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Clinical Pathology of Plastic Ingestion in Marine Birds and Relationships with Blood Chemistry

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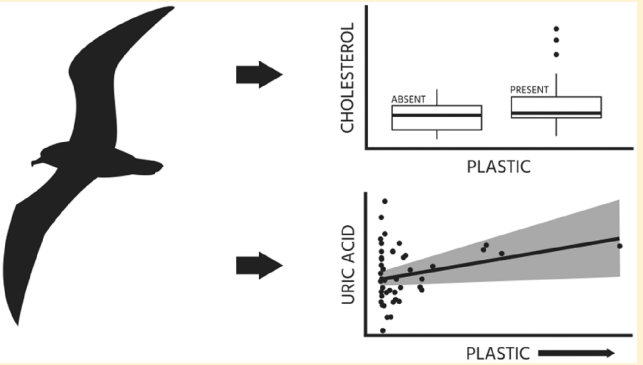
ABSTRACT: Pollution of the environment with plastic debris is a significant and rapidly expanding threat to biodiversity due to its abundance, durability, and persistence. Current knowledge of the negative effects of debris on wildlife is largely based on consequences that are readily observed, such as entanglement or starvation. Many interactions with debris, however, result in less visible and poorly documented sublethal effects, and as a consequence, the true impact of plastic is underestimated. We investigated the sublethal effects of ingested plastic in Flesh-footed Shearwaters (*Ardenna carneipes*) using blood chemistry parameters as a measure of bird health. The presence of plastic had a significant negative effect on bird morphometrics and blood calcium levels and a positive relationship with the concentration of uric acid, cholesterol, and amylase. That we found blood chemistry parameters being related to plastic pollution is one of the few examples to date of the sublethal effects of marine debris and highlights that superficially healthy individuals may still experience the negative consequences of ingesting plastic debris. Moving beyond crude measures, such as reduced body mass, to physiological parameters will provide much needed insight into the nuanced and less visible effects of plastic.



1. INTRODUCTION

Increasing demand for, and production of, plastic products coupled with inadequate waste management and policy contributes to the ongoing and rapidly expanding issue of pollution of our waterways and wildlife.[1,2] Mass production of small plastic items, such as pellets or "nurdles", combined with the breakup of larger items present in the marine environment for decades has led to an increase in the availability of bite-size pieces for wildlife[3,4] and a concomitant increase in the frequency of accidental consumption by marine animals in all of the world's oceans.[5]

The negative consequences resulting from interactions between wildlife and plastic debris are diverse, are often visually striking, and can include nutritional deprivation,[6] entanglement,[7,8] and damage to or obstruction of the gut (e.g., perforations and ulcers[9]). Many of these interactions also include less visible and therefore less well documented effects such as reduced growth and survival rates following ingestion,[6,10,11] and as a consequence, we are drastically underestimating the true impact of plastic waste on our oceans.

Plastic is inherently toxic[12] and becomes increasingly hazardous over time as it accumulates pollutants from the surrounding marine environment.[13,14] Once ingested, the absorbed toxins leach into the animal's bloodstream[6,11,15,16] and contribute to neurological, behavioral, and reproductive problems at all levels of biological organization and, in extreme cases, death of individuals.[11,17,18] At least 43−100% of the world's marine mammal, seabird, and turtle species are at risk from the ingestion of plastic,[19] and this number is expected to increase as plastic production and studies into its effects also rise.[20] How we manage our waste and understand the true scope and severity of impacts this has on wildlife therefore has broad implications for the health of marine ecosystems.

