vitulina*) are associated with higher POP exposure (Brown et al 2014; Castelli et al 2014; No  l et al 2017). The impact of these alterations on whole animal energy balance are unknown. Adipose tissue is not simply an inert storage tissue, but is actively involved in control of appetite, insulin sensitivity, energy balance and inflammation through secretion of an array of adipokines (Klaus 2004). Body fat is also important in marine mammals for thermoregulation (Irving & Hart 1957; Rosen & Renouf 1997), buoyancy (Beck et al 2000), streamlining and as metabolic fuel (Reilly 1991). In addition to relying on blubber stores while foraging in patchy marine environments, some marine mammals rely completely on fat stored in blubber when they fast during reproduction and development after weaning. Such reliance on adipose stores may make them particularly vulnerable to anthropogenic disruption of fat metabolism. Since appropriate regulation of energy balance and fat depots underpins individual health, reproductive fitness, and survival (Hall et al 2002; Lidgard et al 2005; Pomeroy et al 1999; Rolland et al 2016; Young, 1976), POP-induced influences on individual energy balance may ultimately have consequences for wildlife vital rates, and thus their population structure, size and trajectories (Beltran et al 2017; Coetzee & Chown 2015; Green et al 2018; Kight & Swaddle 2007; Pirota et al 2018).

Some POP classes have been implicated as ‘obesogens’ in humans and rodents (Gr  n & Blumberg 2009; Hoppe & Carey 2007; Kim et al 2018; Pestana et al 2017). Most *in vitro* evidence exploring the impact of POPs on fat metabolism, using rodent and human cell lines, support this conclusion (Arsenescu et al 2008; Kim et al 2016; 2018; Wen et al 2019). *In vitro* experimental approaches have also demonstrated that POP mixtures present in animal tissues can induce adipogenic pathways in polar bear (*Ursus maritimus*) pre-adipocytes (Routti et al 2016). POPs alter blubber explant function in grey seals (*Halichoerus grypus*) in a nutritional state dependent manner (Robinson et al 2018). In suckling pups, glucose uptake and lipolysis are reduced by DL-PCBs and the OCP dichlorodiphenyltrichloroethane (DDT) and its metabolites (DDX), respectively (Robinson et al 2018). The impact of these POP-induced blubber function alterations on whole animal energetics must be investigated to understand individual and population level consequences of molecular, cellular and biochemical disruption.

Human epidemiological studies have suggested that POPs are an additional risk factor for obesity and related metabolic disease (Dirinck et al 2014; Lee et al 2011). At the organismal level, *in vivo* rodent studies also provide evidence that POPs are obesogenic, disrupting adipose tissue development and function. Dietary PBDEs (Hoppe & Carey 2007),

PCBs (Arsenescu et al 2008; Baker et al 2013; 2015) and DDE (Pestana et al 2017) alter fat tissue function and insulin sensitivity, stimulate fat gain and induce adipose and systemic inflammation. In contrast, negative correlations exist between POPs and body condition in harbour porpoise (*Phocoena phocoena* (Hall et al 2006)), but such associations can result from increased concentration of lipophilic POPs in blubber during fat mobilisation and may not be causal (Bustnes et al, 2010; Debier et al 2003; Hall et al 2008; Louis et al 2014; Polischuk et al 2002). However, some POP classes can cause adipose depletion at the whole animal level, particularly at high concentrations. For example, dietary exposure to organochlorine pesticides (OCPs) is associated with wasting syndrome in bats (Swanepoel et al 1999). These opposing findings at different levels of organisation and using different approaches can be difficult to reconcile.

Challenges in identifying consequences of POP impacts on cell and tissue function for whole animal energy balance and fat accumulation can arise because POPs impact on numerous, counteracting mechanisms. For example, many POPs impair mitochondrial function (Pereira et al 2013; Rainey et al 2017) and increase metabolism (Swanepoel et al 1999), which triggers fat mobilisation from adipose and thus opposes activation of molecular adipogenic pathways. TH production and signalling are also well-established targets of POP disruption (Brouwer et al 1989; Hallgren et al 2001; Hallgren & Darnerud 2002; Hoppe & Carey 2007; van der Plas et al 2001) and POP levels are often negatively related to TH in marine mammals in the wild (Chiba et al 2001; S  rmo et al 2005; Tabuchi et al 2006; Villanger et al 2011; 2013). TH induce adipocyte differentiation and adipogenesis (Flores-Delgado et al 1987; Oppenheimer et al 1991), but also stimulate metabolism and thus fat mobilisation (Mullur et al 2014; Oppenheimer et al 1991). POPs may thus alter fat accumulation at the organism level via alteration of TH metabolism and signaling (Kassotis et al 2019). The energetic implications of TH-disrupting effects of POPs in marine mammals have been suggested (Tabuchi et al 2006), but have not been explored directly.

Investigating the impact of POP-induced endocrine disruption and metabolic changes at cell and tissue level on whole organism energy balance is an important next step. Establishing the relative importance of POP-induced impacts on processes that facilitate fat accumulation or stimulate fat loss is required. Here, we link *in vitro* and *in vivo* data to predict the effects of current POP levels on energy balance in wild, suckling grey seal pups.

## 2. Methods

### 2.1. Study site, permits and animals

Field work was conducted on the Isle of May, Firth of Forth Scotland (56   11'N, 02   33'W) during October to December 2016–2017. Animal capture and sample collection was performed by personal licence holders under UK Home Office licence 70/7806. This work received ethical approval from Abertay University and the University of St Andrews Animal Welfare and Ethics Committee (AWEC) and was performed in compliance with Animal (Scientific Procedures) Act (ASPA) 1986 and the EU directive on the protection of animals used for scientific purposes (2010/63/EU).

Morphometric measurements were collected from 57 healthy wild mother–pup pairs during the suckling period. Twelve females were represented in both years. Plasma samples for hormone measurements and blubber biopsies for contaminant measurement and explant experiments were obtained from each pup late in the suckling period (day 15).

### 2.2. Morphometric measurements

Mothers that had previously been marked, or were individually recognisable from pelage patterns or scars, were observed from when they came ashore, which allowed an accurate birth date for their pups to be recorded (Pomeroy et al 1999). Mother–pup pairs were captured early

(day 5) and late (day 15) in the suckling period. At each capture, females were anaesthetised using an intramuscular mass-specific dose of Zoletil 100™. Animals were weighed using a load cell ( $\pm 0.2$  kg) mounted on a block and tackle suspended from a tripod (Pomeroy et al 1999). Nose to tail length and axial girth were also measured. After the second capture, daily observation of mother–pup pairs allowed weaning date to be recorded (Bennett et al 2007). Pups were captured within two days from weaning and penned in a large enclosure with access to water (Robinson et al 2018). Pups were weighed 3–5 days after penning to facilitate weaning mass estimation from initial mass loss rates. After a second sample taken postweaning, pups were released from the pen with easy access to the sea to facilitate departure from the colony (Robinson et al 2018).

### 2.3. Plasma samples and thyroid hormone analysis

At the late lactation capture, a blood sample was drawn from the epidural vein into an ethylenediaminetetraacetic acid (EDTA) coated Vacutainer (Becton Dickinson, Oxford, UK) and kept cool in an insulated bag containing an ice pack until it was returned to the laboratory. Plasma was centrifuged at 2000 g for 15 min. The plasma was drawn off and stored at  $-80^{\circ}\text{C}$  until analysis. Total tri-iodothyronine (TT3) and thyroxine (TT4) were measured in triplicate using commercially available Enzyme Linked Immunosorbent Assays (ELISAs; BXE0701B (TT3), BXE0711B (TT4), Fortress Diagnostics Ltd., Antrim, U.K), according to the manufacturer's instructions and using a four parameter log-logistic model in R (R Core Team 2016) to fit the standard curve. Parallelism of a two-fold serial dilution of a grey seal plasma pool to the standard curve was assessed using analysis of covariance (ANCOVA), comparing the slope of the regression lines of the standards and the diluted samples (Kershaw et al 2017). The lack of interaction between the plasma dilution curve and the standard curve confirmed that dilution of the plasma samples ran parallel to the standard curve to 1/8th of neat plasma concentration. Inter and intra assay coefficient of variation was  $< 14\%$  for TT3 and  $< 16\%$  for TT4.

### 2.4. Blubber biopsies

Blubber sampling and storage were performed as described previously (Robinson et al 2018). Briefly, prior to sampling, pups were given a mass-specific dose of intravenous Zoletil 100™ for general anaesthesia and subcutaneous injections of the local anaesthetic, Lignol™, at biopsy sites. Full depth biopsy cores ( $1 \times 6$  mm and  $3 \times 10$  mm) were then taken from the dorso-lateral pelvic region (Bennett et al 2015). Pups were given an intravenous mass specific dose of Carprive® for longer lasting pain relief and as an anti-inflammatory. The 6 mm biopsy was immediately wrapped in foil and frozen at  $-20^{\circ}\text{C}$  for POP analysis (Robinson et al 2018; 2019). The 10 mm biopsies were cut immediately in half cross-sectionally using sterile scissors to give inner (closest to muscle) and outer (closest to skin) sections that were placed in separate pre-warmed Krebs-Ringer solution (pH 7.4; 0.14 M NaCl; 5 mM KCl; 1 mM  $\text{MgSO}_4$ ; 0.4 mM  $\text{K}_2\text{HPO}_4$ ; 5.5 mM glucose; 20 mM HEPES; 1 mM  $\text{CaCl}_2$ ; (Cold Spring Harbour Protocol, 2014)) supplemented with 1% Antibiotic Antimycotic Solution (all supplied by Sigma-Aldrich, Poole, Dorset, UK) and transported to the laboratory within half an hour for explant creation, as described previously (Robinson et al 2018). Biopsy sites were closed with sutures and healing monitored every 5 days.

