Effects of Plastic Ingestion on Blood Chemistry, Gene Expression and Body Condition in Wedge-Tailed Shearwaters (*Ardenna Pacifica*)

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ABSTRACT

Plastic pollution is a global threat and affects almost every marine ecosystem. The amount of plastic in the ocean has increased substantially over the past decade, posing a mounting threat to biodiversity. Seabirds, typically top predators in marine food chains, have been negatively affected by plastic pollution. Here we focused on documenting the sublethal effects of plastic in Wedge-tailed Shearwaters (*Ardenna pacifica*, WTSH) on the island of Maui, Hawai'i. Through analyses of blood chemistry, gene expression, morphometrics and stomach contents, we documented the effects of plastic ingestion on adult WTHS from 3 established colonies. We detected a negative relationship between body weight and the presence of plastic in regurgitated stomach contents. Genes associated with metabolic, biosynthetic pathways, inflammatory responses and ribosome function were upregulated in lighter birds. Birds that had ingested plastic tended to be lighter in weight, in comparison to birds that did not have plastic and tended to weight more. Furthermore, there were 43 genes differentiating males and females that did not have plastic compared to only 11 genes differentiating males and females that had ingested plastic. There was also a marginal negative relationship between lighter birds and blood urea nitrogen levels. We also hope that the morphometric measurements, blood parameters and gene expression data we collected contributes to a database that will be used for future studies on understanding anthropogenic effects on seabird body condition.

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INTRODUCTION

Plastics are the most common form of marine debris. It is estimated that in 2010, approximately 8 million metric tons of plastic entered the ocean (NOAA, 2022). This number has continued to grow exponentially over the past decade as the worldwide production has increased nearly 200-fold since the 1950s (Ritchie & Roser, 2018). The composition of plastic products leads to detrimental environmental consequences because, unlike plant products, they do not biodegrade quickly. Estimates of plastic decomposition range up from decades to several hundreds of years, causing accumulation of plastic in the natural environment (Barnes et al., 2009).

Plastics in the environment

Plastic has several properties that cause it to amass toxins on its surface through physical interactions such as physisorption and non-covalent bonds (Verla, 2019). Microplastics have a low-density and can be found on the surface microlayer of the ocean where they interact with heavy metals and organic chemicals. They also serve as good sorbents for heavy metals and organic chemicals due to a large surface area-to-volume ratio and hydrophobic surfaces. The substantial amount of toxins on the surface of microplastics can serve as vectors for transport of toxins to organisms (Koelmans et al., 2016).

One of the main pathways through which microplastics enter the environment is ingestion by organisms (Gallo et al., 2018). Approximately 43-100% of the world's marine mammals, seabirds, and turtle species are at risk of ingesting plastic (Lavers et al., 2019). Ingestion endangers these organisms to perforations, entanglements, and other non-visible effects. Many marine organisms ingest microplastics through filter- or deposit-feeding, mistaking them for prey when foraging or by consuming prey that have ingested microplastics (Gallo et al., 2018).

Studies suggest that ingested plastics may cause harm to an organism because of their ability to break down in the body and leach toxins into the bloodstream. There was marked bioaccumulation of the toxins in the digestive glands and gills of mussels and lugworms that were exposed to microplastics contaminated with toxins (polyaromatic hydrocarbons, nonylphenol and phenanthrene, Browne et al., 2013, Gallo et al., 2018). Research shows that exposure to these chemicals disrupt endogenous hormones, a process that can cause reproductive complications (Gallo et al., 2018). Toxins absorbed by plastic have also been linked to neurological or behavioral changes in organisms (Gallo et al., 2018). A study that aimed at assessing the effects of polyethylene microplastics in amphibians, exposed *Physalaemus cuvieri* to 60 mg/dL of polyethylenes for just 7 days and reported visible mutagenic (da Costa Araújo et al., 2020). Other effects included accumulation of polyethylene in the gills, gastrointestinal tract, gastrointestinal tract and in the blood as well as several external morphological changes (da Costa Araújo et al., 2020).

It is acknowledged that plastic pollution has more negative effects on seabird health when compared to other marine vertebrates (Thiel, 2018). Examples of negative effects of plastics on marine vertebrates include nutritional deprivation, reduced body mass, reduced appetite and damage or obstruction to the gut (Wang et al., 2021). In a study conducted in Lord Howe Island, Lavers *et al.* (2019) found that plastic ingestion had “significant negative effect(s) on bird morphometrics and blood calcium levels and a positive relationship with the concentration of uric acid, cholesterol, and amylase” (Lavers et al., 2019). Despite these findings, few experiments have further examined sublethal effects of plastic on physiology, gene expression and overall seabird health and living species. There is a limited understanding of the effects of microplastic exposure on gene expression, particularly in vertebrates. However, experiments

112 have shown possible effects of exposure on gene expression in Zebrafish (*Danio rerio*).
113 Disruptions in reproduction were shown in breeding groups of Zebrafish that were exposed to
114 environmentally significant concentrations of Bisphenol - A, a chemical used in plastic
115 production, for 15 days (Liang et al., 2016). More studies are needed on natural populations to
116 understand the mechanistic connections between plastic pollution and gene expression in the
117 affected organisms.
118 In this study, we focused on detecting possible effects of microplastic exposure on gene
119 expression, morphometrics, and blood analytics in three established colonies of Wedge-tailed
120 Shearwaters (*Ardenna pacifica*, WTSH, Fig.1A). WTSH, highly pelagic seabirds that range
121 across the tropical and subtropical areas of the Pacific and Indian Ocean, engage in feeding
122 behaviors such as contact dipping and surface-seizing (Adams et al., 2020). It is predicted that
123 WTSH and other seabirds with similar feeding behavior are susceptible to ingesting the floating
124 pieces of plastic on the surface of the ocean (Boersma & Groom, 1993). Plastic ingestion by
125 WTSH in Hawai□i has been documented; however, sub-lethal effects were not evaluated
126 (NOAA). To evaluate possible sub-lethal effects of plastic ingestion in WTSH, we collected
127 morphometric data and blood samples to measure the health of the seabird. Blood chemistry can
128 be used as an indicator for overall health and morphometric data are widely used in ecological
129 studies to determine body condition (Harr, 2002, Mallory et al., 2010, Labocha & Hayes, 2012).
130 While ornithologists debate over which morphometrics provide the best estimates for body
131 condition, literature suggests gathering multiple proxies to fully understand body condition in
132 seabirds (Mallory et al., 2010, Labocha & Hayes, 2012). Along with collecting several
133 morphometric measurements such as weight and blood chemistry, we used transcriptome
134 analysis to characterize activity of actively expressed genes under various conditions. More

specifically, transcriptome analysis provided a mechanism to compare snapshots of which genes were turned on or off in seabirds that had ingested plastic and those that did not have ingested plastic. Knowledge gaps and questions remain about the intrinsic aspects of plastic, severity of impact on human health and marine organisms, effective mitigation measures, and biomagnification across the food webs (Bonanno & Orlando-Bonaca, 2018, Galloway, 2020). Therefore, the aim of this project is to provide more information to this overarching question of how plastic debris affects marine organisms.

