Blood kinetics of lipophilic and proteinophilic pollutants during two types of long-term fast in king penguins

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Margaux Mollier[1], Paco Bustamante[1], Ignacio Martinez-Alvarez[2], Quentin Schull[3], Pierre Labadie[2], Hélène Budzinski[2], Yves Cherel[4], Alice Carravieri*[1,4]

* Corresponding author: alice.carravieri@gmail.com

[1] Littoral Environnement et Sociétés (LIENSs), UMR 7266 CNRS-La Rochelle Université, 2 rue Olympe de Gouges, 17000 La Rochelle, France

[2] CNRS, UMR 5805 EPOC (LPTC Research Group), Université de Bordeaux, 351 Cours de la Libération, F-33405 Talence Cedex, France

[3] MARBEC, Université de Montpellier, IFREMER, IRD, CRNS, Avenue Jean Monnet CS 30171 34203 Sète, France

[4] Centre d'Etudes Biologiques de Chizé (CEBC), UMR 7372 CNRS-La Rochelle Université, 405 Route de Prissé la Charrière, 79360 Villiers-en-Bois, France

Abstract: In vertebrates, fasting is an intricate physiological process associated with strong metabolic changes, yet its effect on pollutant residues variation is poorly understood. Here, we quantified long-term changes in plasma concentrations of 20 organochlorine and 16 perfluoroalkyl pollutants in king penguins *Aptenodytes patagonicus* during the breeding and molting fasts, which are marked by low and high levels of protein catabolism, respectively, and by strong lipid use. The profile of measured pollutants in plasma was dominated by perfluorooctanesulfonic acid (PFOS, initial relative contribution of 60%). Initial total pollutant concentrations were similar in molting (3.3-5.7 ng g^{-1} ww) and breeding penguins (range 4.2-7.3 ng g^{-1} wet weight, ww). Long-term fasting (25 days) for molting and breeding led, respectively, to a 1.8- and 2.2-fold increase in total plasma pollutant concentrations, though the rate and direction of change was compound-specific. Hexachlorbenzene (HCB) and PFOS concentrations increased in plasma (net mobilization) during both types of fast, likely due to lipid use. Plasma perfluoroundecanoate (PFUnDA) and perfluorotridecanoate (PFTrDA) concentrations increased in breeders (net mobilization), but decreased in molting individuals (net excretion), suggesting a significant incorporation of these pollutants into feathers. This study is a key contribution to our understanding of pollutant variation in blood during long-term fasting in wildlife.

Keywords: Subantarctic, breeding, molt, PFAS, plasma, POPs, seabird, weight loss

Synopsis: Fasting king penguins showed net excretion of some perfluoroalkyl carboxylic acids from blood (plasma) during molt, suggesting potential deposition into feathers.

1. Introduction

Most persistent organochlorine compounds (OCs) and some perfluoroalkyl substances (PFAS) (*i.e.*, perfluorooctanesulfonic acid, PFOS, perfluorooctanoic acid, PFOA, perfluorohexanesulfonic acid, PFHxS, and related compounds) are regulated by the Stockholm Convention,[1] because they are persistent, mobile, biomagnifying and toxic,[2,3] and are thus key targets of ecotoxicological monitoring worldwide. Marine food webs are particularly threatened by OCs and PFAS,[2,3] which can be found in very high concentrations in marine predators (*e.g.*, ref 4,5). Seabirds have been used effectively to monitor marine contaminants for decades.[4,6,7] Diet is the main exposure route to contaminants in seabirds and feeding ecology is a key extrinsic factor driving variation in their concentrations (*e.g.*, ref 8–10). Variation due to intrinsic traits (*e.g.*, age class, sex) has also received considerable attention (*e.g.*, ref 11–13), while the importance of other physiological variables, such as energy use and metabolic changes, is far less understood.[6,14–16]

Fasting is a common physiological process in the life-cycle of seabirds, with most species fasting during breeding, incubation, migration or molting.[17,18] OCs are lipophilic compounds that are preferentially stored in adipose tissues and mobilized in association with lipid metabolism, as shown in marine mammals and humans.[5,19,20] In seabirds, relationships between body condition (*i.e.*, fat stores) and circulating OC concentrations are often attributed to lipid use (*e.g.*, ref 21,22). However, only three studies, all on the same species, have quantified this directly in fasting individuals, showing a strong increase in plasma OC concentrations in female common eiders *Somateria mollissima* during egg incubation.[23–25] Unlike OCs, PFAS are amphiphilic molecules that have high affinity for proteins, such as plasma albumin, organic anion transporters and fatty acid binding proteins,[26,27] and tend to accumulate in protein-rich compartments such as plasma, liver and kidneys.[28–30] PFAS concentrations have been related to body condition in humans,[31,32] wild mammals[33,34] and

seabirds,[35] with mixed results. Yet, to the best of our knowledge, there has been no prior attempt to measure longitudinal changes in PFAS concentrations during fasting in wildlife.

Molting is another key physiological process in birds, necessary to maintain the quality and function of feathers (flight, waterproofing, thermoregulation, ornament, camouflage, *e.g.*, ref 36). Molting is a well-known driver of changes in blood concentrations of mercury, a nonessential metal that is excreted into feathers while they are bound to the bloodstream during their growth (*e.g.*, ref 37,38). The plumage can also contain OCs[39,40] and PFAS[41–43] as a result of incorporation during feather growth, and/or of preen oil and atmospheric deposition onto the feather surface.[39,40,44] Quantifying the changes in OCs and PFAS in blood during molt could thus shed new light on their incorporation into feathers. However, no studies have measured molt-related changes in blood concentrations of OCs or PFAS in seabirds, because most species renew their plumage at sea, where they are not accessible.[36] Studying the changes in blood OC and PFAS concentrations throughout fasting and molting is essential to 1) quantify contaminant variation due to intrinsic rather than environmental influences, and thus enhance the use of seabird tissues as bioindicators, and 2) improve our understanding of toxicity risks during fasting.