### 2.5. Blubber contaminant analysis

POP analysis was performed at the Centre for Analytical Research and Technology (CART) at the University of Liège, Belgium in the Department of Chemistry (CBs and PBDEs) or Laboratory of Animal Ecology and Ecotoxicology (OCPs), as described previously (Robinson et al 2019). Six non dioxin-like PCBs (NDL-PCBs) (PCB28, 52, 101, 138, 153, 180), eight dioxin-like PCBs (DL-PCBs) (PCB105, 114, 118, 123,

156, 157, 167, 189), nine PBDEs (PBDE28, 47, 66, 85, 99, 100, 153, 154, 183) and four OCPs (DDT (summed op'DDT and pp'DDT), and its metabolites, dichlorodiphenyldichloroethane (pp'DDD), dichlorodiphenyldichloroethylene (pp'DDE) were measured.

### 2.6. Blubber glucose uptake and glycerol production

Blubber explant protocols and determination of glucose uptake and lipolytic rates are detailed in Bennett et al (2017) and Robinson et al (2018). Briefly, tissue was rinsed with 1 mL pre-warmed Krebs ringer and hair and blood removed. Tissue was minced into 5–10 mg pieces, weighed out into 100 mg portions and dispensed into pre-prepared 12-well cell culture plates (Corning, Sigma Aldrich) containing 1.5 mL medium 199, Hanks' Balanced Salts supplemented with 1% Antibiotic Antimycotic Solution, 1% fatty acid supplement and 5% charcoal stripped fetal bovine serum. Inner and outer explants were run in duplicate from each animal. Plates were placed in a humidified incubator maintained at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  (Thermo Scientific, Perth, UK, Midi 40  $\text{CO}_2$  Incubator, model: 3404) for 24 h. Media was drawn off after 24 h and frozen at  $-80^{\circ}\text{C}$ . Glucose in media was measured using a Randox (County Antrim, UK) kit (GL364) using either an RX Monza (Randox) Clinical Chemistry analyser (Model: 328–14-0914), as described previously (Bennett et al 2017; Robinson et al 2018) or in a 96 well plate format using 2.5  $\mu\text{L}$  plasma and 250  $\mu\text{L}$  reagent and measured in a BioTek (Swindon, UK) ELx800 plate reader. Glycerol in media was measured using Randox kit GY105 (2016) or using Sigma kit MAK117 (2017) according to manufacturers' instructions. Internal quality control measurements lay within  $\pm 15\%$ . Within and between run coefficient of variation (CV) was  $< 5\%$  for each assay. Inter assay CV between assay formats was 7%. Blubber glucose uptake and glycerol production rates ( $\mu\text{moles } 100 \text{ mg}^{-1} \text{ h}^{-1}$ ) were calculated and mean values for inner and outer blubber from each animal used in analysis.

### 2.7. Calculation of whole animal mass changes

Pup mass at birth and weaning, maternal postpartum (MPPM) and departure masses were estimated, as described previously (Pomeroy et al 1999). However, the weaning mass estimate using this method was up to 4 kg lighter than the actual measured mass immediately after weaning. The underestimate is likely because there were up to 10 days between the second suckling capture and weaning (mean =  $3 \pm 2$  days), which reduces accuracy. We therefore used a second method to estimate weaning mass: mass at penning was added to the product of initial fasting mass loss rate (rate of mass loss between penning and the first post-penning capture) and number of days between weaning and penning. The weaning mass estimate derived from the method that used a measured mass on a date closest to weaning was used in further analysis.

### 2.8. Statistical analysis

Data were analysed using R 3.5.2 (R Core Team 2016). We used *t* tests to examine differences in pup and maternal characteristics, TH and blubber function variables between years. We used paired *t* tests to examine between year differences in data from mothers that had been sampled in both years.

#### 2.8.1. Model formulation and selection

We investigated POP, TH and blubber metabolic characteristics effects on whole-animal energy balance parameters. Normal distribution of response variables (daily mass gain rate during suckling and weaning mass) were tested using Anderson-Darling tests in the *norstest* package (Gross and Ligges 2015). Data were analysed using linear mixed effects models (lme) in the *nlme* package (Pinheiro et al 2018) because some pups were the offspring of the same mothers in different years, and mothers can show repeatability in contaminant profiles of milk

(Pomeroy et al 1996) or lactation performance, including milk composition and output (Lang et al 2009). Year, sex, POPs, blubber metabolic properties and TH were included as candidate variables to explain mass gain rates. Mass gain rate was then included as an additional candidate explanatory variable in the model to explain variance in weaning mass as well as those listed above. Since TH were explanatory variables in models explaining energy balance, the impact of POPs on TH levels were also investigated.

Colinear variables were identified for each global model by calculating variance inflation factor using the *vif* function from the *car* package (Fox & Weisberg 2011). Each variable with *vif* value above 3 was excluded sequentially until the *vif* for all variables included in the full model was  $< 3$  (Zuur et al 2010). Initial backwards model selection from the full model was then performed using the *dredge* function in *MuMIn* (Barton 2018). Each of the candidate models identified by *dredge*, for which  $\Delta AIC_c$  was  $< 2$ , was then assessed for model fit using residual and Q-Q plots. The proportion of support for each of the candidate models was assessed using the model Aikake weights (Wagenmakers & Farrell 2004). The top model was used in each case, rather than model averaging, even when the top model was not the most parsimonious and model weights were low or similar between competing models, because top model outputs were subsequently used to predict POP effects on each of the response variables and this takes the most precautionary approach with regard to potential POP effects. Conditional and marginal  $R^2$  were calculated for the top model in *piecewiseSEM* (Lefcheck 2016). When the conditional  $R^2$  was not greater than marginal  $R^2$ , the random effect was dropped and the linear model (*lm*) reported. Each POP class was included in a separate model selection process. For *lm*,  $\eta^2$  (the amount of variance explained by each variable when all others are kept constant) was calculated using *etasq* in *heplots* (Friendly 2007; Fox et al 2018) to give an index of effect size. In LMEs,  $\eta^2$  cannot be calculated. Instead, we calculated the likelihood ratio (*L*-ratio) for each model, which provides the odds of the likelihood of the data under the model that includes that variable compared to the simpler model.

### 2.8.2. 'Low POP' predictions

We then estimated the size of the impact of current POP levels on energy balance for each of the individuals in our study, acting through the potential mechanisms explored here (altered glucose uptake by blubber, altered TT3 and altered mass gain from some unidentified effect of PBDEs). We used the model produced previously (Robinson et al 2018) to determine the percentage reduction ( $\pm 95\%$ CI) in glucose uptake experienced for the reduction in DL-PCBs that would be experienced by each pup if its levels were at the lowest observed here, and applied this reduction ( $\pm 95\%$ CI) to inner blubber glucose uptake values for all animals. We used this value to estimate mass gain rate ( $\pm 95\%$ CI) under a low  $\Sigma$ DL-PCB scenario using the model that best predicted mass gain rate. We used the estimate from the model for mass gain rate here ( $\pm 95\%$ CI) to predict mass gain rate for each pup if its PBDE levels were at the lowest observed here. We then combined the effect of both reduced  $\Sigma$ DL-PCBs and reduced  $\Sigma$ PBDEs to produce predictions of mass gain rate if these POPs were reduced. We generated predictions using the upper and lower 95% CI of the estimates for each parameter for error propagation to create 'best and worst case scenarios', which also helped explore how the different apparent POP effects may offset each other.

We modelled the effect of POPs on TT3 and used it to generate predictions of TT3 levels ( $\pm 95\%$ CI) if POP levels were fixed at the lowest levels seen in our dataset. TT3 was log transformed prior to analysis to improve model fit. We used these new TT3 values to predict weaning mass ( $\pm 95\%$ CI) under a 'low POP' scenario. We also combined the impact on TT3 with the DL-PCB effect on glucose uptake to explore the extent to which they offset each other, including error propagation as described above. Finally, we combined the effect of all three mechanisms through which POPs may influence mass gain rate and weaning mass to estimate the difference in weaning mass ( $\pm 95\%$  CI produced

from all 3 mechanisms combined) between current and low POP scenarios and to establish which mechanism is likely to have the greatest influence on weaning mass.