Materials and Methods

Study site

The collection of blood samples occurred at three different sites on the island of Maui, Hawai'i. These sites were chosen based on known established colonies of WTSH at these locations (Fig. 1B). Although all populations are located in protected areas, the three sites were on the seashore near frequently visited beaches. Kamaole Park III is located on the southern part of the island. Ho'okipa Beach located on the northern part of the island. Finally, Hawea Point is located on the western part of the island; this location also had the largest WTSH colony.

These populations of WTSH along with other seabird populations are vulnerable to microplastic exposure given that there are significant centers of plastic accumulation around the Hawaiian archipelago. Andrés Cózar et al. (2014) synthesized data to create a global map that approximates the magnitude of plastic pollution in the open ocean. Using the coordinates and data from Cózar et al. (2014), we mapped the magnitude of plastic pollution near the island of Maui (Fig. 1B). The concentration near Maui is sizeable compared to other sampled locations, with a non-adjusted concentration of 225,115 items per km^2. Despite our collection of samples from three different locations, we do not expect any significant genetic differentiation between

158 the populations. Whereas marked genetic differentiation has been found in WTSH in populations
159 breeding in different islands and archipelagos along the Pacific coast of North America, there is
160 no evidence for deep evolutionary divergences (Herman et al., 2022). The three WTSH colonies
161 were treated as one population when we analyzed the data.

162 *Gut Sample Testing*

163 We used the procedure outlined by Duffy et al. (1986) for gut sample testing to collect
164 possible ingested plastic. The procedure outlined by Duffy et al., involves a stomach pump
165 system where the seabird is filled with seawater through a gavage and then tipped over a bowl to
166 promote and collect regurgitation. We completed sampling of the gut contents for 28 out of the
167 29 birds.

168 *Blood Sampling*

169 We collected blood samples first to avoid a signal of stress response in the analyses of
170 gene expression and blood chemistry from being handled. Blood sample collection was possible
171 in 28 out of the 29 birds for gene expression analysis, and in 25 out of the 29 birds for chemical
172 analysis. This lack was due to not enough blood collection during sampling.

173 We collected approximately 200µl of blood using a syringe from the medial metatarsal
174 vein. We used styptic powder to stop bleeding when necessary. We added 100µl of blood to a
175 vial containing RNAlater buffer. We stored 20µl of blood in heparin tubes for iStat cartridge
176 analysis. The iStat Chem8+ provided us with the following blood analytics; sodium (Na
177 mmol/L), potassium (K mmol/L), chloride (Cl mmol/L), ionized calcium (iCa mmol/L), total
178 carbon dioxide (TCO2), glucose (Glu mg/dL), urea nitrogen/urea (BUN mg/dL), creatinine (Crea
179 mg/dL), hematocrit (Hct %PCU), hemoglobin (Hb g/dL), anion gap (AnGap mmol/L). We used
180 Qiagen QiAMP DNA Blood kit for DNA purification to proceed to the PCR reaction. We

used the universal method outlined by Fridolfsson and Ellegren (1999) for sexing in birds with PCR reaction. The 2- primer system is as follows:

2550F: 5'-GTTACTGATTCGTCTACGAGA-3’

2718R: 5'-ATTGAAATGATCCAGTGCTTG-3’

Using this primer system, we employed standard PCR on the templates of DNA extracted from unknown-sex *A. pacifica* species. The PCR mixture (15 µl) contained 1.5 µl of 10X buffet, 0.5 µl of dNTP (10 pmol), 0.5 µl of forward primer (10 pmol), 0.5 µl of reverse primer (10 pmol), 0.1 µl of NEB Ta1 (5U/uL), and 9.4 µl of H_2O. 2.5 µl of the DNA extraction was used. The PCR program was as follows: 94°C for 5 min, 94°C for 30 sec, 60°C for 30 sec *touchdown, –1.0°C/cycle x 10 cycles, 72°C for 30sec, 94°C for 30 sec, 50°C for 30 sec x 30 cycles, 72°C for 30 sec, 72°C for 5 min, 4°C hold. We used molecular graded H_2O as a negative control. A negative control was essential for possible misinterpretation due to contamination or other factors. We separated the PCR product through electrophoresis on a 2% agarose gel at 90 V for about 1 hour. We stained the gel with *GelRed*™ - a fluorescent nucleic acid gel stain that replaces the highly toxic ethidium bromide (EtBr) - and we used a gel imaging camera.

Morphometric Data

We completed collection of morphometric measurements for 28 out of 29 of the birds, using a Pesola scale. We measured tarsus length, bill length, nares depth and width using calipers and we measured wing chord length using a ruler. We banded each bird and recorded the number if the bird was a recapture.

RNA Isolation and Sequencing

Before initiating RNA isolation, we removed RNAlater through centrifuging aliquots at 20,800 x g (RCF). We removed supernatants from the remaining pellets containing

cell material. We used a Qiagen RNeasy Plus Universal Mini Kit for blood isolation. At the Harvard Bauer Sequencing Core Facility, we used KAPA mRNA Hyperprep kit and a NOVASeq SP platform to sequence paired end reads of 150 bp length, yielding between 20 and 30 million reads per sample.

Data analysis

We calculated several values to assess the quality of the RNA. We analyzed RNA integrity score (RIN) values, and calculated Phred Scores to assess the quality of our sequencing. RIN values assign a numerical value to the quality of the RNA that we worked with to evaluate the integrity of 18S and 28S rRNSs (Puchta et al., 2020). A RIN value of 8 and above indicated higher quality and integrity of RNA and values below 5 indicated some levels of RNA degradations. Phred scores are similar in that they assess the quality of sequences. Similar to RIN value, a high Phred score (90% and above) indicated better quality sequences (Scholz, 2021).

We aligned sequences to the publicly available reference genome of Cory's Shearwater (*Calonectris borealis,* accession # PRJNA545868, Feng et al., 2019*)* with the RNA sequence mapper STAR (Spliced Transcripts Alignment to a Reference) (Dobin et al. 2013). Followed by a transcript quantification with RSEM (Li & Dewey, 2011) and a differential gene expression analysis with DESeq2 (Love et al., 2014) in R programming. We created heatmaps to determine if there were observable patterns between gene expression and presence of plastic. Combined with clustering methods, heatmaps can help determine there are similar changes in gene expression based on their activity. In all of the tests, we used both a conservative p-value of 0.05 and a less conservative p-value of 0.1 to determine the significance of results. The summary of all tests we ran can be seen in supplementary table 1.