King penguins *Aptenodytes patagonicus* are exceptional study organisms to evaluate pollutant toxicokinetics during long-term fasting and molt. King penguins' life cycle encompasses two periods of prolonged fasting (approximately 4-5 weeks) on land every year: one for renewing their entire plumage ("molting fast"), and one during courtship ("breeding fast") a few weeks later.[45–48] Both fasting periods are preceded by hyperphagia at sea on similar marine prey (myctophid fish)[49,50] to build up large nutrient stores.[51] King penguins therefore experience a natural alternation of periods of obesity and weight loss (up to 58% of their initial body mass),[46] marked by strong physiological changes. The molting fast is associated with a high metabolic rate due to feather synthesis and the decrease in thermal insulation.[47,48] Unlike

the breeding fast, the molting fast involves a large mobilization of proteins as well as lipids, because amino acids are required for the synthesis of feather keratin.[46,47,52] The breeding fast is characterized by protein sparing and lipid mobilization only to sustain energy requirements. Given the protracted reproductive cycle of king penguins (~one year) and individual variation in breeding onset, molting and courtship individuals are present simultaneously on breeding colonies. This offers an ideal opportunity to study blood toxicokinetics of OCs and PFAS during two types of fast involving the mobilization of different macromolecules within the same temporal and environmental conditions.

The aim of this study was to quantify and compare the change of blood OC and PFAS concentrations throughout 25 days of fasting in molting and breeding wild king penguins from the Crozet Islands, southern Indian Ocean. We tested whether changes were due to pollutant mobilization, pollutant excretion, or body mass loss. We expected: (1) similar initial pollutant concentrations in molting and breeding individuals, because of similar diet in the two groups; (2) a net mobilization of OCs to the blood (plasma) during both types of fast, because lipids are the main energy source; (3) a higher mobilization of PFAS to the blood (plasma) during the molting than the breeding fast, due to protein breakdown for feather synthesis.

2. Material and methods

Study site and blood sampling

This study was conducted in November-December 2014 on molting and breeding king penguins from the Baie du Marin colony, Possession Island (46°25'S, 51°45'E), Crozet Archipelago. Molt takes place in the austral spring after a period of 2-3 weeks of hyperphagia at sea. After molting, birds return to the sea for another period of hyperphagia (2-3 weeks), before returning on land for courtship and breeding. The egg-laying period extends from November to February on the Crozet Islands.[53] King penguins initiating their molt can be easily

identified. A first group of molting individuals (N=12, four males, eight females) was selected based on the wear and tear of the plumage and their visibly high body mass. A second group of breeding male penguins (N=12) was selected based on their courtship song and their renewed plumage.[54,55] Penguins were captured at the periphery of the colony, upon their arrival from the ocean. Each group was housed in open wooden pens of 3x4 m within 10 meters of the colony. Consequently, the birds were exposed to natural climatic conditions and the ambient sounds of the colony. Birds were individually marked using spray animal dye (Porcimark®) and a flipper band (semi-rigid P.V.C Darvic bands; 25.8 mm wide, 1.9 mm thick, 7.4 g). Birds of both groups were kept captive for 25 days, during which they were regularly weighted and blood sampled (5 ml at days (D) 0, 3, 6, 10, 15, 20, and 25). Body mass change followed closely the known pattern of fasting in king penguins (Section S1 and Fig. S1 in the Supporting Information, SI). Blood samples were centrifuged to separate blood cells and plasma within two hours of sampling, and thereafter kept at -20°C until laboratory analyses. At the end of the experiment, birds were released where captured.

OC and PFAS quantification

OCs and PFAS were measured in plasma at the laboratory Environnements et Paléoenvironnements Océaniques et Continentaux, Physico- et Toxico-Chimie de l'environnement (EPOC-LPTC), Bordeaux, France, given their preferential association with plasma lipids and proteins, respectively.[27,56] Targeted OCs included seven indicator polychlorinated biphenyls and 13 organochlorine pesticides, and were quantified using gas chromatography coupled with electron capture detection (GC-ECD)[57]. Targeted PFAS included seven perfluoroalkyl carboxylic acids (PFCAs), four perfluoralkane sulfonamides and five sulfonates (PFSAs). PFAS analysis was carried out by on-line solid phase extraction coupled to high performance liquid chromatography negative electrospray ionization tandem mass

spectrometry.[58] Further details about targeted pollutants, sample preparation, analysis, and quality assurance and quality control are available in the SI (Section S2, Table S2, S3, S4, S5).

Data analysis

Data treatment, figure preparation and statistical analyses were carried out using R Version 4.0.4.[59] Significance was set at $\alpha = 0.05$ for all tests. Pollutants were included in statistical analyses if at least 70% of the individuals of a group ("molting" or "breeding") had concentrations above the limit of quantification (LQ) throughout the fast (Table S4). For these pollutants, any value below the limit of detection (LD) was replaced by a randomly-selected value (*runif* function, R environment) in the range between zero and the LD. Similarly, any value below the LQ was replaced by a randomly-selected value between the LD and LQ. Substitutions concerned 4,4'-DDE, FOSA, PFNA, and PFTrDA, while HCB, L-PFOS and PFUnDA were quantified in 100% of individuals throughout both types of fast (Table S4). Differences of pollutant concentrations between molting and breeding individuals were tested at D0 and D25 through Mann-Whitney tests. The latter approach was also used to test differences in plasma pollutant concentrations between males and females at D0 and D25 in the molting group (Table S6). Given weak sexual differences, the small sample size, and the lack of females in the breeding group, the effect of sex was not included in the following steps of the statistical analysis. In seabirds, sexual differences in contamination are often the consequence of sexual differences in diet or feeding areas.[10,60] Sexual differences in contamination can also result from contaminant excretion into the egg(s), but that effect is usually weak in long-lived species that lay a single egg over ≥12 months.[12]

Pollutant concentration changes throughout fasting were tested in two steps.