## 3. Results

### 3.1. Contaminant load summary

Within each year, pups exhibited a 4 to 10-fold range in  $\Sigma$ NDL-PCBs, an 8-fold range in  $\Sigma$ DL-PCBs, a 9–14 fold range in  $\Sigma$ PBDEs and a 3–5 fold range in  $\Sigma$ OCPs (Table 1). Pups in 2017 were 3.4–4.7 kg lighter at capture and had a wider contaminant concentration range compared to pups in 2016, despite parity in timing of sampling during suckling. There was no correlation between mass at capture at late suckling and log  $\Sigma$ NDL-PCB ( $t = -1.03$ ;  $r = -0.14$ ;  $p = 0.31$ ,  $df = 55$ ), log  $\Sigma$ DL-PCB ( $t = -1.22$ ;  $r = -0.16$ ;  $p = 0.23$ ;  $df = 55$ ), log  $\Sigma$ PBDE ( $t = -0.60$ ;  $r = -0.08$ ;  $p = 0.55$ ;  $df = 55$ ) or log  $\Sigma$ DDX ( $t = -0.63$ ;  $r = -0.08$ ;  $p = 0.53$ ;  $df = 55$ ).

### 3.2. TH and blubber metabolic properties

Table 2 shows circulating TT3 and TT4 levels, metabolic properties of blubber tissue and energy balance characteristics of the pups in 2016 and 2017. There was no difference between years in TT3 or TT4. Glucose uptake was significantly higher in 2016 compared to 2017 in both blubber layers, whereas glycerol production was higher in 2017 in both inner and outer blubber (Table 2). Neither birth mass, MPPM nor lactation duration differed between the cohorts. Although pup mass gain rates were approximately  $0.25 \text{ kg day}^{-1}$  greater in 2016, there was no difference in weaning mass between years. These results were largely mirrored in the data from only the females that had been sampled in both years: MPPM ( $T = 1.37$ ;  $p = 0.20$ ), lactation duration ( $T = 1.88$ ;  $p = 0.09$ ) and birth mass ( $T = 1.05$ ;  $p = 0.32$ ) did not differ between years, but both mass gain rate ( $T = 3.92$ ;  $p = 0.002$ ) and weaning mass ( $T = 3.05$ ;  $p = 0.011$ ) were lower in 2017. Similarly, glucose uptake in inner ( $T = 7.30$ ;  $p < 0.001$ ) and outer blubber ( $T = 5.98$ ;  $p < 0.001$ ) were lower and glycerol production in inner ( $T = 2.89$ ;  $p = 0.014$ ) and outer blubber ( $T = 2.41$ ;  $p = 0.029$ ) were higher in 2017. Neither TT3 ( $T = 0.88$ ;  $p = 0.40$ ) nor TT4 levels ( $T = 1.61$ ;  $p = 0.14$ ) were different between years.

### 3.3. Drivers of mass gain rate and weaning mass

The candidate variables included at each step of the analysis for each response variable, those retained after exclusion by *vif*, those then retained in the top model and those that were significant in each top model are summarised in Table 3. *Dredge* outputs and model selection details at each step, including model weights, are given in supplementary material.

MPPM = maternal postpartum mass. -Indicates where variable was not appropriate to include; GM indicates variable included in global model; VIF indicates variable also retained after variance inflation factor applied; DREDGE indicates variable also retained in one of models with  $AIC_c$  within  $< 2$  of the top model; TM indicates variable also retained in top model; TM-S indicates effect of variable is also significant in top model ( $p < 0.05$ ). Note that each of the POP class variables, highlighted in grey, were not included in any model at the same time as the others, but were each tested separately. POP class combinations were tested when the models for individual POP classes produced similar fits and directions of relationship. Details of model selection and model output for each dependent variable are given in supplementary material. Maternal identity was included as a random effect, and only dropped when conditional  $R^2 < 5\%$  greater than marginal  $R^2$ . \* indicates where the random effect of maternal identity was retained in the model.

#### 3.3.1. Mass gain rate

The model that best described mass gain rate included birth mass,

**Table 1**

Median (range) blubber concentrations of individual and summed POP congeners, and % blubber lipid; mean ( $\pm$ se) body mass and age at capture, and sample size (n) of suckling grey seal pups from the Isle of May in 2016 and 2017. All concentrations are in ng g<sup>-1</sup> lipid.

|                           | 2016                      | 2017                       |
|---------------------------|---------------------------|----------------------------|
| CB28                      | 1.46<br>(0.4–6.6)         | 0.4<br>(0.4–2.1)           |
| CB52                      | 10.1<br>(1.8–38.5)        | 6.2<br>(0.8–25.9)          |
| CB101                     | 17.8<br>(10.7–68)         | 15.0<br>(8.9–43.8)         |
| CB138                     | 110.8<br>(58.2–223.3)     | 93.3<br>(47.7–388.7)       |
| CB153                     | 189.3<br>(99.1–390.7)     | 159.4<br>(75.9–794.1)      |
| CB180                     | 29.9<br>(9.9–102.1)       | 27.8<br>(11.5–330.3)       |
| $\Sigma$ NDL-PCB          | 354.29<br>(194.21–790.45) | 297.43<br>(151.89–1571.41) |
| CB105                     | 1.69<br>(0.84–8.73)       | 3.04<br>(1.09–7.30)        |
| CB114                     | 0.77<br>(0.45–2.15)       | 0.51<br>(0.21–4.83)        |
| CB118                     | 4.93<br>(2.43–25.12)      | 7.54<br>(2.83–17.31)       |
| CB123                     | 0.02<br>(0.02–0.24)       | 0.02<br>(0.02–0.31)        |
| CB156                     | 2.09<br>(1.20–6.62)       | 3.03<br>(1.35–13.46)       |
| CB157                     | 0.54<br>(0.29–1.37)       | 0.82<br>(0.38–4.16)        |
| CB167                     | 0.06<br>(0.01–0.45)       | 0.12<br>(0.01–0.49)        |
| CB189                     | 0.41<br>(0.12–1.20)       | 0.34<br>(0.09–2.71)        |
| $\Sigma$ DL-PCBs          | 10.75<br>(5.47–45.62)     | 15.38<br>(6.23–49.59)      |
| BDE28                     | 0.1<br>(0.1–0.3)          | 0.1<br>(0.03–0.4)          |
| BDE47                     | 11.4<br>(5.2–48.6)        | 9.2<br>(2.8–44.9)          |
| BDE66                     | 0.005<br>NA               | 0.005<br>NA                |
| BDE85                     | 0.02<br>(0.005–0.15)      | 0.06<br>(0.005–0.3)        |
| BDE99                     | 0.4<br>(0.1–4.1)          | 0.3<br>(0.01–3.6)          |
| BDE100                    | 1<br>(0.5–3.1)            | 0.8<br>(0.2–2.5)           |
| BDE153                    | 0.3<br>(0.13–1.3)         | 0.2<br>(0.005–1.7)         |
| BDE154                    | 0.3<br>(0.2–0.7)          | 0.3<br>(0.01–0.9)          |
| BDE183                    | 0.01<br>(0.005–0.11)      | 0.01<br>(0.005–0.03)       |
| $\Sigma$ PBDE             | 13.81<br>(6.44–57.87)     | 11.08<br>(3.57–50.94)      |
| DDT                       | 14.26<br>(5.70–46.94)     | 13.48<br>(5.14–57.35)      |
| DDD                       | 5.7<br>(2.81–17.21)       | 3.94<br>(1.19–10.57)       |
| DDE                       | 145.89<br>(87.01–356.63)  | 144.33<br>(70.48–310.58)   |
| $\Sigma$ DDX              | 169.09<br>(98.49–420.77)  | 159.33<br>(79.12–378.50)   |
| % lipid                   | 80.12<br>(67.79–85.51)    | 81.99<br>(75.50–96.92)     |
| Body mass at capture (kg) | 41.8<br>(1.12)            | 38.4<br>(1.0)              |
| Age at capture (days)     | 15.6 (0.13)               | 15.4 (0.13)                |
| n                         | 27                        | 30                         |

**Table 2**

. Mean ( $\pm$ se) plasma thyroid hormone levels (TT3 and TT4), blubber metabolic characteristics and whole animal energy balance parameters for grey seal pups from the Isle of May, Firth of Forth in 2016 and 2017. MPPM = maternal post-partum mass. Bold highlights between year differences (T test:  $p < 0.05$ ).