The Flesh-footed Shearwater (*Ardenna carneipes*) is one of the most heavily impacted marine vertebrates with regards to plastic pollution,[6] containing a mixture of macro-, micro-, and ultrafine debris.[21] Populations across the species' range are in decline[22−24] with the ingestion of plastic implicated in the downward trend due to its negative effect on chick growth and survival and its capacity to act as a vector for contaminants.[6,25] The mechanisms by which plastic negatively influences shearwater and other wildlife populations are poorly understood,[18] are likely present at the molecular or cellular levels of organization, and may not result in organisms' death, but in their poor health.[18,26] To better understand the often invisible and sublethal effects of ingested plastic waste, we examined the

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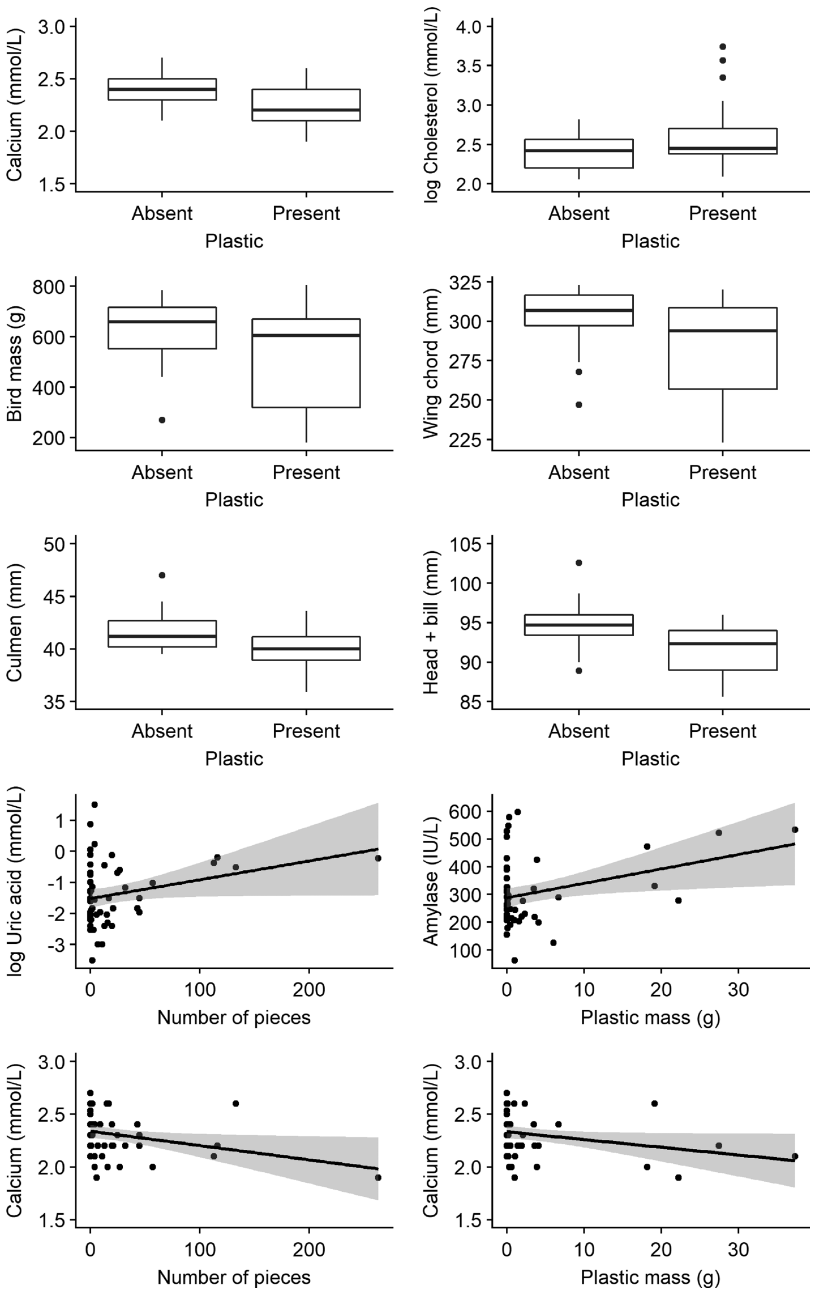

Figure 1. Blood chemistry parameters and measures of body condition that were significantly related to plastic ingestion in Flesh-footed Shearwaters (see also Table 2). In boxplots, solid lines are medians, boxes are the interquartile range (25th and 75th percentile), whiskers are the 5th and 95th percentile, and dots are final outliers. In other plots, solid lines are regressions with the model confidence interval shaded in gray.

clinical pathology of ingested plastic through blood chemistry and body condition in Flesh-footed Shearwaters.