First, we applied a mixed model analysis of variance on paired data (*rstatix* package), with individual identity as a random factor, to check for significant differences in plasma

concentration between days, for each type of fast. The normality of model residuals was checked through QQ plots and Shapiro-Wilk tests, while the homogeneity of variances with plots of model residuals *versus* fitted values, and Levene tests. Post-hoc Tukey multiple comparison tests (Tukey honestly significant difference, HSD) were used to know which days were significantly different from each other.

Second, we tested whether observed changes in pollutant concentrations throughout each type of fast were due to a net mobilization and accumulation in blood, a net excretion from blood, or mass loss alone. To this end, we modeled the *predicted concentration* of a compound that each individual would have if the quantity of the compound circulating in blood was constant throughout the fast-related decline of body mass. The predicted concentration on day t (C_t) was calculated with the following equation: $C_t = \frac{C_0 * M_0}{M_t}$, where C_0 is the plasma pollutant concentration at D0, M_0 is the body mass of the individual at D0, and M_t is the body mass of the individual on day t. This calculation assumes that the ratio between blood mass and body mass remains constant while fasting, as shown by unchanged hematocrit throughout fasting phase II in king penguins.[45,46] Predicted and observed concentrations were compared each day through paired-sample t-tests, for each type of fast, after checking for normality (QQ plot and Shapiro-wilk test) and homoscedasticity (Levene test). We interpreted observed changes in plasma concentrations throughout fasting as a result of (i) mass loss alone, if there was no difference between predicted and observed concentrations; (ii) net mobilization from internal tissues into the blood and subsequent accumulation there, if the observed concentrations were higher than the predicted ones; and (iii) net excretion from blood (towards other tissues or excrements), if the observed concentrations were lower than the predicted ones. Similar predicted and observed concentrations (interpreted as mass loss dependency) could also arise from equal amounts of pollutants being mobilised into, and excreted from the bloodstream.

3. Results

3.1. OC and PFAS concentrations in plasma of king penguins

Among the 36 targeted OCs and PFAS, 26 were detected in king penguins' plasma (Fig. S2, Table S2, S3, S4). Among OCs, only hexachlorbenzene (HCB) and 4,4' dichlorodiphenyldichloroethylene (DDE) concentrations were included in statistical analyses because they had a quantification frequency above 70% in both fasting groups. Among PFAS, linear PFOS (L-PFOS), perfluorooctane sulfonamide (FOSA), perfluorononanoate (PFNA), perfluoroundecanoate (PFUnDA), perfluorotridecanoate (PFTrDA) had a quantification frequency above 70% in both fasting groups, and were the only PFAS included in statistical analyses. Branched PFOS (Br-PFOS), perfluorodecasulfonate (PFDS), perfluorooctane sulfonamidoacetic acids, (FOSAA, MeFOSAA and EtFOSAA) and PFOA were not detected. Perfluoroheptasulfonate (PFHpS) and perfluorohexanesulfonic acid (PFHxS) had high quantification frequency (≥80%) only on D25 in both fasting groups. Perfluorodecanoate (PFDA) had high quantification frequency during the breeding (90%), but not the molting fast (≤50%). Perfluorododecanoate (PFDoDA) and perfluorotetradecanoate (PFTeDA) quantification frequency decreased during the molting fast and increased during the breeding fast (up to 80% and 50%, respectively, Fig. S2, Table S3).

The profile of measured pollutants in plasma was dominated by PFAS (relative contributions: molting individuals: 90.6% and 87.8%, at D0 and D25, respectively; breeding individuals, 87.3% and 85.6%, at D0 and D25, respectively; Table S4). L-PFOS was the dominant pollutant throughout both types of fast (median concentrations; molting: 2.64 and 5.96 ng g^{-1} ww, at D0 and D25, respectively; breeding: 3.5 and 7.5 ng g^{-1} ww at D0 and D25, respectively; Fig. 1, Table S2, S7). HCB contributed more than 4,4'-DDE to the total pollutant burden in both groups (median concentrations; molting: 0.32 and 0.73 ng g^{-1} ww at D0 and D25 respectively; breeding, 0.50 and 1.16 ng g^{-1} ww at D0 and D25, respectively; Fig. 1, Table S2,

S7). At D0, PFUnDA and PFTrDA had large relative contributions to the pollutant burden in both groups (up to 9.8% and 20.5%, respectively, Fig. 1, Table S7), yet they decreased by a factor of two to five in molting birds at D25 (Table S7).

At D0, molting and breeding individuals had similar total pollutant concentrations (Wilcoxon test, $W = 2048$, $p = 0.48$, Fig. 1, Table S2). In contrast, total pollutant concentrations were 1.5 times higher in breeding than molting individuals at D25 ($W = 1640$, $p = 0.01$, Fig. 1, Table S2). No significant differences were observed between the two groups at D0 and D25 for HCB and 4,4'-DDE concentrations (W ranged 25–33, all $p > 0.05$). Conversely, PFTrDA concentrations at D0 were higher in molting than breeding individuals ($W = 80$, $p = 0.003$), while the opposite was true for L-PFOS concentrations ($W = 11$, $p = 0.004$). At D25, L-PFOS concentrations were similar in breeding and molting individuals ($W = 29$, $p = 0.21$), while PFUnDA and PFTrDA concentrations were significantly higher in breeding penguins (both $W = 1$, $p < 0.001$). Within the molting group, males and females had similar organohalogen compounds concentrations at both D0 and D25, with two exceptions: males had lower plasma PFTrDA concentrations at D0 and higher plasma 4,4'-DDE concentrations at D25 than females (Table S6).