|                                                                                            | 2016                     | 2017                    | T           | p                |
|--------------------------------------------------------------------------------------------|--------------------------|-------------------------|-------------|------------------|
| TT3 (ng ml <sup>-1</sup> )                                                                 | 0.88 (0.03)              | 0.84<br>(0.03)          | 0.87        | 0.39             |
| TT3 (nmol l <sup>-1</sup> )                                                                | 1.35 (0.05)              | 1.29<br>(0.04)          |             |                  |
| TT4 ( $\mu$ g dl <sup>-1</sup> )                                                           | 2.65 (0.21)              | 2.20<br>(0.14)          | 1.79        | 0.08             |
| TT4 (nmol l <sup>-1</sup> )                                                                | 73.24<br>(5.77)          | 60.72<br>(3.93)         |             |                  |
| Inner blubber glucose uptake<br>( $\mu$ mol 100 mg <sup>-1</sup> h <sup>-1</sup> )         | <b>0.045</b><br>(0.003)  | <b>0.020</b><br>(0.002) | <b>6.53</b> | <b>&lt;0.001</b> |
| Outer blubber glucose uptake<br>( $\mu$ mol 100 mg <sup>-1</sup> h <sup>-1</sup> )         | <b>0.041</b><br>(0.003)  | <b>0.015</b><br>(0.003) | <b>8.01</b> | <b>&lt;0.001</b> |
| Inner blubber glycerol<br>production ( $\mu$ mol 100 mg <sup>-1</sup><br>h <sup>-1</sup> ) | <b>0.015</b><br>(0.0007) | <b>0.021</b><br>(0.001) | <b>4.72</b> | <b>&lt;0.001</b> |
| Outer blubber glycerol<br>production ( $\mu$ mol 100 mg <sup>-1</sup><br>h <sup>-1</sup> ) | <b>0.017</b><br>(0.001)  | <b>0.024</b><br>(0.001) | <b>4.68</b> | <b>&lt;0.001</b> |
| Birth mass (kg)                                                                            | 14.42<br>(0.42)          | 13.67<br>(0.20)         | 0.20        | 0.84             |
| MPPM (kg)                                                                                  | 188.73<br>(3.28)         | 184.45<br>(4.47)        | 0.77        | 0.44             |
| Lactation duration (days)                                                                  | 19 (0.4)                 | 19.5 (0.5)              | 1.61        | 0.12             |
| Daily rate of mass gain (kg d <sup>-1</sup> )                                              | <b>1.92 (0.08)</b>       | <b>1.68</b><br>(0.09)   | <b>1.97</b> | <b>0.05</b>      |
| Estimated weaning mass (kg)                                                                | 45.08<br>(1.19)          | 42.00<br>(7.67)         | 1.80        | 0.08             |
| n                                                                                          | 27                       | 30                      |             |                  |

**Table 3**

Variables used and retained at each step in the modelling of mass gain rate, weaning mass and TT3 levels.

|                          |                                | Mass gain<br>rate | Weaning<br>mass | TT3         |
|--------------------------|--------------------------------|-------------------|-----------------|-------------|
| Explanatory<br>variables | Sex                            | GM                | TM              | TM          |
|                          | Year                           | GM                | GM              | VIF         |
|                          | log $\Sigma$ DL-PCB            | VIF               | VIF             | <b>TM-S</b> |
|                          | log $\Sigma$ NDL-PCB           | VIF               | TM              | DREDGE      |
|                          | log $\Sigma$ sumPCB            | VIF               | DREDGE          | DREDGE      |
|                          | log $\Sigma$ DDX               | VIF               | DREDGE          | DREDGE      |
|                          | log $\Sigma$ BDE               | <b>TM-S</b>       | DREDGE          | DREDGE      |
|                          | log $\Sigma$ POP               | VIF               | DREDGE          | DREDGE      |
|                          | TT3                            | VIF               | <b>TM-S</b>     | –           |
|                          | TT4                            | GM                | VIF             | –           |
|                          | Glucose uptake<br>(Inner)      | <b>TM-S</b>       | VIF             | –           |
|                          | Glucose uptake<br>(Outer)      | GM                | GM              | –           |
|                          | Glycerol production<br>(Inner) | VIF               | DREDGE          | –           |
|                          | Glycerol production<br>(Outer) | GM                | GM              | –           |
|                          | MPPM                           | <b>TM-S</b>       | <b>TM-S</b>     | –           |
|                          | Lactation duration             | <b>TM-S</b>       | <b>TM-S</b>     | –           |
|                          | Birth mass                     | TM                | <b>TM-S</b>     | –           |
|                          | Mass gain rate                 | –                 | <b>TM-S</b>     | –           |
|                          | Maternal identity              | *                 |                 |             |

lactation duration, MPPM, glucose uptake in inner blubber tissue and log  $\Sigma$ PBDE (Table S1 and 2). Support for this model was low (0.112), but the model including log  $\Sigma$ PBDE was 1.96 times more likely than the more parsimonious model using model weights (Table S1). Pups with heavier mothers gained mass faster: the predicted difference in mass gain rate of pups from the lightest and heaviest mothers was 1.29 kg day<sup>-1</sup> (95%CI: 1.08–1.50 kg day<sup>-1</sup>) assuming all other variables remained constant. Pups with higher birth mass tended to gain mass

more slowly, with a predicted difference of  $0.81 \text{ kg day}^{-1}$  (95% CI:  $0.62\text{--}1.01 \text{ kg day}^{-1}$ ) across the range of birth masses, assuming all other variables remained the same. Pups that had longer suckling duration tended to gain mass more slowly, with a predicted difference of  $0.62 \text{ kg day}^{-1}$  (95% CI:  $0.44\text{--}0.80 \text{ kg day}^{-1}$ ) across the range of suckling durations. In addition to these well-established effects of maternal size, birth mass and lactation duration (Pomeroy et al 1999), daily mass gain rate was positively related to glucose uptake in inner blubber (Fig. 1). Glucose uptake by inner blubber was retained in both models identified by dredge with a  $\Delta\text{AIC}$  of  $< 2$  (Table S1). Pups that had higher glucose uptake rate in inner blubber gained mass faster, with a predicted difference of  $0.43 \text{ kg day}^{-1}$  ( $0.37\text{--}0.48$  95% CI) across the range of glucose uptake rates measured. Pups with a greater blubber  $\Sigma\text{PBDE}$  concentration also gained mass faster, with a predicted difference of  $0.59 \text{ kg day}^{-1}$  ( $0.44\text{--}0.74$  95% CI) across the range of log  $\Sigma\text{PBDE}$  values seen here. Other POPs, when substituted for PBDEs in the model selection, were not retained in the top candidate models by dredge (Table 3).

### 3.3.2. Weaning mass

In the model that best described weaning mass, pups were heavier at weaning if they were larger at birth, had larger mothers, undertook a longer suckling period and gained mass more rapidly (Table S3 and S4). There were 11 models with  $\Delta\text{AIC} < 2$ , all with low support ( $< 0.1$ ). Along with birth mass, lactation duration, MPPM and pup mass gain rate, TT3 was included in all models and the negative association was significant (Fig. 2). TT3 levels were associated with a  $4.37 \text{ kg}$  lower weaning mass in animals with highest TT3 values compared with those that had the lowest when all other variables were fixed. In addition, both log  $\Sigma\text{NDL-PCBs}$  and sex were retained as non-significant variables. The model with log  $\Sigma\text{NDL-PCBs}$  was only 1.01 times more likely than the model without

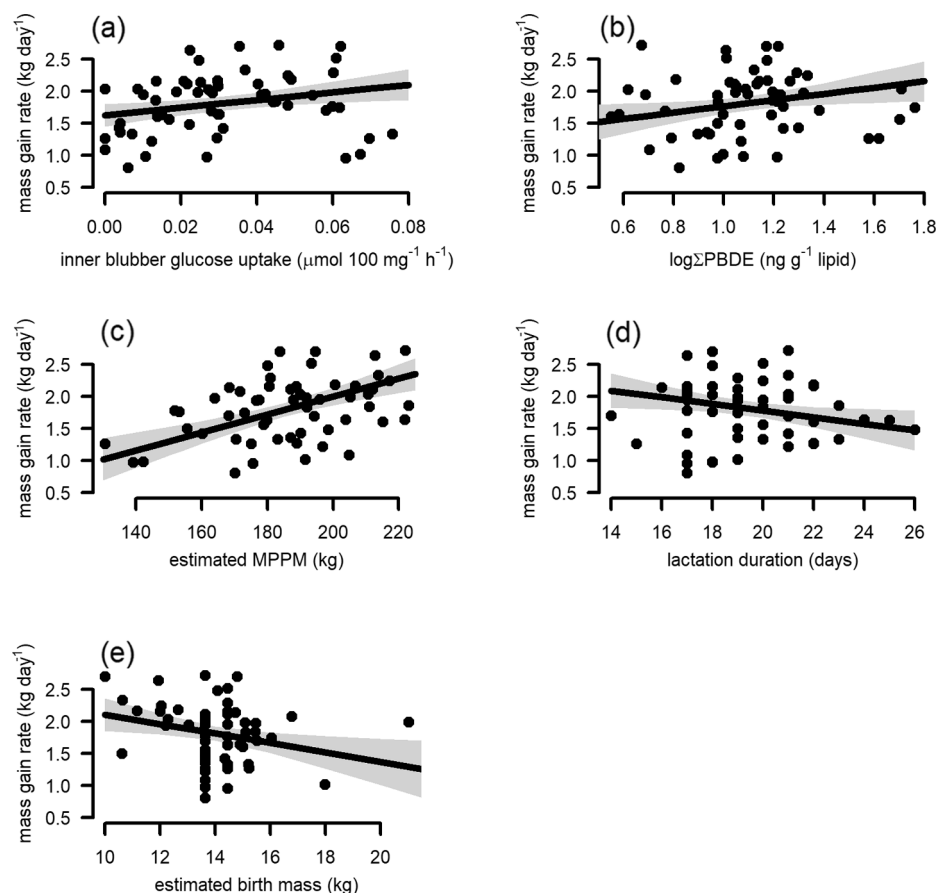
this parameter, and the model that included sex was 1.9 times more likely than the model that excluded it. The 95% confidence intervals of the co-efficient estimate for both sex and NDL-PCBs overlapped zero, suggesting that these variables had no important biological effect on weaning mass, and they were excluded from predictions.