We conducted gene ontology analysis using R 3.5.1 (R Core Team 2018) and the package ggprofiler2 with *Gallus gallus*, *Taeniopygia guttata* and *Mus musculus* as the model systems in the search database. We used Ggplot2 and plotly for plotting as outlined in Kolberg et al. (2021). We separated terms into Gene Ontology, KEGG pathways and Reactome databases.

We ran multiple tests to analyze possible relationships between the variables of sex, presence of plastic and blood chemistry levels. We ran all analyses using R 3.5.1 (R Core Team 2018). We used a t-test to compare the means between blood analytes of birds that had ingested plastic and those that had no. All blood parameters were analyzed independently. Parameters were plotted against the presence of plastic. Differences were considered statistically significant when $p < 0.07$. We ran principal component analysis (PCA) to determine if the categories of plastic and sex where clustering together according to the morphometric, blood chemical and genetic data. We first ran PCA in relation to sex to control for possible differences in sex. We then ran PCA in relation to the presence of plastic to determine if individuals would cluster due to physiological differences caused by the presence of plastic. Packages used for these analyses included devtools, ggplot2 and ggbiplot. Finally, we used a general linear model (glm) to study the association between the variables, such as the morphometric measurements and blood parameters, and the conditions, which was either presence or absence of plastic. Differences were considered statistically significant when $p < 0.05$. and we considered effect size as well.

Results

Gut samples

Plastic or other unidentified hard pieces were found in 12 of the 28 birds sampled for plastic (Fig. 1C). These included fishing line and pieces of microplastics. A summary of which birds contained plastic, their sex and what blood samples were available for each bird can be

found in Fig. 2. Four out of the ten males contained plastic or other unidentified hard pieces. Seven out of 17 females contained plastic or other unidentified hard pieces. Bird N004 contained plastic or other unidentified hard pieces, but the sex of this bird was unknown due to insufficient blood sample to carry out analyses (Fig. 2).

Sex Determination through PCR

Our sample consisted of 10 males and 17 females. Bird N001 was a female, but gut samples were not collected for this bird (Fig. 2).

Blood Analytics, Morphometric and Gut Sampling

We found minimal relationships between the presence of plastic and the measurements listed above. Significant values from t-test analyses included levels TCO2 (Fig. 3e.) in the presence of plastic. There were no other significant values for other blood analytes in our t-tests and t-values ranged from .05789 to .87 (Fig. 3).

Our PCA results showed minimal relationships for both blood chemistry and morphometric measurements when the independent variable is set as either sex or presence of plastic (Fig. 4). The absence of clustering when using blood chemistry and morphometrics provides a control for sexual dimorphism in our samples. Wedge-tailed Shearwaters are not sexually dimorphic, which is consistent with our results.

Weight deviated from the other variables in the presence or absence of plastic under PCA 3 and 4 (Fig. 4b). There was a negative relationship between weight and presence of plastic when using a general linear model; birds that had ingested plastic tended to weigh less whereas birds that did not have ingested plastic tended to weigh more (Fig. 5a). When weight was the independent variable, urea nitrogen/urea, hematocrit and potassium demonstrated significant p-

values (Fig. 6). A summary of the averages, standard deviation and p-values from t-tests is found in Supplementary figure S2.

R NA Isolation and Sequencing

The RIN value used to assess RNA integrity during isolation was around 8.4 - 6 for most samples (Supplementary figure S9). Sample N005 had a lower RIN value of 4.8. Phred Scores were used to assess sequencing quality (Supplementary figure S4). On average, all samples reached > 30 Phred Score, which indicated good quality for downstream analyses. Supplementary figure S4 depicts reads per sample, which fell between 48,000,000 and 80,000,000 reads per sample.Uniquely mapped reads were all above 40%, with the highest being slightly above 60% (Supplementary figure S5). Low values could be due to the rate of degradation of blood RNA (Dobin & Gingeras, 2015) or because we are not using a species-specific reference genome for the transcriptome alignment. Multiple mapped reads, which read mapping to multiple locations in the genome, occurred at a rate of 1.3% to 2.7% (Supplementary figure S6, Pantano, 2018). We were able to distinguish four genes that separate the samples into two groups, discernible by upregulation and downregulation activity. Shades of blue represent downregulation activity while shades of red indicate upregulation of the gene (Fig. 7A).

There were no discernible differences in gene expression under the presence and absence of plastic. These results suggested that the clustering by presence of plastic does not explain the differential gene expression profiles (see Fig. 7A). We did not obtain significant results with either the conservative p-value or less conservative p-value.

We divided the analysis into sex; females in one analysis and males in the other with and without plastic as the variable (Supplementary figure S7). In this analysis we identified one gene that differentiated females with and without plastic when using the less conservative p-value.

Fourteen genes differentiated males with and without plastic under the more conservative p-value. The presence of plastic in males was associated with the upregulation of the top four genes. Eleven genes differentiated males and females that contained plastic (Supplementary figure S8A). Forty-three genes differentiated males and females that did not have plastic (Supplementary figure S8B).

We analyzed DE genes that examined weight with and without plastic. We divided weight into 3 factors: low, medium and high. Birds that did have ingested plastic, tended to be heavier and showed a downregulation in the expression of the top 18 differentiating genes (Figure 8A). Birds that had ingested plastic, tended to be lighter and showed an upregulation in the expression of the same genes. These distinctions were able to be made while using a conservative p-value for the top four genes and marginal p-value for the remaining genes. The genes responsible for differentiation when the samples were not separated into variables were associated with transcriptional activation, body fluid secretion and protein transportation activity (Fig. 7B). The top two genes responsible for weight differentiation, Ankrd11_1 and Hsph 1 (Supplementary figure S10A), were upregulated in heavier birds. Genes that were upregulated in heavier birds were associated with trimethylation and cell cycle function (Supplementary figure S10B). The top twelve genes that were upregulated in lighter birds (Fig. 8A) have been associated with several metabolic and biosynthetic processes, ribosome function and pathway response associated with COVID-19 (Supplementary figure S10).

DISCUSSION

Our overarching question was whether ingested plastics from the environment have a sublethal effect in seabirds, gut samples, morphometric measurements and blood samples from Wedge-tailed Shearwaters in Maui, Hawai'i as a test case. Using PCA analysis, general linear

317 models and analysis of DE genes, we quantified the effect of microplastic load on the overall
318 health of the birds. We found a marginal, negative correlation between body weight and plastic.
319 When using body weight as an indirect measure for the effect of plastic on the bird, we found
320 associations between upregulation of metabolic and biologic pathways and lighter birds. Birds
321 with lower weight tended to contain plastic.