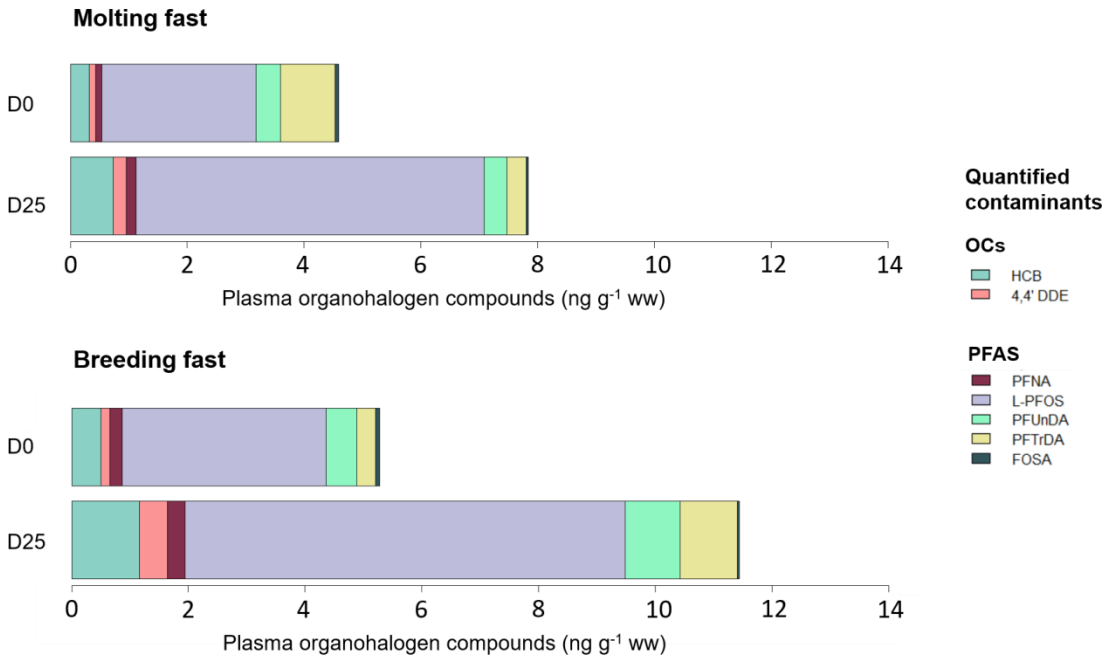

Figure 1. Stacked bar plot of organohalogen compounds (OCs and PFAS) in plasma at D0 and D25 of the molting (N=9) and breeding fasts (N=10) in king penguins from the Crozet Islands. Values correspond to median concentrations.

3.2. Plasma OC concentrations during the breeding and molting fasts – observed data

Observed HCB concentrations increased significantly throughout both the molting (F_{ANOVA} = 3.75, p = 0.0039) and breeding fasts (F_{ANOVA} = 5.61, $p < 0.001$) (Fig. S3AB). HCB concentrations were significantly higher at D25 than D0 and D3, for both groups (Tukey HSD; all $p < 0.05$). HCB concentrations were also significantly higher at D6 than D0 (p = 0.049) in breeding individuals. 4,4'-DDE concentrations changed significantly throughout the molting (F_{ANOVA} = 4.78, $p < 0.001$) and breeding fasts (F_{ANOVA} = 6.73, $p \leq 0.0001$), with concentrations higher at D25 than all other days (all $p < 0.05$) except D20 for the molting, and D6 for the breeding fasts (Fig. S3CD).

3.3. Plasma OC concentrations during the molting and breeding fasts – predicted data

During molt, observed HCB concentrations were significantly higher than predicted ones at D6, D10 and D20 (t ranged 2.53–2.95, all $p < 0.05$), while they were similar the other days (Fig. 2A). During the breeding fast, observed HCB concentrations were higher than predicted ones from D6 to D25, with significant differences at D6 (t = 7.18, $p < 0.001$), D15 (t = 3.80, $p < 0.01$), and D25 (t = 3.51, $p < 0.01$) (Fig. 2B). During molt, observed and predicted 4,4'-DDE concentrations were similar throughout the fast, except at D15 when observed concentrations were significantly lower than predicted ones (t = -3.03, $p < 0.05$, Fig. 2C). Conversely, in breeders, observed 4,4'-DDE concentrations were significantly higher than predicted ones on D25 (t = 4.15, $p < 0.01$, Fig. 2D).