2. MATERIALS AND METHODS

2.1. Ingested Plastic Sampling. Flesh-footed Shearwater fledglings (n = 53) were captured by hand at night on the colony surface on Lord Howe Island, New South Wales (31.554°S, 159.085°E; for more information see Lavers, Hutton, and Bond[24]) from 1 to 9 May 2014−2017 when the birds were approximately 90 days of age. Body mass (±10 g) was determined using a spring balance, wing chord (flattened; ±1 mm) using a stopped ruler, and culmen and head + bill length using Vernier callipers (±0.1 mm). Ingested plastic was collected by stomach flushing following procedures outlined by Duffy and Jackson[27] and detailed in Lavers et al.[28] In brief, seawater (approximately 150 mL) at ambient temperature was gently pumped into the proventriculus through a tube, thus displacing any ingested items. Once fluid and stomach contents began to flow back up the esophagus (i.e., once the stomach was filled completely), the bird was inverted over a container to collect anything expelled. Plastic items were dried and weighed to the nearest 0.001 g using an electronic balance.

2.2. Blood Sampling. Approximately 0.5 mL of whole blood was collected from the brachial vein of each bird using a 26-gauge needle and stored in lithium heparin vacutainer tubes (Grenier MiniCollect, Austria). A drop of blood was smeared onto a microscope slide immediately after collection and air-dried. Samples were collected between 20:00 and 21:30 Lord

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Table 1. Flesh-Footed Shearwater Fledgling Blood Components, Morphometrics, and Ingested Plastic on Lord Howe Island during 2014-2017

parameter	units	mean	SD	min	max	*n*	log-transformed
blood composition							
packed cell volume	L/L	0.45	0.50	0.31	0.56	33	no
white blood cells	10^9/L	5.41	4.82	0.20	21.5	41	yes
% heterophils	%	53	21	16	88	41	NA
heterophils	10^9/L	2.7	2.8	0.1	14.7	41	yes
% lymphocytes	%	39	22	6	82	41	NA
lymphocytes	10^9/L	2.2	2.9	0.1	13.1	41	yes
% monocytes	%	7	6	0	32	41	NA
monocytes	10^9/L	0.4	0.5	0.0	2.3	41	no
% eosinophils	%	1	2	0	8	41	NA
eosinophils	10^9/L	<0.1	<0.1	0.0	0.2	41	no
% basophils	%	1	2	0	10	41	NA
basophils	10^9/L	0.1	0.1	0.0	0.6	41	no
liver function							
aspartate aminotransferase	IU/L	439	854	78	6176	53	yes
glutamate dehydrogenase	IU/L	10	8	1	46	53	yes
bile acids, fasting	mmol/L	13	11	0	41	52	no
kidney function							
urea	mmol/L	1.7	1.6	0.1	11.1	53	yes
uric acid	mmol/L	0.4	0.7	0.0	4.5	53	yes
blood proteins							
total protein	g/L	33	10	15	64	49	yes
albumen	g/L	13	5	5	26	50	yes
globulin	g/L	20	6	10	43	49	yes
albumen:globulin	ratio	0.6	0.2	0.1	1.0	49	NA
other blood parameters							
calcium	mmol/L	2.3	0.2	1.9	2.7	50	no
creatine kinase	IU/L	4602	13374	521	94466	52	yes
cholesterol	mmol/L	12.0	7.0	3.7	42.1	53	yes
amylase	IU/L	305	121	63	596	53	no
glucose	mmol/L	14.0	4.8	1.2	34.5	53	yes
bird morphometrics							
bird mass	g	562	175	180	805	53	no
wing chord	mm	289	30	223	323	51	no
culmen	mm	40.7	2.1	35.9	47.0	51	no
head + bill	mm	92.6	3.8	84.6	102.6	51	no
ingested plastic							
plastic mass	g	3.1607	7.5600	0	37.3267	53	no
plastic count	items	21	45	0	263	53	NA