322 The main effects to gene expression were attributed to i) upregulation of biosynthetic and
323 metabolic pathways in lighter birds and ii) downregulation of biosynthetic pathways in heavier
324 birds. Analysis of DE genes showed an upregulation of genes involved in biosynthetic processes
325 in lighter birds. Biosynthetic and metabolic processes are responsible for body mass
326 accumulation. Disruptions to energy and lipid metabolism pathways have been documented in
327 Zebrafish (*D.rerio*) (Limonta et al., 2019, Lu et al., 2016), and African Catfish that were
328 exposed to microplastics (*Clarias gariepinus*) (Karami et al., 2016). Toxins found in
329 microplastics, such as BPA and BP, are associated with disruption of metabolic and biosynthetic
330 pathways (Sun et al., 2022). Sprague-Dawley Rats exposed to BPA experienced weight loss (Sun
331 et al., 2022). Adult Zebrafish, also exposed to BPA, experienced a decrease in oxidative stress
332 and body weight change (Jordan et al., 2012, Lu et al., 2014, Huang et al., 2019, Sun et al.,
333 2022). Previous physiological suggestions in seabirds include reduction in the functional volume
334 of the gizzard; this reduces digestive capability causing weight loss (Furness & Monaghan,
335 1987).

336 Lighter birds also displayed upregulation of genes involved in organonitrogen compound
337 metabolic processes. This process is associated with the formation of compounds that are directly
338 linked to a nitrogen atom. Our general linear model revealed a marginal relationship between
339 lighter birds and higher BUN (blood urea nitrogen) levels. Higher BUN levels have been used to

infer dehydration in birds (Hochleither). Higher urea levels were associated with European pond turtles (*Emys orbicularis*) exposed to varying doses of microplastics (Banaee et al., 2020). These results could suggest a possible relationship between the presence of plastic, and disruption of genes involved in metabolic and biosynthetic processes.

There were only 11 genes that differentiated females and males among individuals with plastic (Supplementary figure S8A). Meanwhile, there were 43 genes that differentiated females and males among individuals without plastic (Supplementary figure S8B). Several studies have found that when bisphenol-a (BPA), a chemical used in plastic, is leached from plastic, it can activate estrogen receptors in mammals (Bittner et al., 2009, Gao et al., 2015). These findings suggest that males would present a detectable level of estrogen, causing the genetic differentiation to be less defined between males and females. More research is needed on the effects of plastic toxins on sex hormones to support this hypothesis.

One of our significant findings included genes associated with a pathway in response to a COVID-19 infection (Supplementary figure S10B). This pathway is associated with an inflammatory response, organ failure, and hypercoagulability (Harrison et al., 2020). We cannot be sure that this has to do with a Sars-CoV-2 infection in birds, but this pathway response in birds may be associated with a similar infection leading to an inflammatory response. Male mice exposed to PCBs, DDE (dichlorodiphenyldichloroethylene) and HCB (hexachlorobenzene), suffered liver injury and systemic inflammation (Deng et al., 2019). PCBs, DDE and HCB are chemicals found in plastic. Presence of microplastics in zebrafish have been linked to enhanced immune responses (Limonta et al., 2019), and other disruptions to the immune system (Powell et al., 2010).

Challenges in study design

One significant challenge in our study was the possibility of false negative results. All bird populations we sampled are on the island near a big source of plastic. It could be the case that those birds without plastic in their gut at the moment of sampling, were already exposed at some level to plastic and we were not able to detect it. The flushing technique for gut sampling we used does not allow for full collection of gut samples. Other methods of obtaining sampling might be more effective but could also increase the risk of death and injury to the birds. A study that does not rely on destructive sampling also allows for repeated sampling and continued monitoring of bird health. Destructive sampling only allows for a single observation (Provencher et al., 2019).

Using weight as an indirect metric

With a marginal p-value, lighter birds tended to be associated with the presence of plastic while heavier birds tended to be associated with the absence of plastic (Fig. 5a). More importantly, the relationships that we detected have been noted in previous studies examining plastic load and its effect on different organisms (Ryan 1986, Sievert & Sileo 1993, Pierce et al. 2004). A study on *Physalaemus cuvieri* tadpoles that were exposed to polyethylene microplastics at significant concentrations for 7 days reported accumulation of microplastics in the internal organs of which led to morphological and mutagenic changes; these changes can have effects on health and development (da Costa Araújo et al., 2020). In the same study, abnormalities were observed in nuclear erythrocytes and on external morphological traits such as mouth-cloaca distance.

Despite the challenges presented by experimental design, we were able to detect marginal indications of possible effects of microplastic toxins in relation to genetic expression, weight and

386 blood analytes. Perhaps the challenges of conducting an ecological study in the field cause the
387 associations to not be as strong as they could have been in a laboratory setting.

388
389 **CONCLUSIONS**

390 Our results indicate that there are signs of sublethal effects of ingested plastic in Wedge-
391 tailed Shearwaters. We were able to detect a negative relationship between the presence of
392 plastic and weight. When using weight as an indirect measurement for the effect of ingested
393 microplastics, there is some evidence that plastic affects metabolic and biosynthetic processes in
394 Wedge-tailed Shearwaters. Whereas there was no direct relationship between load of plastic
395 collected and DE genes, there was upregulation of genes involved in biosynthetic processes and
396 ribosome function in lighter birds. Birds that had ingested plastic tended to be lighter. There
397 were more genes that differentiated females and males that had not ingested plastic than females
398 and males that had ingested plastic. Furthermore, there is the possibility of false negatives (in
399 absence of plastic loads during gut sampling). The experiment contributes to an understanding of
400 the relationship between plastic and sublethal effects in seabirds. Given the finding of a COVID-
401 19 response pathway, we can further ask more questions on how anthropogenic diseases are
402 translating into wildlife. In seabirds specifically, this raises the question of whether ingested
403 plastic is having an impact on seabirds' immune system.

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Data Availability Statement

The sequence data will be available upon publication. Data sets associated with this paper will be available upon acceptance.

References

Adams J, Felis J, Czapanskiy M. 2020. "Habitat Affinities and At-Sea Ranging Behaviors among Main Hawaiian Island Seabirds: Breeding Seabird Telemetry, 2013–2016." *BOEM*

Barnes DKA, Galgani F, Thompson RC, Barlaz M. 2009. Accumulation and fragmentation of plastic debris in global environments. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 364:1985–1998. DOI: 10.1098/rstb.2008.0205.

Barrett R, Camphuysen K. 2008. Sampling methods for seabird dietary studies. *ICES Journal of Marine Science*.

Barry ME, Pinto-González D, Orson FM, McKenzie GJ, Petry GR, Barry MA. 1999. Role of endogenous endonucleases and tissue site in transfection and CpG-mediated immune activation after naked DNA injection. *Human Gene Therapy* 10:2461–2480. DOI: 10.1089/10430349950016816.

Basu M, Wang K, Ruppin E, Hannenhalli S. 2021. Predicting tissue-specific gene expression from whole blood transcriptome. *Science Advances* 7:eabd6991. DOI: 10.1126/sciadv.abd6991.