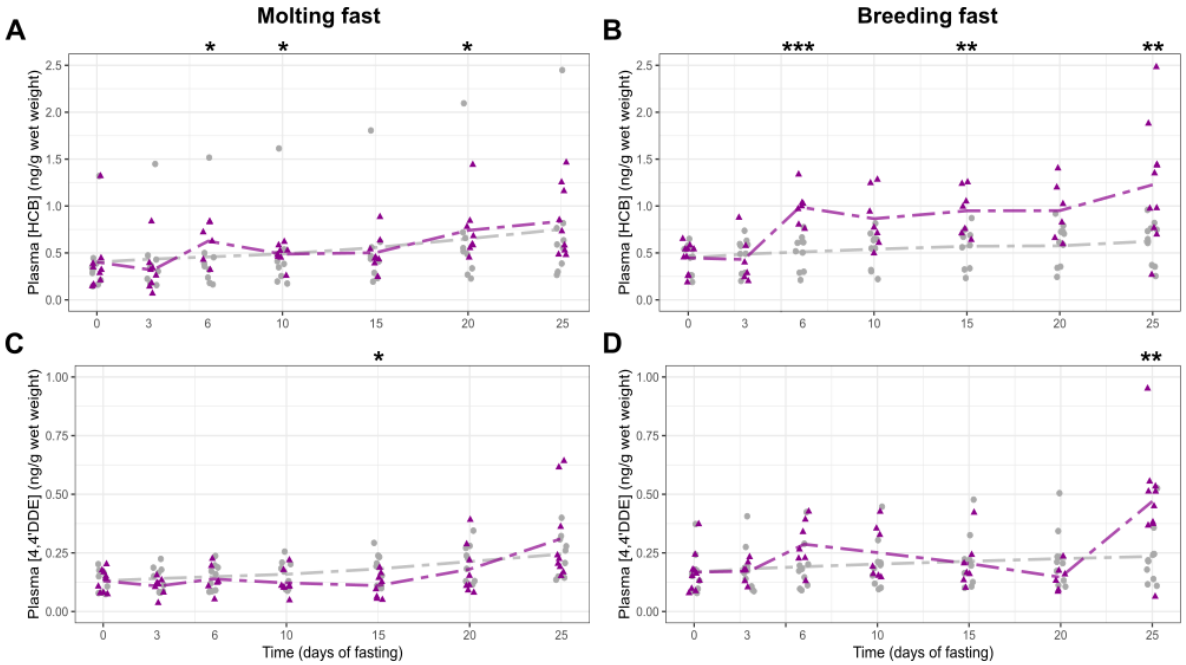

Figure 2. Predicted (grey) and observed (purple) concentrations of HCB (A, B) and 4,4'-DDE (C, D) in plasma of king penguins form the Crozet Islands throughout the molting (left) and breeding fasts (right). Significant differences between the daily mean of predicted and observed values are represented by stars (t-tests, * p<0.05; ** p<0.01; *** p<0.001).

3.4. Plasma PFAS concentrations during the molting and breeding fasts – observed data

Observed L-PFOS concentrations increased throughout both the molting (F_{ANOVA} = 36.48, p < 0.001; D0 = D10 < D25, p < 0.01) and the breeding fasts (F_{ANOVA} = 30.08, p < 0.001; D0 < D10 < D25, Tukey HSD, p < 0.01) (Fig. S4AF). FOSA concentrations tended to decrease during both types of fast, but not significantly so (breeding, F_{ANOVA} = 3.09, p = 0.064; molting, F_{ANOVA} = 1.87, p = 0.177, Fig. S4BG). PFNA concentrations increased at the end of the molting fast (F_{ANOVA} = 5.60, p = 0.012; D0 = D10 < D25, p < 0.05 Fig. S4C) and throughout the breeding fast (F_{ANOVA} = 4.25, p < 0.001, D0 ≤ D10 ≤ D25, p < 0.001, Fig. S4H). No significant changes were observed for plasma PFUnDA concentrations for molting individuals (F_{ANOVA} = 0.501, p = 0.612, Fig. S4D), while they increased significantly at D25 of the breeding fast (F_{ANOVA} = 16.2, p < 0.001, D0 = D10 < D25, all p < 0.001, Fig. S4I). PFTrDA concentrations changed significantly during both types of fast, but in opposite directions; molting individuals had lower concentrations at the end of the fast (F_{ANOVA} = 11.49, p < 0.001; D0 = D10 > D25, all p < 0.01;

Fig. S4E), while breeding individuals had higher concentrations (F_{ANOVA} = 11.11, $p < 0.001$; D0 = D10 < D25, all $p < 0.01$; Fig. S4J).

3.5. Plasma PFAS concentrations during the molting and breeding fasts – predicted data

Observed L-PFOS concentrations were higher than predicted ones at D25 of the molting fast (t = 4.25, $p < 0.01$; Fig. 3A) and at D10 and D25 of the breeding fast (t = 5.34 and 4.14, both $p < 0.01$; Fig. 3F). Conversely, observed FOSA concentrations were significantly lower than predicted ones at D25 during both types of fast (t = -8.78 and -6.16, both $p < 0.001$; Fig. 3BG). Observed PFNA concentrations were slightly, but significantly lower than predicted ones at D10 of the molting fast (t = -2.45, $p < 0.05$, Fig. 3C), while they were similar to predicted ones throughout the breeding fast (t ranged between -1.88–1.66, $p > 0.05$, Fig. 3H). Observed PFUnDA concentrations were significantly lower than predicted ones throughout the molting fast (D10, t = -4.35, $p < 0.01$; D25, t = -6.64, $p < 0.001$) (Fig. 3D), while they were higher than predicted ones at D25 of the breeding fast (t = 3.98, $p < 0.01$, Fig. 3I). Similar results were observed for PFTrDA, with significantly lower observed than predicted concentrations at the end of the molting fast (t = -6.07, $p < 0.001$, Fig. 3E) and significantly higher observed than predicted concentrations at the end of the breeding fast (t = 3.12, $p < 0.01$, Fig. 3J).