### 3.4. Effects of reduced POPs on weaning mass

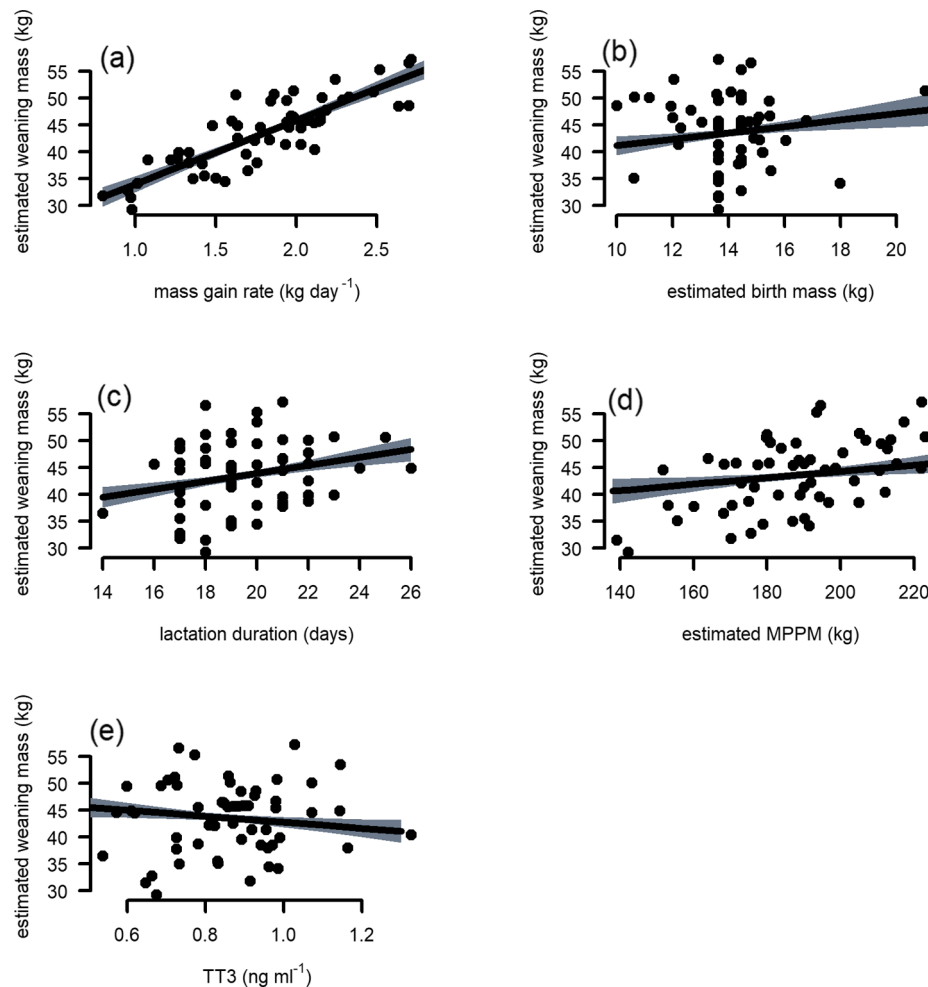
We predicted the impact on weaning mass of reducing POPs for each animal. We examined the relative impact of the three ways in which POPs may influence weaning mass identified here: 1. through the previously established negative relationship between DL-PCB and blubber glucose uptake (Robinson et al 2018), which impacts mass gain rate; 2. through the potential PBDE effect on mass gain rate and 3. through the negative relationship between DL-PCBs and TT3, which in turn influences weaning mass. We examined these effects individually and in combination to determine their relative influence.

#### 3.4.1. POP impacts through altered mass gain

A previous model generated from the 2016 data (Robinson et al 2018) predicted DL-PCBs reduced inner blubber glucose uptake by 43.92% (95%CI: 36.91–50.92%) across the range of log  $\Sigma\text{DL-PCBs}$  here. We predicted how much mass gain rate would be altered by a reduction in log  $\Sigma\text{DL-PCBs}$  by adjusting the glucose uptake values by these amounts. Predicted mass gain rate was altered by  $-0.28\text{--}0.63 \text{ kg day}^{-1}$  (lower 95% CI =  $-0.61\text{--}0.32$ ; upper 95% CI =  $-0.17\text{ to }0.94 \text{ kg day}^{-1}$ ). Alleviation of the DL-PCB effect on glucose uptake was thus predicted to increase mass gain rate in 65% (37 [range from upper and lower CI estimates = 12–53]) of the animals. If DL-PCBs only reduce blubber glucose uptake by 37%, mass gain rate was predicted to be altered by



**Fig. 1.** Relationship between pup mass gain rate and (a) inner blubber glucose uptake; (b) log  $\Sigma\text{PBDE}$ ; (c) MPPM; (d) lactation duration and (e) estimated birth mass in grey seal pups ( $n = 57$ ). Lines and shading show model prediction and 95% confidence intervals when all other covariates are held at the mean value.



**Fig. 2.** Relationship between estimated weaning mass and (a) mass gain rate, (b) estimated birth mass, (c) lactation duration, (d) estimated MPPM, and (e) TT3. Lines and shading show model prediction and 95% confidence intervals when all other covariates were held at the mean value.

$-0.30$ – $0.61 \text{ kg day}^{-1}$  (lower 95% CI =  $-0.62$  –  $0.31$ ; upper 95% CI =  $-0.23$  to  $0.77 \text{ kg day}^{-1}$ ) resulting in 37 (range from upper and lower CI estimates = 10–45) animals with faster mass gain. If DL-PCBs reduce inner glucose uptake by as much as 51%, mass gain rate was predicted to be altered by  $-0.27$ – $0.66 \text{ kg day}^{-1}$  (lower 95% CI =  $-0.60$  –  $0.33$ ; upper 95% CI =  $-0.21$  to  $0.84 \text{ kg day}^{-1}$ ) resulting in 68% (39 [range from upper and lower CI estimates = 14–55] of animals experiencing faster mass gain rate.

If both DL-PCBs and PBDEs were reduced to the lowest levels seen here, mass gain rate was altered by  $-0.59$ – $0.43 \text{ kg day}^{-1}$  (lower 95% CI =  $-0.97$  –  $0.02$ ; upper 95% CI =  $-0.31$  to  $0.84 \text{ kg day}^{-1}$ ) and just 14 (range from upper and lower CI estimates = 1–43) animals were predicted to increase their mass gain rate. The effects on mass gain rate of reducing PBDEs thus strongly counteracted by the effects of alleviating the DL-PCB impact on blubber function.

We then predicted the effects of altered mass gain rate on weaning mass. The effect of a 43% increase in blubber glucose uptake on mass gain rate, as a result of lower DL-PCBs, was predicted to increase weaning mass by  $1.08 \pm 0.62 \text{ kg}$ , a gain of  $3.7 \pm 1.59\%$  (sem). Predicted differences ranged from 7.47 kg lighter up to 12.93 kg heavier. The predicted consequences for weaning mass and number of animals predicted to be heavier under more extreme scenarios, when POP effects were set to their respective upper and lower CI, are shown in Table 4. Combining the effects on mass gain rate of a reduction in DL-PCBs and PBDEs produced an average reduction in weaning mass of  $1.99 \pm 0.63 \text{ kg}$ , a difference of  $3.51 \pm 1.54\%$  (sem), such that the amelioration of the DL-PCB effect on glucose uptake offset about half the effect of PBDE

reduction. Only if the DL-PCB effect on glucose uptake is assumed to be as large as 51% does it ameliorate the PBDE effect on mass gain rate in most animals (Table 4). Clearly, if the effect of PBDEs on mass gain is causal, it is large enough to overcome effects of DL-PCB-induced blubber glucose uptake inhibition.

Alleviation of POP effects were predicted for each mechanism individually (DL-PCB reduction of glucose uptake; DL-PCB reduction of TT3 and PBDE elevation of mass gain rate) and combined. Mean effects are reported in text. Scenarios (1–12) were generated from the 95% CI of estimates of POP effects in each case to allow error propagation where dependent variables from one model are used as predictors in another, and facilitate exploration of most and least conservative estimates. Bold indicates scenarios predicted to decrease weaning mass in most animals. 1. Smallest predicted effect of reduced DL-PCBs on glucose uptake rate producing least benefit to mass gain rate; 2. Greatest predicted effect of reduced DL-PCBs on glucose uptake rate producing largest benefit to mass gain rate; 3. Greatest increase in TT3 from reduced DL-PCBs, producing biggest detriment to weaning mass; 4. Smallest increase in TT3 from reduced DL-PCBs producing least detriment to weaning mass; 5. Greatest predicted effect of reduced PBDEs on mass gain rate, producing biggest detriment to weaning mass; 6. Smallest predicted effect of reduced PBDEs on mass gain rate producing least detriment to weaning mass; 7. Smallest predicted effect of reduced DL-PCBs on glucose uptake rate combined with greatest effect of PBDEs on mass gain rate, producing least offset for weaning mass; 8. Greatest predicted effect of reduced DL-PCBs on glucose uptake rate combined with smallest effect of PBDEs on mass gain rate, producing greatest offset for weaning