Howe Standard Time (LHST; UTC-10:30), refrigerated at 4 °C overnight, and then express posted to IDEXX Laboratory (Rydalmere, NSW, Australia) by airplane and courier the following afternoon (i.e., within 18 h of collection). At the laboratory, two slides were prepared from each sample using two separate techniques to evaluate hematological parameters. The first technique involved diluting and mixing 20 μL of whole blood with 620 μL of Eosin stain solution. The mixture was then used to fill two chambers of a hemocytometer for manual count of heterophils and eosinophils through a microscope. The second technique required a May-Grunswald/Giemsa stain to perform a differential stain, calculate a total white blood cell count, and assess morphology of the red blood cells, white blood cells, and platelets. The proportion of different types of leucocytes (lymphocytes, heterophils, monocytes, eosinophils, and basophils) was assessed based on an examination of 100 leucocytes under oil immersion (1000× magnification). Blood components (glucose (mmol/L)), urea (mmol/L), calcium (mmol/L), total protein (g/L), albumin (g/L), globulin (g/L), aspartate aminotransferase (AST; IU/L), creatine kinase (IU/L), cholesterol (mmol/L), amylase (IU/L), glutamate dehydrogenase (GLDH; IU/L), uric acid (mmol/L), and bile acids (umol/L)) were measured using an Olympus AU680 chemistry analyzer (Beckman-Coulter, Japan). A packed cell volume (PCV; hematocrit) was obtained by spinning whole blood in heparinised capillary pipettes in a microhematcrit centrifuge for 5 min. If the PCV was below the reference range for seabirds, a slide was stained with methylene blue and a reticulocyte count on the red blood cells was performed. All QA/QC and analytical procedures were undertaken by IDEXX Laboratories under ISO/IEC 17025 accreditation (No. 10166).

2.3. Statistical Methods. Data were tested for normality using Shapiro−Wilk test[29] and, where needed, log-transformed to improve normality. Parameters were grouped by physiological function: those associated with the liver (aspartate aminotransferase, glutamate dehydrogenase, bile acids), blood proteins (total protein, albumen, globulin), those with kidney/

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Table 2. Results from Linear Models Examining the Relationships between Plastic Ingestion and Clinical Pathology or Body Condition in Flesh-Footed Shearwaters[a]

	plastic presence			no. of pieces			plastic mass		
parameter	test statistic	p-value	$\beta \pm$ SE	test statistic	p-value	$\beta \pm$ SE	test statistic	p-value	$\beta \pm$ SE
blood composition									
packed cell volume	1.64	0.21		1.67	0.21		2.90	0.10	
white blood cell count	1.35	0.25		0.37	0.55		0.63	0.43	
WBC composition	0.88	0.47		0.89	0.52		0.96	0.91	
liver function (AST, GLDH, bile acids)	0.95	0.49		0.97	0.73		0.95	0.46	
kidney function (urea, uric acid)	0.99	0.91		0.87	0.05		0.90	0.07	
urea				0.20	0.66				
uric acid				**4.05**	**0.05**	**0.006 ± 0.003**			
blood proteins (albumen, globulin)	0.98	0.64		0.99	0.87		0.98	0.66	
other blood parameters									
calcium	**5.62**	**0.022**	**−0.133 ± 0.056**	**5.06**	**0.029**	**−0.001 ± 0.001**	**4.096**	**0.048**	**−0.007 ± 0.004**
creatine kinase	3.56	0.07		2.68	0.11		1.72	0.20	
cholesterol	**4.66**	**0.036**	**0.276 ± 0.128**	0.33	0.57		0.13	0.72	
amylase	0.25	0.62		1.68	0.20		**6.037**	**0.018**	**5.190 ± 2.112**
glucose	1.97	0.17		0.14	0.71		0.05	0.83	
bird morphometrics									
bird mass	**4.45**	**0.040**	**−101.061 ± 47.933**	0.03	0.86		0.28	0.60	
wing chord	**7.71**	**0.008**	**−22.131 ± 8.973**	0.08	0.79		0.30	0.59	
culmen	**10.22**	**0.002**	**−1.757 ± 0.550**	0.01	0.97		0.06	0.81	
head + bill	**13.28**	**<0.001**	**−3.528 ± 0.968**	0.03	0.96		0.01	0.92	

[a]Test statistics from multivariate tests are Wilk's λ, and those from univariate tests are F-statistics. Significant results ($p < 0.05$) include parameter estimates ($\beta \pm$ SE) and are in bold.

renal function (urea, uric acid), and white blood cell types (numbers of heterophils, lymphocytes, monocytes, eosinophils, and basophils). Other parameters (glucose, calcium, cholesterol, creatine kinase, amylase) and the number of white blood cell types were analyzed individually.