Bittner GD, Yang CZ, Stoner MA. 2014. Estrogenic chemicals often leach from BPA-free plastic products that are replacements for BPA-containing polycarbonate products. *Environmental Health* 13:41. DOI: 10.1186/1476-069X-13-41.

Blumenberg M, Blumenberg M. 2019. *Introductory Chapter*. IntechOpen. DOI: 10.5772/intechopen.85980.

Boersma D, Groom M. 1993. Conservation of storm-petrels in the North Pacific. University of Washington.

Bonanno G, Orlando-Bonaca M. 2018. Ten inconvenient questions about plastics in the sea. *Environmental Science & Policy* 85:146–154. DOI: 10.1016/j.envsci.2018.04.005.

Bond AL, Lavers JL. 2013. Effectiveness of emetics to study plastic ingestion by Leach's Storm-petrels (Oceanodroma leucorhoa). *Marine Pollution Bulletin* 70:171–175. DOI: 10.1016/j.marpolbul.2013.02.030.

Borrelle SB, Ringma J, Law KL, Monnahan CC, Lebreton L, McGivern A, Murphy E, Jambeck J, Leonard GH, Hilleary MA, Eriksen M, Possingham HP, De Frond H, Gerber LR, Polidoro B, Tahir A, Bernard M, Mallos N, Barnes M, Rochman CM. 2020. Predicted growth in plastic waste exceeds efforts to mitigate plastic pollution. *Science* 369:1515–1518. DOI: 10.1126/science.aba3656.

Bost, C. A., and LeMaho, Y.: 1993, 'Seabirds as bio-indicators of changing marine ecosystems: new perspectives', *Acta Oecologica* 14, 463–470.

Browne MA, Niven SJ, Galloway TS, Rowland SJ, Thompson RC. 2013. Microplastic Moves Pollutants and Additives to Worms, Reducing Functions Linked to Health and Biodiversity. *Current Biology* 23:2388–2392. DOI: 10.1016/j.cub.2013.10.012.

da Costa Araújo AP, de Melo NFS, de Oliveira Junior AG, Rodrigues FP, Fernandes T, de Andrade Vieira JE, Rocha TL, Malafaia G. 2020. How much are microplastics harmful to the health of amphibians? A study with pristine polyethylene microplastics and Physalaemus cuvieri. *Journal of Hazardous Materials* 382:121066. DOI: 10.1016/j.jhazmat.2019.121066.

Cózar A, Echevarría F, González-Gordillo JI, Irigoien X, Úbeda B, Hernández-León S, Palma ÁT, Navarro S, García-de-Lomas J, Ruiz A, Fernández-de-Puelles ML, Duarte CM. 2014. Plastic debris in the open ocean. *Proceedings of the National Academy of Sciences* 111:10239–10244. DOI: 10.1073/pnas.1314705111.

DNA Microarray Technology Fact Sheet. *Available at https://www.genome.gov/about-genomics/fact-sheets/DNA-Microarray-Technology* (accessed November 8, 2022).

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. 2013. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics (Oxford, England)* 29:15–21. DOI: 10.1093/bioinformatics/bts635.

Dobin A, Gingeras TR. 2015. Mapping RNA-seq Reads with STAR. *Current Protocols in Bioinformatics* 51:11.14.1-11.14.19. DOI: 10.1002/0471250953.bi1114s51.

Duffy DC, Jackson S. 1986. Diet Studies of Seabirds: A Review of Methods. *Colonial Waterbirds* 9:1–17. DOI: 10.2307/1521138.

Ekblom R, Wennekes P, Horsburgh GJ, Burke T. 2014. Characterization of the house sparrow (Passer domesticus) transcriptome: a resource for molecular ecology and immunogenetics. *Molecular Ecology Resources* 14:636–646. DOI: 10.1111/1755-0998.12213.

Estrogen's Effects on the Female Body. 2022. *Available at https://www.hopkinsmedicine.org/health/conditions-and-diseases/estrogens-effects-on-the-female-body* (accessed November 8, 2022).

Feng S. et al. Dense sampling of bird diversity increases power of comparative genomics. Nature 587:252–257. DOI: 10.1038/s41586-020-2873-9.

Furness R. W., Monaghan P. 1987. *Seabird Ecology*. Blackie□; Published in the USA by Chapman and Hall.

Franchini P, Irisarri I, Fudickar A, Schmidt A, Meyer A, Wikelski M, Partecke J. 2017. Animal tracking meets migration genomics: transcriptomic analysis of a partially migratory bird species. *Molecular Ecology* 26:3204–3216. DOI: 10.1111/mec.14108.

Fridolfsson A-K, Ellegren H. 1999. A Simple and Universal Method for Molecular Sexing of Non-Ratite Birds. *Journal of Avian Biology* 30:116–121. DOI: 10.2307/3677252.

Laist DW. 1997. Impacts of Marine Debris: Entanglement of Marine Life in Marine Debris Including a Comprehensive List of Species with Entanglement and Ingestion Records. In: Coe JM, Rogers DB eds. *Marine Debris: Sources, Impacts, and Solutions*. Springer Series on Environmental Management. New York, NY: Springer, 99–139. DOI: 10.1007/978-1-4613-8486-1_10.

The Problem of Marine Plastic Pollution. 2016. *Available at https://www.cleanwater.org/problem-marine-plastic-pollution* (accessed November 8, 2022).

Clinical Pathology of Plastic Ingestion in Marine Birds and Relationships with Blood Chemistry | Environmental Science & Technology. *Available at https://pubs.acs.org/doi/full/10.1021/acs.est.9b02098* (accessed November 8, 2022).

Coe JM, Rogers DB. 1997. Introduction. In: Coe JM, Rogers DB eds. *Marine Debris: Sources, Impacts, and Solutions*. Springer Series on Environmental Management. New York, NY: Springer, 5–6. DOI: 10.1007/978-1-4613-8486-1_1.

Fine-scale population genetic structure and barriers to gene flow in a widespread seabird (Ardenna pacifica) | Biological Journal of the Linnean Society | Oxford Academic. *Available at https://academic.oup.com/biolinnean/article/137/1/125/6650017* (accessed November 8, 2022).

Gall SC, Thompson RC. 2015. The impact of debris on marine life. *Marine Pollution Bulletin* 92:170–179. DOI: 10.1016/j.marpolbul.2014.12.041.

Gallo F, Fossi C, Weber R, Santillo D, Sousa J, Ingram I, Nadal A, Romano D. 2018. Marine litter plastics and microplastics and their toxic chemicals components: the need for urgent preventive measures. *Environmental Sciences Europe* 30:13. DOI: 10.1186/s12302-018-0139-z.