**Table 4**

Predicted impact of alleviation of POP effects on blubber glucose uptake, T3 and mass gain rate on  $\Delta$  % weaning mass ( $\pm$ sem) and number of animals predicted to be heavier (upper and lower CI) using 95% CI of estimated POP effects from each model.

| Scenario | Assumed POP impact                                     |                                        |                                                | Effect if POP effect is alleviated<br>$\Delta$ % weaning mass | # animals heavier at weaning |
|----------|--------------------------------------------------------|----------------------------------------|------------------------------------------------|---------------------------------------------------------------|------------------------------|
|          | DL-PCB-induced $\downarrow$ in blubber glucose uptake  | DL-PCB induced $\downarrow$ in TT3     | PBDE induced $\uparrow$ in mass gain rate      |                                                               |                              |
| 1        | $\downarrow$ 37%                                       | –                                      | –                                              | $3.29 \pm 1.58$                                               | 32 (19–37)                   |
| 2        | $\downarrow$ 51%                                       | –                                      | –                                              | $4.01 \pm 0.62$                                               | 34 (25–39)                   |
| 3        | –                                                      | $\downarrow$ by upper CI of PCB effect | –                                              | $-3.05 \pm 0.30$                                              | 4 (0–41)                     |
| 4        | –                                                      | $\downarrow$ by lower CI of PCB effect | –                                              | $-0.65 \pm 0.29$                                              | 22 (3–51)                    |
| 5        | –                                                      | –                                      | $\uparrow$ by upper CI estimate of PBDE effect | $-14.86 \pm 1.78$                                             | 7 (5–15)                     |
| 6        | –                                                      | –                                      | $\uparrow$ by lower CI estimate of PBDE effect | $2.81 \pm 2.01$                                               | 27 (23–32)                   |
| 7        | Lower CI of effect on mass gain of $\downarrow$ by 37% | –                                      | $\uparrow$ by upper CI estimate of PBDE effect | $-13.49 \pm 1.82$                                             | 10 (6–15)                    |
| 8        | Upper CI of effect on mass gain $\downarrow$ by 51%    | –                                      | $\uparrow$ by lower CI estimate of PBDE effect | $6.19 \pm 2.17$                                               | 34 (27–37)                   |
| 9        | Lower CI of effect on mass gain of $\downarrow$ by 37% | $\downarrow$ by upper CI of PCB effect | –                                              | $-6.97 \pm 1.90$                                              | 16 (15–20)                   |
| 10       | Upper CI of effect on mass gain $\downarrow$ by 51%    | $\downarrow$ by lower CI of PCB effect | –                                              | $7.21 \pm 1.73$                                               | 32 (30–38)                   |
| 11       | Lower CI of effect on mass gain of $\downarrow$ by 37% | $\downarrow$ by upper CI of PCB effect | $\uparrow$ by upper CI estimate                | $-16.54 \pm 1.80$                                             | 7 (4–12)                     |
| 12       | Upper CI of effect on mass gain $\downarrow$ by 51%    | $\downarrow$ by lower CI of PCB effect | $\uparrow$ by lower CI estimate                | $-14.13 \pm 1.83$                                             | 8 (7–13)                     |

mass; 9. Smallest predicted effect of reduced DL-PCBs on glucose uptake rate combined with greatest increase in TT3 producing smallest offset between these two mechanisms; 10. Greatest predicted effect of reduced DL-PCBs on glucose uptake rate combined with smallest increase in TT3 producing greatest offset between these two mechanisms; 11. Smallest predicted effect of reduced DL-PCBs on glucose uptake rate combined with greatest increase in TT3 and greatest predicted effect of reduced PBDEs on mass gain rate producing most extreme negative effect of lowering POPs on weaning mass when all three mechanisms are combined; 12. Greatest predicted effect of reduced DL-PCBs on glucose uptake rate combined with smallest increase in TT3 and smallest predicted effect of reduced PBDEs on mass gain rate, producing least detrimental effect on weaning mass when all three mechanisms are combined.

### 3.4.2. POP impacts through altered TT3

PCBs were associated with lower TT3. Log  $\Sigma$ DL-PCBs produced the model with the lowest AICc and all other POPs produced  $\Delta$ AICcs that

were much greater than 2, including  $\Sigma$ NDL-PCBs, which are more abundant, and  $\Sigma$ PCBs (Table S5 and S6). Log TT3 was negatively related to log  $\Sigma$ DL-PCBs (Fig. 3). Sex was retained in the top model for TT3, but the model weights were similar when sex was excluded and the 95% CI for sex overlapped zero. Sex was not considered an informative variable and was dropped from the model. TT3 ( $\pm$ 95% CI) values were then predicted for every animal if  $\Sigma$ DL-PCBs were at lowest levels observed levels in this study. Log  $\Sigma$ DL-PCB was predicted to reduce TT3 by 0.135 (CI = 0.04–0.233) ng ml<sup>-1</sup>, a reduction of 20% (CI = 9.3–31.7) and 50 animals (range from upper and lower CI estimates = 35–53) were predicted to have higher TT3 if DL-PCBs were reduced. The predicted effect of increasing TT3 by reducing DL-PCB was to lower weaning mass by  $0.75 \pm 0.11$  kg ( $1.79 \pm 0.29\%$ ) and 50 animals were predicted to be lighter at weaning when TT3 was adjusted to a ‘low POP’ scenario. Table 4 shows the predictions generated from upper and lower CI estimates of DL-PCB impacts on TT3.

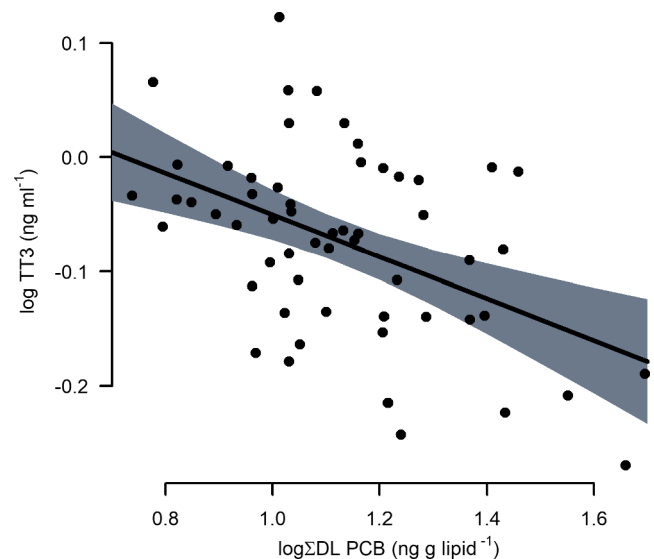
We then considered the effects mediated by DL-PCBs together because these both have a mechanistic underpinning, are both produced by the same POP class, but are predicted to produce opposite effects on weaning mass. Simultaneous alleviation of the DL-PCB effect on TT3 levels and blubber function together had a small, generally positive, effect on weaning mass. Pups were, on average,  $0.33 \pm 0.63$  kg heavier, a difference of  $1.86 \pm 1.60\%$ . This ranged from 8.67 kg lighter to 11.09 kg heavier and 28 of the pups were predicted to be heavier.

### 3.4.3. Combined effects of all POPs

Combining all the routes we have identified through which POPs may alter weaning mass we show that a reduction in PCB and PBDEs to the lowest levels seen here would reduce weaning mass by  $2.74 \pm 0.65$  kg ( $5.3 \pm 1.56\%$ ), ranging from 12.55 kg lighter to 8.64 kg heavier, and 13 animals were predicted to be heavier. Predictions generated from 95% CI estimates of POP effects are shown in Table 4. The predicted impact on weaning mass is highly dependent on how well the effects on both TT3 and glucose uptake are predicted, the extent to which the suppressive effects of the DL-PCBs are alleviated when their levels drop and whether or not the relationship between PBDEs and mass gain rate is causal.

### 3.5. Impacts of POP reduction on weaning mass are size dependent

There was a negative linear relationship between observed weaning mass and the percentage difference in predicted weaning mass when the



**Fig. 3.** Relationship between log TT3 and log  $\Sigma$ DL-PCB. Line and band shows model predictions and 95% confidence intervals.

impact of POPs was reduced. In all scenarios, smaller pups experienced the greatest gains when POP effects on energy balance were reduced, whereas the largest pups were predicted to be lighter (Fig. 4). When only the PCB effects on mass gain rate through altered blubber glucose uptake were reduced, pups of < 47 kg were predicted to be heavier by up to 20%, whereas larger pups were predicted to be up to 10% lighter (LM: % difference in weaning mass  $\sim 55.77 + (-1.20 \times \text{weaning mass})$ ;  $T = -6.45$ ;  $F_{(1,54)} = 41.65$ ;  $p < 0.001$ ;  $\text{Adj } R^2 = 0.425$ ; Fig. 4a). When combined effects of DL-PCBs on both TT3 and glucose uptake rate were reduced, pups of < 46 kg were predicted to be heavier by up to 18%, whereas larger pups were predicted to be up to 12% lighter (LM: % difference in weaning mass  $\sim 50.76 + (-1.22 \times \text{weaning mass})$ ;  $T = -5.75$ ;  $F_{(1,54)} = 33.12$ ;  $p < 0.001$ ;  $\text{Adj } R^2 = 0.369$ ; Fig. 4b). Finally, when the effects of PBDEs on mass gain rate were also reduced, pups of < 38 kg were predicted to be heavier by up to 9%, whereas larger pups were predicted to be up to 15% lighter (LM: % difference in weaning mass  $\sim 36.31 + (-0.95 \times \text{weaning mass})$ ;  $T = -4.69$ ;  $F_{(1,54)} = 21.98$ ;  $p < 0.001$ ;  $\text{Adj } R^2 = 0.276$ ; Fig. 4c).