Relationships between measurements of body condition or blood parameters and plastic ingestion were explored using a series of univariate (reporting F-statistics) or multivariate linear models (reporting Wilk's λ, followed by univariate tests when results were significant). Analyses were done using R 3.5.1 (R Core Team 2018), differences were considered statistically significant when $p < 0.05$, and values are reported as mean ± SD.

3. RESULTS

We found no relationship between the presence, number, or mass of plastics and packed cell volume, white blood cell count, or white blood cell composition (Table 2). Similarly, there was no relationship between any measure of plastic burden and molecules associated with liver function (aspartate aminotransferase, glutamate dehydrogenase, or bile acids; Table 2).

The only significant relationship between plastic burden and measures of kidney function was a positive association between the number of plastic pieces and concentration of uric acid (Figure 1, Table 2). There were negative relationships between plastic presence, number, and mass with blood calcium, and positive relationships between plastic presence and cholesterol, as well as plastic mass and amylase concentration (Table 2).

The presence of plastic had significant negative relationships with all morphometrics, though there was no link between bird size and the number or mass of ingested plastic (Figure 1, Table 2).

4. DISCUSSION

There is scant information on blood composition for free-living birds, especially seabirds, many of which have been identified as threatened species. Flesh-footed Shearwaters have experienced considerable mortality through human actions (e.g., fisheries bycatch) which contributed to a reduction in population numbers in recent decades.[24,31] Our data therefore provide much needed blood chemistry reference values for Flesh-footed Shearwaters, a species that was recently up-listed to Near Threatened[30] and is declining across its range.[22−24] Blood chemistry data also provide valuable information on other threats that may affect the health of individuals, including plastic debris.

Blood cell volume (PCV) varied considerably among individual Flesh-footed Shearwaters and is known to be influenced by age or physiological status in other Procellariiforms.[32,33] The body mass recorded for some shearwater fledglings (180−270 g) was well below the range typically observed in this species (500−750 g),[34] suggesting these birds were in poor condition. This agrees with our visual observations, which indicated these individuals were emaciated and often lethargic. However, PCV values do not reliably reflect condition in some seabird species, even when birds are experimentally handicapped.[35,36] In light of this, and the variability in blood cell count parameters we observed, it is unsurprising that we failed to detect a relationship between PCV and plastic presence, mass, or number of ingested items (Table 2).

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Table 3. Blood Chemistry Parameters (Mean ± SD, Range in Parentheses) for Juvenile Seabirds from the Literature