Gao H, Yang B-J, Li N, Feng L-M, Shi X-Y, Zhao W-H, Liu S-J. 2015. Bisphenol A and Hormone-Associated Cancers: Current Progress and Perspectives. *Medicine* 94:e211. DOI: 10.1097/MD.0000000000000211.

Gornicka A, Morris-Stiff G, Thapaliya S, Papouchado BG, Berk M, Feldstein AE. 2011. Transcriptional Profile of Genes Involved in Oxidative Stress and Antioxidant Defense in a Dietary Murine Model of Steatohepatitis. *Antioxidants & Redox Signaling* 15:437–445. DOI: 10.1089/ars.2010.3815.

gprofiler2 -- an R package for gene list functional... | F1000Research. *Available at https://f1000research.com/articles/9-709/v2* (accessed November 8, 2022).

Handling multi-mapped reads — seqcluster 1.2.4a7 documentation. *Available at https://seqcluster.readthedocs.io/multi_mapped.html* (accessed November 8, 2022).

Harr KE. 2002. Clinical Chemistry of Companion Avian Species: A Review. *Veterinary Clinical Pathology* 31:140–151. DOI: 10.1111/j.1939-165X.2002.tb00295.x.

Harrison AG, Lin T, Wang P. 2020. Mechanisms of SARS-CoV-2 Transmission and Pathogenesis. *Trends in Immunology* 41:1100–1115. DOI: 10.1016/j.it.2020.10.004.

538 Herman RW, Winger BM, Dittmann DL, Harvey MG. 2022. Fine-scale population genetic
539 structure and barriers to gene flow in a widespread seabird (Ardenna pacifica). *Biological*
540 *Journal of the Linnean Society* 137:125–136. DOI: 10.1093/biolinnean/blac091.

541 Hirt N, Body-Malapel M. 2020. Immunotoxicity and intestinal effects of nano- and
542 microplastics: a review of the literature. *Particle and Fibre Toxicology* 17:57. DOI:
543 10.1186/s12989-020-00387-7.

544 Hochleithner. "Biochemistries." *Avian Medicine: Principles and Applications*, pp. 243-245

545 Jâms IB, Windsor FM, Poudevigne-Durance T, Ormerod SJ, Durance I. 2020. Estimating the
546 size distribution of plastics ingested by animals. *Nature Communications* 11:1594. DOI:
547 10.1038/s41467-020-15406-6.

548 Jax E, Müller I, Börno S, Borlinghaus H, Eriksson G, Fricke E, Timmermann B, Pendl H, Fiedler
549 W, Klein K, Schreiber F, Wikelski M, Magor KE, Kraus RHS. 2021. Health monitoring in birds
550 using bio-loggers and whole blood transcriptomics. *Scientific Reports* 11:10815. DOI:
551 10.1038/s41598-021-90212-8.

552 Jax E, Wink M, Kraus RHS. 2018. Avian transcriptomics: opportunities and challenges. *Journal*
553 *of Ornithology* 159:599–629. DOI: 10.1007/s10336-018-1532-5.

554 Kain EC, Lavers JL, Berg CJ, Raine AF, Bond AL. 2016. Plastic ingestion by Newell's (Puffinus
555 newelli) and wedge-tailed shearwaters (Ardenna pacifica) in Hawaii. *Environmental Science and*
556 *Pollution Research* 23:23951–23958. DOI: 10.1007/s11356-016-7613-1.

557 Karami A, Romano N, Galloway T, Hamzah H. 2016. Virgin microplastics cause toxicity and
558 modulate the impacts of phenanthrene on biomarker responses in African catfish (Clarias
559 gariepinus). *Environmental Research* 151:58–70. DOI: 10.1016/j.envres.2016.07.024.

560 Kenyon KW, Kridler E. 1969. Laysan Albatrosses swallow indigestible matter. *The Auk* 86:339–
561 343. DOI: 10.2307/4083505.

562 Koelmans AA, Bakir A, Burton GA, Janssen CR. 2016. Microplastic as a Vector for Chemicals
563 in the Aquatic Environment: Critical Review and Model-Supported Reinterpretation of Empirical
564 Studies. *Environmental Science & Technology* 50:3315–3326. DOI: 10.1021/acs.est.5b06069.

565 Krjutškov K, Koel M, Roost AM, Katayama S, Einarsdottir E, Jouhilahti E-M, Söderhäll C,
566 Jaakma Ü, Plaas M, Vesterlund L, Lohi H, Salumets A, Kere J. 2016. Globin mRNA reduction
567 for whole-blood transcriptome sequencing. *Scientific Reports* 6:31584. DOI: 10.1038/srep31584.

568 Labocha MK, Hayes JP. 2012. Morphometric indices of body condition in birds: a review.
569 *Journal of Ornithology* 153:1–22. DOI: 10.1007/s10336-011-0706-1.

570 Laing LV, Viana J, Dempster EL, Trznadel M, Trunkfield LA, Uren Webster TM, van Aerle R,
571 Paull GC, Wilson RJ, Mill J, Santos EM. 2016. Bisphenol A causes reproductive toxicity,
572 decreases dnmt1 transcription, and reduces global DNA methylation in breeding zebrafish
573 (Danio rerio). *Epigenetics* 11:526–538. DOI: 10.1080/15592294.2016.1182272.

574 Li B., Dewey C.N. 2021."RSEM: accurate transcript quantification from RNA-Seq data with or
575 without a reference genome". *BMC Bioinformatics* 12, 323.
576
577 Limonta G, Mancia A, Benkhalqui A, Bertolucci C, Abelli L, Fossi MC, Panti C. 2019.
578 Microplastics induce transcriptional changes, immune response and behavioral alterations in
579 adult zebrafish. *Scientific Reports* 9:15775. DOI: 10.1038/s41598-019-52292-5.

580 Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for
581 RNA-seq data with DESeq2. *Genome Biology* 15:550. DOI: 10.1186/s13059-014-0550-8.

582 Lu Y, Zhang Y, Deng Y, Jiang W, Zhao Y, Geng J, Ding L, Ren H. 2016. Uptake and
583 Accumulation of Polystyrene Microplastics in Zebrafish (Danio rerio) and Toxic Effects in
584 Liver. *Environmental Science & Technology* 50:4054–4060. DOI: 10.1021/acs.est.6b00183.

585 Mallory ML, Robinson SA, Hebert CE, Forbes MR. 2010. Seabirds as indicators of aquatic
586 ecosystem conditions: A case for gathering multiple proxies of seabird health. *Marine Pollution*
587 *Bulletin* 60:7–12. DOI: 10.1016/j.marpolbul.2009.08.024.

588 Meitern R, Andreson R, Hõrak P. 2014. Profile of whole blood gene expression following
589 immune stimulation in a wild passerine. *BMC Genomics* 15:533. DOI: 10.1186/1471-2164-15-
590 533.