#### 4. Discussion

We have estimated the contribution of blubber metabolic properties, TH, and POP concentrations to energy balance in grey seal pups. Inner blubber glucose uptake was a significant predictor of mass gain rates, in addition to established biological drivers (Pomeroy et al 1999), suggesting impacts on this aspect of blubber function can have substantial consequences for whole animal energy balance. Lipolytic rate was not related to the energy balance parameters measured here. TT3 was negatively related to weaning mass. The TH reduction associated with

DL-PCBs thus offset the predicted decrease in weaning mass associated with DL-PCB-induced suppression of blubber glucose uptake. PBDEs were positively associated with mass gain rate, which, in suckling grey seal pups, is largely a result of increased fat mass (Bennett et al 2007). Our data thus suggest PBDEs could promote fat gain in most animals, consistent with experimental data (Wen et al 2019), whereas DL-PCBs tend to reduce it, despite their negative influence on TH and experimental evidence that they can act as obesogens (Arsenescu et al 2008). Organohalogen impacts on whole-animal energy balance in grey seal pups appear to partially offset each other through opposing effects on different mechanisms. POP impacts were particularly detrimental for smaller pups irrespective of the mechanism through which they altered energy balance. These POP impacts were apparent despite the overriding and well-established effect of maternal characteristics on pup growth characteristics during suckling (Pomeroy et al 1999). Our data show the extent to which POPs may alter energy balance in marine mammals and highlight the need to understand the mechanistic underpinning and improve the predictive power of observed relationships.

##### 4.1. Relationship between blubber metabolic properties and whole animal energetics

There was a positive relationship between glucose uptake rates from inner blubber explants *in vitro* and whole animal mass gain rate. This effect was apparent in addition to the well-established positive effect of maternal size on suckling mass gain rate (Mellish et al 1999; Pomeroy et al 1999). Mass gain rate was, in turn, a strong positive driver of weaning mass, as expected (Mellish et al 1999; Pomeroy et al 1999). Glucose uptake by blubber thus appears to represent an important component of

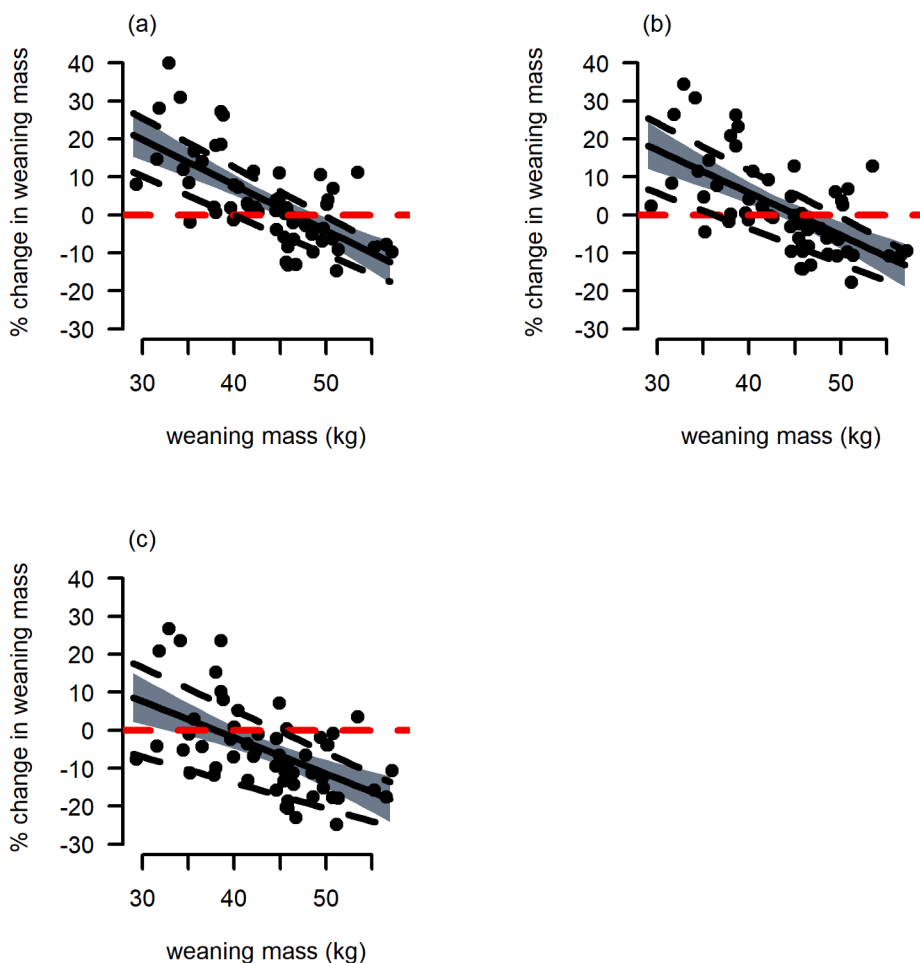


Fig. 4. Relationship between observed weaning mass for each animal, and the percentage change in their weaning mass predicted when a. the effect of DL-PCB on blubber glucose uptake was reduced; b. both the effect of DL-PCB on blubber glucose uptake and TT3 were reduced and c. the effect of DL-PCB on blubber glucose uptake and TT3 levels, and the PBDE effect on mass gain rate were all reduced. Black solid line and grey shading represent model prediction and 95% confidence intervals. Black dashed lines represent the prediction when using 95% confidence intervals combined for each scenario. Red line indicates where animals would experience no change in weaning mass. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

energy balance that is poorly understood in seals. The fate of the glucose taken up by blubber explants has not been determined. Insulin, which is positively related to mass gain in suckling pups (Bennett et al 2015), may stimulate blubber glucose and fatty acid uptake, and synthesis of adipose glycerol-3-phosphate and triglyceride in seals as it does in other mammals, including humans (Ballard et al 1967; Dimitriadis et al 2011). Blubber glucose uptake may thus have an even larger contribution to energy balance *in vivo* than suggested here from measurements taken under basal conditions. While *in vitro* lipolytic rate was not related to whole animal metrics measured here, it may be more important during mass loss stages of life history.

Observed annual differences in blubber metabolic properties are unlikely to be a result of differences in methodology to measure glucose uptake and glycerol production because assay performance was similar between methods. Instead, they appear to be real biological differences reflected in annual variation in mass at capture and mass gain rate that were not accounted for by maternal characteristics, lactation duration or birth mass. Other stressors may influence mass gain through altered blubber function. For example, annual variation in local weather patterns and climatic conditions may have physiological consequences for a whole cohort, but were not measured here. Effects of blubber glucose uptake on mass gain rate need to be explored in other age groups and species to determine the extent of the predictive power of *in vitro* measurements for whole animal energetics. High rates of blubber glucose uptake in pups leading to rapid mass gain in suckling pups may make this age class particularly vulnerable to stressors that impact glucose metabolism, such as DL-PCBs (Robinson et al 2018). Increased knowledge of blubber glucose uptake regulation and its disruption will help to predict how natural and anthropogenic stressors affect mass gain rate and body condition in marine mammals.

#### 4.2. Relationship between TH and whole animal energetics

TT3, but not TT4, levels were associated with lower weaning mass. The negative effect of TT3 on weaning mass is consistent with studies that show stimulatory effects of TT3 on energetic costs and lipolytic rates rodents and humans (Mullur et al 2014; Oppenheimer et al 1991). The constraining effect of TT3 on weaning mass may limit fuel available to pups to sustain them until they start to feed, which has a greater impact on smaller pups. Whole animal studies in rodents show TH can increase both lipolysis and lipogenesis, but the net effect is to increase fat loss (Oppenheimer et al 1991). How TT3 exerted the weaning mass-reducing effect here remains unknown but better understanding of this relationship will improve the ability to predict the impacts of stressors that target TH.

#### 4.3. DL-PCB effects on weaning mass through blubber function and thyroid disruption

We have previously shown that DL-PCBs reduce glucose uptake in inner blubber (Robinson et al., 2019). Using these estimates of their influence on blubber function, we predicted that DL-PCBs alter mass gain rate. The size of the estimated impact depends on the assumed magnitude of the DL-PCB effect. The DL-PCB-induced reduction in mass gain rate was predicted to lower weaning mass in about two-thirds of pups. However, most animals were predicted to experience only a 3.6–1.59% reduction in weaning mass. The effect of current POP contaminant levels on blubber function in UK grey seals may therefore have only a small effect on body condition for most animals. However, the impact of reducing POPs on blubber glucose uptake was highly variable and in extreme cases predicted changes in mass gain represented up to ~ 40% of weaning mass. The mechanisms through which POPs and other contaminants can influence blubber glucose uptake, and the impacts on survival of POP-induced changes to blubber function thus need to be investigated. Effects of POPs and other contaminants on glucose uptake by blubber and other tissues also need to be explored in

age groups and species in which concentrations are higher than seen here.