parameter	units	*Pterodroma sandwichensis*	*Diomedea immutabilis*	*Oceanites oceanicus*	stranded *Ardenna pacifica*	healthy *Ardenna pacifica*
blood composition		(*n* = 56; 1994)	(*n* = 40; 1993)	(*n* = 17; 2000)	(*n* = 45; 1994)	(*n* = 69; 1994)
packed cell volume	L/L			0.39 ± 0.47 (0.3−0.4)		
white blood cells	10^9/L	18.1 ± 11.1 (5.2−65.1)	33.9 ± 10.4 (21.3−55.3)		14.3 ± 8.4 (3.0−54.4)	29.8 ± 13.9 (9.5−76.6)
heterophils	10^9/L	2.3 ± 1.3 (0.2−6.0)	6.7 ± 2.1 (3.8−9.9)		8.4 ± 5.4 (2.1−32.9)	2.5 ± 0.9 (0.9−5.0)
lymphocytes	10^9/L	13.9 ± 10.6 (1.3−58.6)	25.5 ± 9.8 (14.9−42.9)		5.4 ± 4.3 (0.4−21.2)	26.0 ± 13.5 (7.6−70.0)
monocytes	10^9/L	0.09 ± 0.16 (0.00−0.77)	0.00 ± 0.00 (0.00−0.00)		0.36 ± 0.61 (0.00−3.36)	0.2 ± 0.2 (0.0−0.77)
eosinophils	10^9/L	0.68 ± 0.58 (0.09−2.73)	1.24 ± 1.21 (0.00−4.49)		0.04 ± 0.09 (0.00−0.58)	0.74 ± 2.21 (0.00−14.81)
basophils	10^9/L	1.16 ± 0.68 (0.18−3.26)	0.77 ± 0.50 (0.13−1.74)		0.15 ± 0.14 (0−0.48)	0.34 ± 0.44 (0−2.68)
liver function						
aspartate aminotransferase	IU/L	106 ± 28 (71−169)	98 ± 21 (57−133)		366 ± 231 (169−1740)	153 ± 41 (95−274)
kidney function						
uric acid	mmol/L	2.2 ± 0.6 (1.2−3.5)	3.5 ± 1.7 (1.4−7.6)		7.3 ± 5.5 (1.4−24.7)	6.2 ± 4.3 (1.2−15.8)
blood proteins						
total protein	g/L	2.7 ± 0.9 (1.7−6.5)	3.9 ± 0.8 (2.7−5.0)	2.6 ± 0.1 (1.7−3.6)	3.2 ± 0.6 (2.1−4.4)	3.2 ± 0.4 (2.6−4.2)
albumen	g/L	1.3 ± 0.4 (1.0−3.3)	1.5 ± 0.3 (1.1−1.9)		1.5 ± 0.2 (1.1−2.0)	1.7 ± 0.2 (1.4−2.2)
globulin	g/L	1.3 ± 0.5 (0.7−3.2)	2.4 ± 0.5 (1.5−3.2)		1.6 ± 0.4 (1.0−2.4)	1.5 ± 0.2 (1.2−2.0)
other blood parameters						
calcium	mmol/L	10.1 ± 0.8 (8.5−12.2)	12.2 ± 0.7 (11.3−13.5)		11.0 ± 1.9 (5.1−14.0)	11.8 ± 1.2 (7.8−13.8)
greatine kinase	IU/L	114 ± 90 (23−344)	534 ± 266 (194−1146)		1091 ± 1142 (65−6000)	899 ± 899 (147−4473)
glucose	mmol/L	316 ± 35 (222−375)	136 ± 23 (83−175)		133 ± 44 (22−248)	202 ± 23 (145−255)
source		Work[33]	Work[33]	Quillfeldt et al.[67]	Work and Rameyer[68]	Work and Rameyer[68]

Changes in blood parameters have been evaluated in some seabirds well adapted to seasonal fasting (e.g., penguins[37]). Fasting in birds occurs in three phases with the final phase indicating the depletion of fat reserves, when body proteins are catabolized, resulting in a higher rate of body-mass loss, which is often indicative of a critical limit.[38] Low total protein (<25 g/L) is considered a good indicator of starvation in birds but can also signify chronic disease or stress.[39] In Flesh-footed Shearwaters, total protein was only slightly above this threshold (33 ± 10 g/L; Table 1) and was not related to plastic presence, mass, or number of ingested items (Table 2). However, uric acid, cholesterol, and amylase values all increased in response to the presence of ingested plastic (Figure 1, Table 2). Increased production of uric acid is commonly reported in birds during this final phase of fasting as it minimizes water loss while allowing nitrogen to be excreted.[37,40] However, high concentrations of uric acid in the blood can also indicate renal failure in birds and contribute to diseases such as kidney stones and diabetes.[41] Thus, increased uric acid associated with the ingestion of plastic is potentially problematic. Renal issues are also associated with increases in amylase which plays a key role in digestion in birds.[42] We detected a positive relationship between plastic presence and mass and amylase concentration in shearwaters. Similar patterns have been reported in tropical fish where exposure to microplastics significantly increased amylase concentrations.[43] While very little has been published on amylase in seabirds, this reinforces the possible role that ingested plastic may play in renal health.