591 Microplastics | National Geographic Society. *Available at*
592 *https://education.nationalgeographic.org/resource/microplastics* (accessed November 8, 2022).

593 Mootha VK, Lindgren CM, Eriksson K-F, Subramanian A, Sihag S, Lehar J, Puigserver P,
594 Carlsson E, Ridderstråle M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR,
595 Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D, Groop LC. 2003. PGC-1α-
596 responsive genes involved in oxidative phosphorylation are coordinately downregulated in
597 human diabetes. *Nature Genetics* 34:267–273. DOI: 10.1038/ng1180.

598 Morphometric indices of body condition in birds: a review | SpringerLink. *Available at*
599 *https://link.springer.com/article/10.1007/s10336-011-0706-1* (accessed November 8, 2022).

600 Nania TG, Shugart GW. 2021. Are plastic particles reduced in size in seabirds' stomachs?
601 *Marine Pollution Bulletin* 172:112843. DOI: 10.1016/j.marpolbul.2021.112843.

602 Peng Y, Wu P, Schartup AT, Zhang Y. 2021. Plastic waste release caused by COVID-19 and its
603 fate in the global ocean. *Proceedings of the National Academy of Sciences* 118:e2111530118.
604 DOI: 10.1073/pnas.2111530118.

605 Pierce KE, Harris RJ, Larned L, Pokras M. 2004. Obstruction and starvation associated with
606 plastic ingestion in a Northern Gannet Morus bassanus and a Greater Shearwater Puffinus gravis.
607 *Marine Ornithology* 32:187–189.

608 Prata JC, da Costa JP, Lopes I, Duarte AC, Rocha-Santos T. 2020. Environmental exposure to
609 microplastics: An overview on possible human health effects. *Science of The Total Environment*
610 702:134455. DOI: 10.1016/j.scitotenv.2019.134455.

611 Provencher JF, Borrelle SB, Bond AL, Lavers JL, van Franeker JA, Kühn S, Hammer S, Avery-
612 Gomm S, Mallory ML. 2019. Recommended best practices for plastic and litter ingestion studies
613 in marine birds: Collection, processing, and reporting. *FACETS* 4:111–130. DOI: 10.1139/facets-
614 2018-0043.

615 Puchta M, Boczkowska M, Groszyk J. 2020. Low RIN Value for RNA-Seq Library Construction
616 from Long-Term Stored Seeds: A Case Study of Barley Seeds. *Genes* 11:1190. DOI:
617 10.3390/genes11101190.

618 Ritchie H, Roser M. 2018. Plastic Pollution. *Our World in Data*.

619 Ryan PG. 1987. The effects of ingested plastic on seabirds: Correlations between plastic load and
620 body condition. *Environmental Pollution* 46:119–125. DOI: 10.1016/0269-7491(87)90197-7.

621 Scholz M. 2021. "Metagenomics - Phred score (Q score)." *Metagenomics wiki*,
622 https://www.metagenomics.wiki/pdf/definition/phred-base-quality.
623

624 Science-Based Solutions to Plastic Pollution. 2020. *One Earth* 2:5–7. DOI:
625 10.1016/j.oneear.2020.01.004.

626 Sievert P.R., Sileo L. 1993. "The effects of ingested plastic on growth and survival of albatross
627 chicks. In: Vermeer, K., Briggs, K.T., Morgan, K.H. & Siegal–Causey, D. (Eds). The status,
628 ecology, and conservation of marine birds of the North Pacific." *Ottawa: Canadian Wildlife*
629 *Service Special Publication*, pp. 212–217.
630
631 Signa G, Mazzola A, Vizzini S. 2021. Seabird influence on ecological processes in coastal
632 marine ecosystems: An overlooked role? A critical review. *Estuarine, Coastal and Shelf Science*
633 250:107164. DOI: 10.1016/j.ecss.2020.107164.

634 Stafford R, Jones PJS. 2019. Viewpoint – Ocean plastic pollution: A convenient but distracting
635 truth? *Marine Policy* 103:187–191. DOI: 10.1016/j.marpol.2019.02.003.

636 Sun J, Fang R, Wang H, Xu D-X, Yang J, Huang X, Cozzolino D, Fang M, Huang Y. 2022. A
637 review of environmental metabolism disrupting chemicals and effect biomarkers associating
638 disease risks: Where exposomics meets metabolomics. *Environment International* 158:106941.
639 DOI: 10.1016/j.envint.2021.106941.

640 Tanaka K, Takada H, Yamashita R, Mizukawa K, Fukuwaka M, Watanuki Y. 2013.
641 Accumulation of plastic-derived chemicals in tissues of seabirds ingesting marine plastics.
642 *Marine Pollution Bulletin* 69:219–222. DOI: 10.1016/j.marpolbul.2012.12.010.

643 "Terms and Definitions – ENCODE." *encode*, https://www.encodeproject.org/data-
644 standards/terms/.
645

646 Tilman D. 1989. Ecological Experimentation: Strengths and Conceptual Problems. In: Likens
647 GE ed. *Long-Term Studies in Ecology: Approaches and Alternatives*. New York, NY: Springer,
648 136–157. DOI: 10.1007/978-1-4615-7358-6_6.

649 Valle CA, Ulloa C, Regalado C, Muñoz-Pérez J-P, Garcia J, Hardesty BD, Skehel A,
650 Deresienski D, Lewbart GA. 2020. HEALTH STATUS AND BASELINE HEMATOLOGY,
651 BIOCHEMISTRY, AND BLOOD GAS VALUES OF GALAPAGOS SHEARWATERS
652 (PUFFINUS SUBALARIS). *Journal of Zoo and Wildlife Medicine* 50:1026–1030. DOI:
653 10.1638/2019-0035R.

654 Verla AW, Enyoh CE, Verla EN, Nwarnorh KO. 2019. Microplastic–toxic chemical interaction:
655 a review study on quantified levels, mechanism and implication. *SN Applied Sciences* 1:1400.
656 DOI: 10.1007/s42452-019-1352-0.

657 Verlis KM, Campbell ML, Wilson SP. 2018. Seabirds and plastics don't mix: Examining the
658 differences in marine plastic ingestion in wedge-tailed shearwater chicks at near-shore and
659 offshore locations. *Marine Pollution Bulletin* 135:852–861. DOI:
660 10.1016/j.marpolbul.2018.08.016.

661 Wang L, Nabi G, Yin L, Wang Y, Li S, Hao Z, Li D. 2021. Birds and plastic pollution: recent
662 advances. *Avian Research* 12:59. DOI: 10.1186/s40657-021-00293-2.

663 Weimerskirch H, Grissac S de, Ravache A, Prudor A, Corbeau A, Congdon BC, McDuie F,
664 Bourgeois K, Dromzée S, Butscher J, Menkes C, Allain V, Vidal E, Jaeger A, Borsa P. 2020. At-
665 sea movements of wedge-tailed shearwaters during and outside the breeding season from four
666 colonies in New Caledonia. *Marine Ecology Progress Series* 633:225–238. DOI:
667 10.3354/meps13171.