TT3 was reduced by 20% across the range of DL-PCB values observed in this study, which are well below the toxic thresholds identified for marine mammals (Kannan et al 2000). When TT3 levels were increased by removing the predicted DL-PCB suppression, weaning mass was predicted to be lower, by up to 6.4%, in 86% of the animals. The impacts of DL-PCBs on weaning mass through both blubber function (Robinson et al 2018) and TT3 thus offset each other. However, DL-PCB had a greater effect on weaning mass through their effect on blubber glucose uptake than their impact on TT3. Pups may be able to offset reductions in TT3 levels by altering TH sensitivity, feedback and metabolism, and changing behaviours such as suckling intensity, frequency and activity. Effects of TH disruption on energy balance in more contaminated populations and species may be more severe and likely to continue due to slow POP elimination from top predators (Robinson et al 2019). POP-suppressed TH levels are likely to alter processes other than those measured here, such as immune, skeletal or neural development, which may have greater detrimental consequences (Gouveia et al 2018; Koibuchi & Chin 2000; Montesinos & Pellizas 2019).

#### 4.4. Association between PBDEs and mass gain rate

Blubber ΣPBDE concentration was associated with faster mass gain rate. A reduction in PBDEs was thus predicted to reduce weaning mass by  $3.18 \pm 0.82$  kg ( $6.02 \pm 1.86\%$  (sem) %). The effects of PBDEs on mass gain rate completely offset the effect of DL-PCBs on weaning mass. The greater mass gain in pups with higher PBDE content could reflect a correlation as a result of bioaccumulation in fat tissue. However, if this were the case, more abundant POP classes, such as PCBs, would be more likely to show a positive relationship with mass gain rate. Conversely, rapid mass gain rate can also result in a dilution effect as the fat tissue expands more rapidly than the rate of accumulation of POPs, despite an overall higher POP burden (Hall et al 2008; Peterson et al 2014; 2015), producing a negative association between the two. Here we saw no relationship in late suckling between PBDE levels (or other POP classes) and body mass, or between PBDEs and weaning mass, suggesting the negative association with mass gain is not simply a result of their dilution. PBDEs could impact on energetics by disrupting mechanisms we have not measured here, but through which they have been shown to act as ‘obesogens’, such as adipogenesis, lipogenesis, secretion and/ or sensitivity of other hormones, mitochondrial biogenesis or function, or induction of inflammatory pathways that alter adipose function (Elmore & La Merrill 2019; Jackson et al 2017; Regnier & Sargis 2014; Routti et al 2016). Although fatness is a strong driver of first year survival in grey seal pups (Hall et al 2001; 2002), an ‘obesogenic’ impact of PBDEs is not likely to be beneficial. Indeed, there is a negative relationship between PBDEs and survival probability in this age group (Hall et al 2009). If PBDEs promote fat accumulation they could be detrimental by impairing essential lipid mobilisation during fasting. Establishing whether the relationship between fat accumulation and PBDEs is causal, and identifying the mechanisms involved is essential to estimate their true impact.

The predicted impact of POPs on weaning mass varied dramatically depending on the assumed routes through which they disrupted energy balance. Fat accumulation may thus partially depend on the cocktail of POPs and other contaminants present.

#### 4.5. Size dependence of POP effects

POPs effects on weaning mass are generally small for most animals in this population, and other external drivers may have greater influence over their energy balance during suckling. However, in all cases, the pups that were smallest for other reasons, such as small birth mass, lactation duration or maternal mass, experienced the greatest negative effects of POP exposure and were predicted to be heavier at weaning

when POPs were reduced. It is possible that POPs disrupt fat accumulation differently depending on nutrient availability and the hormonal milieu of the animal. Pups of 46–47 kg and lower were predicted to be heavier, on average, if DL-PCBs were reduced, which represents pups up to and more than average weaning mass in this study. If DL-PCB and PBDEs were both lower, this threshold mass was  $\sim 38$  kg. Although we have no survivorship data for the animals in this study, previous studies suggest a 9 to 18% increase in weaning mass could make a dramatic difference to survival of the smallest pups, particularly males (Hall et al 2001; 2009). A 10% reduction in the largest animals may not be proportionately detrimental because the relationship between survival and mass is not linear.

#### 4.6. Model performance and predictions

The models presented have relatively low support, suggesting that factors not measured here contribute to variation in the data. We may also have overestimated POP effects by taking the precautionary approach of retaining them where they are included in the top ranked model, even when other competing models that do not include POPs have a similar weight and by assuming the DL-PCB-induced reduction of blubber glucose uptake observed in 2016 is a fixed value. Our data present only a selected number of the array of POPs and other contaminants present in blubber, such that unmeasured contaminants may contribute to or better explain the trends we have reported. Blubber POPs are also only a proxy for whole animal exposure. Circulating contaminant levels and those in other tissues may have more direct effects on the mechanisms measured here or on other processes that influence energetics. In addition, we assumed each contaminant acts independently on each mechanism, but it is likely that POPs have complex interacting effects whereby they may be synergistic, act as competitive inhibitors of each other or of natural ligands (eg Lühmann et al 2020), or produce small additive effects that are not selected in the models. Such complexity cannot be captured in the approach taken here. Removal of individual contaminant classes could not happen in reality and our predictions therefore simplify the complex nature of multiple chemicals acting on multiple pathways, many of which have not been measured here. For example, if PBDEs also disrupt blubber glucose uptake and TT3, but their effects are much smaller than those of PCBs and therefore have not been incorporated, removing their impact only on mass gain does not capture the full extent of their influence on weaning mass. The predictions generated from our models therefore have a high degree of uncertainty. However, the predictions capture the dominant effects of the POPs we have measured on each process we have considered. Since mass at late suckling was part of our selection criteria for inclusion, our data underrepresent the smaller animals that we predict are likely to be most impacted. Our approach nevertheless attempts to address the need for greater linkages between *in vitro* and *in vivo* studies to assess POP effects in wildlife. Our results indicate the magnitude of effects of contaminants on whole energy balance and show that such effects are measurable, even when POP levels are lower than accepted toxic thresholds, representing an important step forward in understanding the extent to which metabolic disruption has organism level energetic consequences.

## 5. Conclusion

Our findings suggest a possible link between POPs and whole-animal energy balance impacts in grey seal pups. This represents an additional mechanism through which POPs may exert their previously identified impacts on survival probability in this species (Hall et al 2009). We showed that glucose uptake rates in inner blubber tissue are strongly related to mass gain rates, which drive weaning mass. The mechanistic underpinning of the relationship between blubber glucose uptake rate and fat gain needs further investigation because first year survival in grey seal pups increases with weaning mass (Hall et al 2002; 2009).

POPs altered mass gain rates and weaning mass, at least partially through impacts on blubber function and TH. Effects of POPs on TH and blubber glucose uptake rate have opposing, relatively minor, effects on whole animal energy balance and may largely offset each other in most pups, but were predicted to reduce weaning mass in pups that are already small for other reasons, which compounds the limited survival probability of low weaning mass (Hall et al 2002). Although PBDEs were predicted to offset the effects of DL-PCBs, mechanistic information on how they promote fat deposition and how this may influence survival is needed. The impacts of POPs seen here occur despite their relatively low concentrations in North Sea grey seals compared to other regions and species (Robinson et al 2019). Population level effects of POPs could thus be most apparent in years when food is scarce, when mothers will be leaner, have a higher POP burden, and thus raise smaller pups to which they will transfer more POPs. These small pups would have more limited reserves to sustain them to their first meal and may be less able to gain fat, potentially exacerbated by higher circulating POPs (Debier et al 2006). For animals, populations or species with higher POPs (eg killer whales and other delphinids (Desforges et al 2018; Jepson et al 2016) and polar bears (Routti et al 2016), the energetic consequences may be more pronounced than those seen here. Quantification of POP effects on other mechanisms that impact energy balance, and greater certainty at each level of modelling will improve reliability of the predictions we have generated. Nevertheless, these data demonstrate *in vitro* measurements can partially predict organismal mass change trajectories, and thus provide an important contribution to the assessment and quantification of impacts of contaminants and other stressors on whole animal energetics.

#### CRediT authorship contribution statement

**Kimberley A. Bennett:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Validation, Writing - original draft, Visualization, Supervision, Project administration, Funding acquisition. **Kelly J. Robinson:** Methodology, Investigation, Data curation, Project administration. **Holly C. Armstrong:** Methodology, Investigation, Data curation. **Simon E.W. Moss:** Methodology, Investigation, Validation. **Georges Scholl:** Methodology, Investigation, Data curation, Validation. **Alexandra Tranganida:** Methodology, Investigation. **Gauthier Eppe:** Conceptualization, Methodology, Investigation, Validation, Supervision, Funding acquisition. **Jean-Pierre Thomé:** Methodology, Investigation, Validation, Supervision. **Cathy Debier:** Conceptualization, Validation, Funding acquisition. **Ailsa J. Hall:** Conceptualization, Methodology, Supervision, Project administration, Funding acquisition.

#### Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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## Appendix A. Supplementary data

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