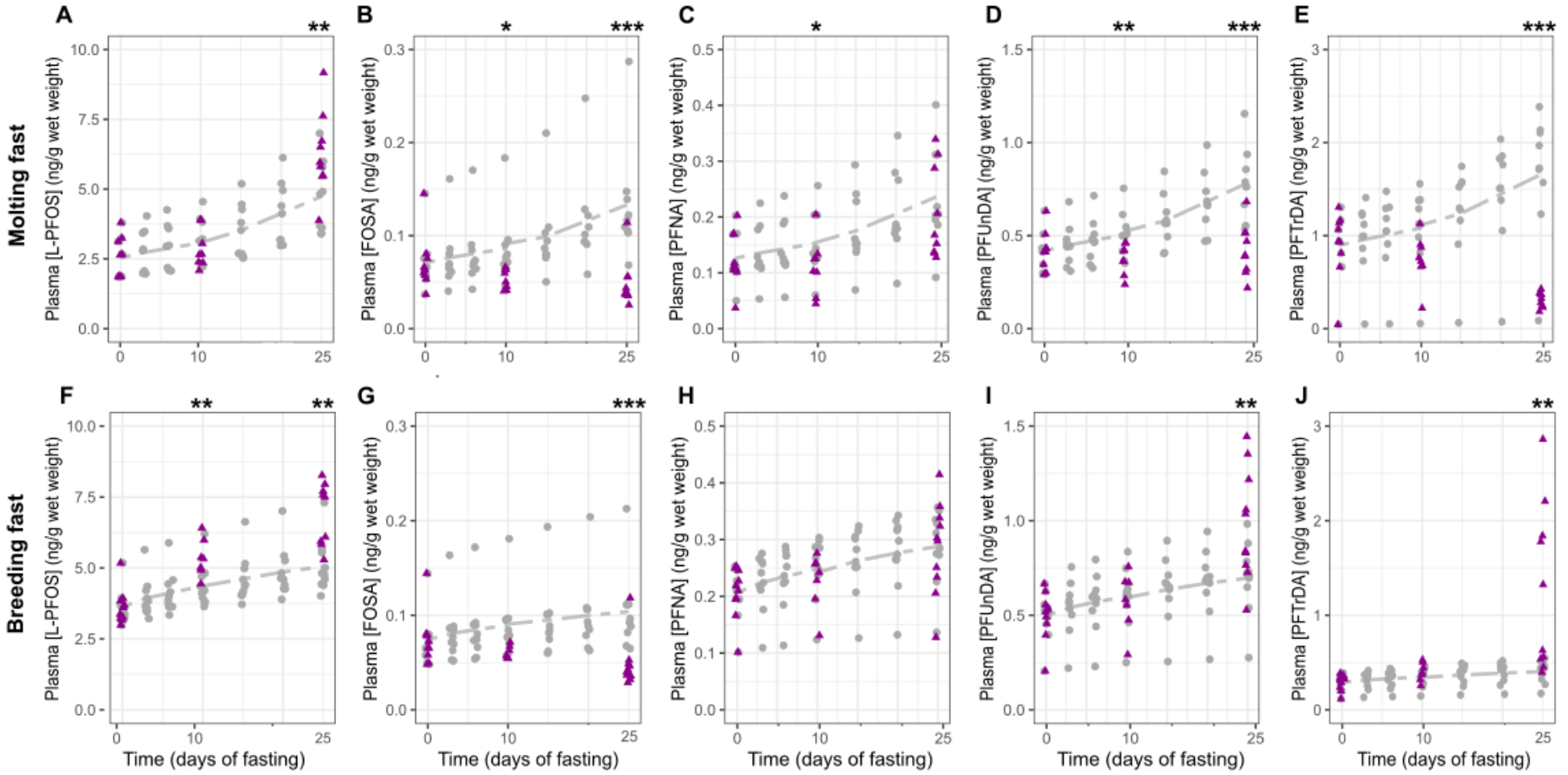

Figure 3. Predicted (grey) and observed (purple) concentration of L-PFOS, FOSA, and three PFCAs (PFNA, PFUnDA, and PFTrDA) in plasma of king penguins from the Crozet Islands during the molting (A, B, C, D, E) and breeding fasts (F, G, H, I, J). Significant differences between the daily mean of predicted and observed values are represented by stars (t-tests, * $p<0.05$; ** $p<0.01$; *** $p<0.001$).

4. Discussion

This is the first study to quantify longitudinal changes in blood PFAS concentrations during fasting in wildlife, and the first report of plasma PFAS contamination in king penguins (Munoz *et al.*[58] erroneously refer to king penguins, while samples were collected on Adélie penguins *Pygoscelis adeliae*; YC personal communication). Long-term fasting for molting and breeding led to a moderate increase in total plasma OC and PFAS concentrations. Yet, concentrations changed in different directions and rates depending on compound and type of fast. The two fasting groups differed mainly in changes of plasma PFAS concentration, suggesting significant transfer of long-chain PFCAs from blood to feathers during molt. This needs direct confirmation from feather PFCA quantification, but is consistent with previous studies documenting large proportions of long-chain PFCAs in feathers.[41–43,61]

4.1. Plasma concentrations and profile of OCs and PFAS in king penguins

The initial total OC and PFAS concentrations were overall comparable in breeding and molting individuals, and between sexes in the molting group, likely following similar dietary exposure[49,50] (confirmed by comparable stable isotopic values in the two groups and sexes, data not shown). Blood OC concentrations can reflect exposure over a few days to several weeks, depending on compound,[62–64] while PFAS have long blood half-lives[65] (230 days in chicken for L-PFOS).[66] Initial PFUnDA and PFTrDA concentrations were different between groups, possibly because of blood bioaccumulation along the year in breeding individuals, and excretion into feathers in molting individuals (see Section 4.3.). A (potentially weak) sex effect cannot be excluded, since molting males and females had different plasma PFTrDA concentrations. When compared to other seabirds, king penguins had relatively low OC and relatively high PFAS concentrations given their ecology and feeding grounds (see Section S3 for detailed comparisons with the literature). Interestingly, PFAS contributed more than OCs to

the total pollutant burden of king penguins' plasma, contrary to results in other Southern Hemisphere seabirds, where PFAS contamination is still low, particularly at high latitudes.[67–70] The PFAS profile was consistent with most other studies in seabird plasma, showing a strong contribution of L-PFOS, and of odd-numbered long-chain PFCAs (*e.g.*, ref 8,35,71). King penguins remain within the limits of the Southern Ocean year-round, where they feed almost exclusively on myctophid fish.[72] Our results thus suggest that myctophid fish might be largely contaminated by PFAS in the Southern Ocean, as recently shown in the northeast Atlantic Ocean.[73] This urges direct investigation, given the pivotal role of myctophid fish in Southern Ocean food webs.[49]