Plasma cholesterol can increase in birds during periods of stress,[44] including during short fasting periods.[45] However, the concentrations we detected in shearwaters were elevated (12.0 ± 7.0 mmol/L; Table 1) compared with other juvenile seabirds experiencing significant food deprivation (7.5 ± 0.2 mmol/L[38]). This suggests that other factors, not just emaciation, may be influencing cholesterol concentrations in this species. The ingestion of plastic could play a key role, with cholesterol levels in Flesh-footed Shearwaters increasing in relation to plastic presence (Table 2). The relationship between ingested plastic and cholesterol is likely complex, as plastic items contribute to emaciation, subsequent stress levels, and exposure to chemicals.[6,46] In marine mammals, plasticizers (chemicals directly associated with plastic) can interfere with cholesterol levels and reproduction by altering transport through the mitochondrial membrane.[47]

Calcium levels in Flesh-footed Shearwater fledgling blood (2.3 ± 0.2 mmol/L; Table 1) were substantially lower than in other juvenile seabirds (10.1−12.2 mmol/L; Table 3) and the reference range for putative healthy, uncontaminated Northern Fulmars (*Fulmarus glacialis*; 6.1 mmol/L[48]). Many factors can influence calcium levels, including gastrointestinal diseases that contribute to malabsorption, hypoparathyroidism, and pancreatitis.[49] The cause of the low calcium in Flesh-footed Shearwaters is unclear,; however, exposure to petroleum-based chemicals has been shown to reduce plasma calcium levels in many waterbirds.[50,51] Seabirds with reduced body fat also exhibit lower blood calcium concentrations.[48] Reduced blood calcium was associated with the ingestion of plastic in Flesh-footed Shearwaters (Table 2), which is attributed to poor fat reserves and increased chemical exposure.[6]

In contrast to previous work,[6] we did not find significant relationships between the amount of plastic and bird

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morphometrics. Rather, simply the presence of plastic had a negative relationship with body mass, wing length, culmen, and head + bill length. Though our sample size is small (n = 38), it does span the range of severity of plastic ingestion.[6,52] This suggests that such effects are likely to be complex, influenced by interannual variation, and could imply a threshold over which additional plastic has little additional effect. Understanding this mechanism is fundamental to the question "how much plastic is bad", which is key for establishing relevant policy targets.

To date, studies of the effect of plastic debris on wildlife have been largely confined to quantifying plastic burden (e.g., Provencher et al.[53]) or relating that burden to gross measures of body condition, such as mass or chick size (e.g., Lavers, Bond, and Hutton[6]). Though these studies remain important, increasingly, the potential for sublethal impacts on birds' physiology is of concern,[54] including the presence of toxic chemicals,[6,15,55] harmful microbiota,[56,57] and the ubiquity of small particles which can be distributed throughout the digestive tract.[21,58] Such relationships have been recently described in marine invertebrates and fish,[59,60] but few data exist for seabirds. This is concerning as birds can compensate for physiological impairments caused by disease or other stressors[49] meaning they can appear healthy for a period of time, which can be misleading when undertaking visual assessments of health and condition or using superficial measures, such as body size.

While quantifying population-level impacts of plastic ingestion on wildlife remains an important goal,[18,61] this will continue to be challenging given the myriad threats faced by seabirds[62] and, as we have demonstrated, the often hidden sublethal effects plastic may be having on wildlife. In seabirds, which spend very little time on land or at breeding colonies in the course of their annual cycle, most mortality occurs at sea where the causes are often unknown and only manifest in the failure of an individual to return in subsequent years.[63] Crucially, mortality of juvenile prebreeding seabirds, which have deferred maturity and may not begin breeding until 5−10 years old, is poorly studied owing to the logistical challenge of measuring survival and determining the fates of individuals away from breeding sites.[64] Consequently, we cannot know at present how, or indeed whether, the physiological challenges faced by fledging birds, such as we measured, might affect demographic rates and the survival of individuals. However, the application of clinical pathology to wildlife conservation (e.g., Dagleish et al.[65]) has the potential to inform management and conservation action, including through the elucidation of the presence and magnitude of pressures on individuals. Moving beyond crude measures such as mortality or reduced body mass to physiological parameters will help us understand the nuanced effects of plastic ingestion and other cumulative stressors.[66]

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Notes

The authors declare no competing financial interest.

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