4.2. Changes in plasma OC concentrations during the molting and breeding fasts

Changes in plasma OC concentrations in king penguins were overall consistent with the hypothesis that prolonged periods of mass loss result in the mobilization of OCs from fat tissues to plasma where they accumulate, as previously shown in seabirds,[16,23,24] marine mammals,[5,19,74] and humans (*e.g.*, ref 20). Both HCB and 4,4'-DDE concentrations increased more during the breeding (factor of 2.7 and 2.9 between D0 and D25, respectively) than the molting fast (factor of 2.1 and 2.4, respectively). The weaker increase in plasma OCs concentrations during the molting fast could also be linked to partial excretion into feathers (*e.g.*, ref 39). Net mobilization was clear for HCB throughout the breeding fast. In contrast, prolonged lipid metabolism seemed to be necessary for a net mobilization of the more hydrophobe 4,4'-DDE (log K_{ow}, octanol-water partition coefficient, of 6.5 *vs* 5.8 for HCB),[75] which was clear only at D25 of the breeding fast. Increasing observed 4,4'-DDE concentrations early during the breeding fast and throughout the molting fast were likely mass-dependent, *i.e.*, (i) they were the result of the concentration of the same quantity of 4,4'-DDE in a smaller volume of blood, or (ii) equal amounts of 4,4'-DDE were mobilized into, and excreted from

blood. The quicker mobilization of HCB than 4,4'-DDE could stem from a larger HCB burden in fat tissues and/or to differences in physico-chemical properties between the two OCs. HCB is a smaller, less lipophilic molecule than 4,4'-DDE,[62] which could be easily released into the circulation from the early stages of lipid metabolism. Conversely, more lipophilic compounds are less efficiently mobilized from fat tissues, and are strongly concentrated within them during fasting, as shown in marine mammals.[19,74] Contrary to our findings, previous results from incubating common eiders showed a stronger mobilization of 4,4'-DDE than HCB into the circulation.[23–25] Moreover, the rate of change of plasma 4,4'-DDE residues was larger in common eiders than in breeding king penguins (8.2- *vs* 2.9-fold increase, respectively), despite similar fasting duration (~20 days) and similar initial plasma 4,4'-DDE concentrations. The strong increase in plasma 4,4'-DDE residues was attributed to large 4,4'-DDE burdens in common eiders' fat tissues.[23,24] Hence, the different fast-related increase of 4,4'-DDE between common eiders and king penguins points to a small 4,4'-DDE burden in king penguins' fat tissues. Conversely, plasma HCB residues showed a stronger increase in king penguins than common eiders (2.7- *vs* 1.7-fold, respectively; this study and ref 23,24), suggesting that a larger HCB burden was present in king penguins (HCB was previously quantified in Antarctica penguins' fat).[76] Here, king penguins were released towards the end of fasting phase II, when fat stores are still available.[45,46] There could be a further release of 4,4'-DDE and other OCs with high log K_{ow} (*e.g.*, other DDT metabolites, highly chlorinated PCBs) into the bloodstream at a later stage of fasting.

4.3. Changes in plasma PFAS concentrations during the molting and breeding fasts

Changes in PFAS concentrations were partially consistent with the hypothesis of stronger release during the molting than the breeding fast, although clear differences were observed between compounds. Similarly to OCs, plasma L-PFOS concentrations increased

during both types of fast, leading to significant net mobilization and accumulation into the bloodstream. Yet, contrary to OCs, the rate of increase of L-PFOS was stronger during the molting (2.5-fold increase between D0 and D25) than the breeding fast (2-fold increase). This suggests that L-PFOS was released into the circulation in association with both lipid and protein metabolism, and that potential incorporation into feathers was weak (but see below). The marked increase of plasma L-PFOS concentrations could have been exacerbated by the biotransformation of FOSA into PFOS,[33,77] which is supported by the significant decrease in observed plasma FOSA concentrations in both fasting groups. Kinetics of the other targeted precursors of PFOS, namely MeFOSAA, EtFOSAA and FOSAA, could also have helped in interpretation, but their concentrations were all below detection. Alternatively, FOSA and L-PFOS concentrations changes were not related, and the decrease in FOSA residues derived from excretion mechanisms to other tissues, including blood cells. The distribution behaviour of FOSA among tissues is known to be unique among PFAS (*e.g.*, higher affinity for blood cells than for plasma in humans),[78] which complicates the interpretation of results. Another challenging pattern to disentangle was the one of PFNA. Previous studies in seabirds indicate a lipid-dependent behaviour of PFNA.[29,35] Here, plasma PFNA concentrations showed a mass-dependent change in both fasting groups, which suggests that PFNA mobilization into, and excretion from blood were weak and/or balanced in king penguins.

A central finding of this study was that plasma concentrations of PFCAs with a chain longer than nine carbons, in particular PFUnDA (C_{11}) and PFTrDA (C_{13}), changed in opposite directions depending on the type of fast: they showed a net excretion during the molting fast and a net mobilization during the breeding fast. The rate of change was particularly strong for PFTrDA, with a three-fold decrease in observed concentrations during molt and a four-fold increase during the breeding fast. PFDoDA (C_{12}) and PFTeDA (C_{14}) showed a similar pattern for their quantification frequency. PFDA (C_{10}) showed a similar trend that could not be

quantified precisely due to low quantification frequency during molt. Hence, we hypothesise, and discuss hereafter, that long-chain C_{10}-C_{14} PFCAs (i) were mobilized mainly in association with lipid metabolism, and (ii) were incorporated into feathers during molt.

The increase in plasma C_{10}-C_{14} PFCA concentrations along the breeding fast could be associated with lipid mobilization from adipose tissues, similarly to L-PFOS. This agrees with previous results showing high plasma PFSA and C_8-C_{10} PFCA concentrations in wild mammals in poor body condition (cross-sectional studies: fasting *vs* feeding female polar bears *Ursus maritinus*;[34] lean *vs* fat Arctic foxes *Vulpes lagopus*[33]). The net excretion observed for C_{10}-C_{14} PFCA in molting, but not breeding individuals could stem from several nonexclusive factors. Long-chain PFCAs have strong bioaccumulative potential, because their structure favors biliary enterohepatic recirculation, and are more hydrophobic than short-chain PFCAs, which can be more easily eliminated through urine.[79,80] Urine production could be exacerbated in molting individuals, which lose two times more water than breeding individuals during fasting.[47] However, renal tubular reabsorption of PFAS into the blood has been shown in humans and laboratory mammals, and is a key driver of the long blood half-life of PFAS.[79,81] Hence, urinary excretion is unlikely to be the main driver of the difference in C_{10}-C_{14} PFCA concentration changes in molting and breeding individuals. In addition to urinary excretion, other potential elimination routes for PFCAs (and other PFAS) include transfer to growing feathers,[41,43,82] preen-oil (mainly PFOS[82]), and the egg(s),[30,83] while biotransformation is thought to be negligible.[3,80] Here, egg transfer can be excluded, because all breeding individuals were males, and molt takes place before the onset of breeding in this species.[47,48] The chemical composition of preen oil can vary with breeding status, among other factors,[84,85] and could thus be different between molting and breeding individuals. This could drive differences in PFAS transfer to preen oil in the two fasting groups. However, and with the exception of PFOS, PFAS transfer to preen oil is thought to be weak.[82,86] While the total amount of synthesized preen oil could

also differ between the two fasting groups, this would likely be negligible when compared to the difference in the total amount of synthesised feathers (approx. 400 g in molting *vs* 0 g in breeding king penguins).[47] Therefore, feather incorporation appears to be the most likely route explaining the excretion of C_{10}-C_{14} PFCAs from blood in molting, but not breeding king penguins. This is in agreement with avian studies showing that long-chain PFCA concentrations are correlated between plasma and feathers, unlike other shorter-chain PFAS.[41–43,61] Here, incorporation into feathers was substantial enough to affect plasma residues of C_{10}-C_{14} PFCAs of molting individuals, possibly because of the large feather mass synthesized at once.[47,87] Interestingly, PFTrDA concentrations in breeding individuals at D0 were significantly lower than those of molting individuals at D0, but similar to those of molting individuals at D25 (similar trend observed for PFUnDA). Breeding individuals had molted ~one month before sampling.[47,48] Conversely, molting individuals had been accumulating pollutants since the previous molt (~one year before). These results suggest that PFTrDA, and possibily PFUnDA, accumulated in blood along the annual cycle, before being excreted into feathers during molt. Repeated PFAS quantification in the same individuals during two successive reproductive cycles should confirm this.

Unlike PFCAs, L-PFOS pattern of change during the molting fast did not indicate excretion into feathers, despite PFOS concentrations being usually high in feathers and correlated to those in blood (*e.g.*, ref 41). Feather excretion might be significant but not sufficient to decrease plasma L-PFOS concentrations, likely due to larger burdens and stronger lipid-driven mobilization of L-PFOS when compared to PFCAs. Previous studies have shown that long-chain PFCAs are preferentially transferred to the eggs in seabirds,[83] and from maternal blood to the placenta in humans,[88] unlike other PFAS including L-PFOS. This has been hypothesised to stem from selective binding of long-chain PFCAs to low density lipoproteins involved in egg- and placenta transfers.[28,30,88] We thus hypothesise that C_{10}-C_{14} PFCAs can also

bind to proteins involved in feather synthesis, and/or directly to keratins, to a larger extent than other PFAS including L-PFOS.

To sum up and conclude, repeated measures of OCs and PFAS in king penguins indicated (i) net mobilization and accumulation in plasma of HCB and L-PFOS in both fasting groups, and of 4,4'-DDE, PFUnDA and PFTrDA in breeding individuals only; (ii) mass-dependent increase in plasma PFNA concentrations in both fasting groups; (iii) net excretion from plasma of FOSA in both fasting groups, and of PFUnDA and PFTrDA in molting individuals only. FOSA concentration changes could also arise from biotransformation into PFOS. OC toxicokinetics were consistent with previous studies, while we showed for the first time strong excretion potential of long-chain PFCAs into feathers. To confirm this, it is warranted to quantify these compounds in king penguins' feathers, preen oil and excrements. A larger sample size could better elucidate the longitudinal change of OC and PFAS concentrations during fasting, notably in relation with body mass loss variation between individuals, as well as potential sexual differences. Profiling of plasma proteins and lipids could also help us disentangle whether plasma pollutant variation is linked to fasting-related changes in levels of specific macromolecules (*e.g.*, low density lipoproteins, albumin, phospholipids, fatty acids, triglycerides). Increases in some compounds throughout fasting were related to mass loss alone, which calls for caution in the interpretation of monitoring data in seabirds of unknown physiological status. The mobilization and accumulation in plasma of L-PFOS and long-chain PFCAs during fasting is worrying, since these highly toxic compounds can be preferentially transferred to sensitive tissues such as eggs and the brain.[29,83] This calls urgently for further studies on deleterious effects of these compounds on physiology and fitness during periods of prolonged fasting in seabirds, particularly when not associated with molting.

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Supporting information

The supporting information includes details, figures and tables about king penguins' fasting phases and their body mass change; pollutant concentrations, quantification frequency, quality assurance and quality control; limits of detection and quantification; sexual differences in contamination in the molting group; and observed concentrations throughout both types of fast.

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