668 Wilcox C, Van Sebille E, Hardesty BD. 2015. Threat of plastic pollution to seabirds is global,
669 pervasive, and increasing. *Proceedings of the National Academy of Sciences* 112:11899–11904.
670 DOI: 10.1073/pnas.1502108112.

671 Xu K, Zhang Y, Huang Y, Wang J. 2021. Toxicological effects of microplastics and
672 phenanthrene to zebrafish (Danio rerio). *Science of The Total Environment* 757:143730. DOI:
673 10.1016/j.scitotenv.2020.143730.

674

675 **Figure Legends**

676 **Fig. 1.** (A) Wedge-tailed Shearwater *(Ardenna pacifica)* in Hawai'i. From U.S. Fish and Wildlife
677 Service by Ian Jones. (B) Sampling Sites. Three sampling sites on the island of Maui in the
678 Hawaiian Islands represented by triangles. Colored circle represents concentrations of plastic
679 (items km-2) found around the Hawaiian Islands based on data from Cózar et al. 2014). (C)
680 Plastic samples. Plastic collected from gut samples from WTSH.

681 **Fig. 2.** Bird ID and data available for each specimen. An ID was appointed for each specimen.
682 The sex is indicated for each specimen. Plastic refers to if that particular specimen had ingested
683 plastic; (+) indicates presence of plastic and (-) indicated that that specimen did not have
684 ingested plastic. Blood sample refers to what information was obtained from the collected blood.
685 Specimens where only genetic information is available is denoted by "Gene only". Specimens
686 where only a chemical blood panel is available is denoted by "Chem only". Specimens where
687 both genetic information and chemical blood panels are available is denoted by "both".

688 **Fig. 3**. Individual t-test analysis of each blood analyte and weight. (a-k) Blood analyte plotted
689 against presence and absence of plastic with error bars. Any test that showed a slight significance
690 value is noted on the right-hand corner of the individual chart. (l) Weight plotted against
691 presence or absence of plastic.

692 **Fig. 4.** PCA analyses of morphometrics and blood analytes. (a) Morphometric measurements
693 with variables of presence of plastic using PCA 1 and PCA 2. (b) Morphometric measurements
694 with variables of presence of plastic using PCA 3 and PCA 4. (c) Morphometrics measurements
695 with variables of sex using PCA 1 and PCA 2. (d) Morphometric measurements with variables of
696 sex using PCA 3 and PCA 4. (e) Blood analytes with variables of presence of plastic using PCA
697 1 and PCA 2. (f) Blood analytes with variables of presence of plastic using PCA 3 and PCA 4.

(g) Blood analytes with variables of sex using PCA 1 and PCA 2. (h) Blood analytes with variables of sex using PCA 3 and PCA 4.

Fig. 5. Significant relationships from general linear model. (a) Relationship between presence or absence of plastic and weight of bird. (b) Relationship between presence or absence of plastic and total carbon dioxide.

Fig. 6. Significant results from general linear model of blood analytes with weight as the variable. (a) Hematocrit as percentage with weight as variable. (b) Urea nitrogen/urea with weight as the variable. (c) Potassium with weight as the variable.

Fig. 7. DE genes and enrichment analysis for the entire data set. (A) Heatmap showing that plastic and size factor of libraries do not explain the DE gene profiles. The heatmap shows the top 20 genes with the highest statistical power. (B) Enrichment analysis and significant terms for the 6 DE genes shown on the heatmap above.

Fig. 8. DE genes with three categories of weight; low, medium and high. (A) Heatmap showing the 18 significantly DE genes. (B) Normalize count in the two top genes showing differences in counts between the three weight categories.

Supporting Information

List of Supplementary figures and Tables. Tables. Supporting Information, Tables are both in the Supporting Information, File (pdf) and in the Supporting Information.

Supporting Information, Fig. S1. Table summarizing differential genetic expression analyses run. The test/experiment column describes the three main analyses that we conducted and the variables that were used for each test. Transcripts represent the count of transcripts that were aligned with the reference genome. Net transcripts represent the count of transcripts that were represented or quantifiable in all of the samples. Outlier transcripts and outlier genes represent the count of transcripts that differentiated within the respective test. P-value represents the two p-values we used to determine significance of the results. We used both a marginal p-value (0.1) and a standard value (0.05).

Supporting Information, Fig. S2. Summary of values from blood analytes**.** This table provides the mean, standard deviation and p-values from the t-tests for each of the blood chemistry analytes and weight. The plus sign (+) indicated the values for individuals with plastic. The minus sign (-) indicates the values for individuals without plastic. The blood analytes measured were sodium (Na mmol/L), potassium (K mmol/L), chloride (Cl mmol/L), ionized calcium (iCa mmol/L), total carbon dioxide (TCO2), glucose (Glu mg/dL), Urea nitrogen/urea (BUN mg/dL), creatine (Crea mg/dL), hematocrit (Hct%PCU), hemoglobin (Hb g/dL), anion gap (AnGap mmol/L) and are ordered respectively in the table.

Supporting Information, Fig. S3. Reads per sample. This measurement assesses an inferred sequence of base pairs that correspond to a single DNA fragment.

Supporting Information, Fig. S4. Phred score for samples. Phred score is used for quality assessment of sequencing.

Supporting Information, Fig. S5. Uniquely mapped reads. Uniquely mapped reads have one exact location within the reference genome which they map to. This is the number of uniquely mapped reads from the prepared library that are aligned to the Cory Shearwater reference genome.

Supporting Information, Fig. S6. Multiple mapped reads. Multiple mapped reads describe reads that map more than once in the genome. This is the number of multiple mapped reads from the prepared library that are aligned to the Cory Shearwater reference genome.

Supporting Information, Fig. S7. DE genes for plastic and sex. (A) Heatmap showing the 14 significantly expressed genes in males. (B) Heatmap showing the 4 significant DE genes in females.

Supporting Information, Fig. S8. DE genes divided into samples that had ingested plastic and samples that did not have plastic. (A) Heatmap showing the 11 significant DE genes in all of the samples containing plastic separated by sex. (B) Heatmap showing the 43 significant DE genes in all samples that do not contain plastic separated by sex.

Supporting Information, Fig. S9. RIN values. RIN values assign a numerical value to the quality of the RNA that we worked with. A RIN value of 8 and above indicated higher quality and integrity of RNA and values below 5 indicated some levels of RNA degradations.

Supporting Information, Fig. S10. Gene enrichment analysis results for bird weight. (A) Manhattan plot showing results of enrichment analysis and the databases used. (B) Terms with significant values from the enrichment analysis in the DE genes between weight categories

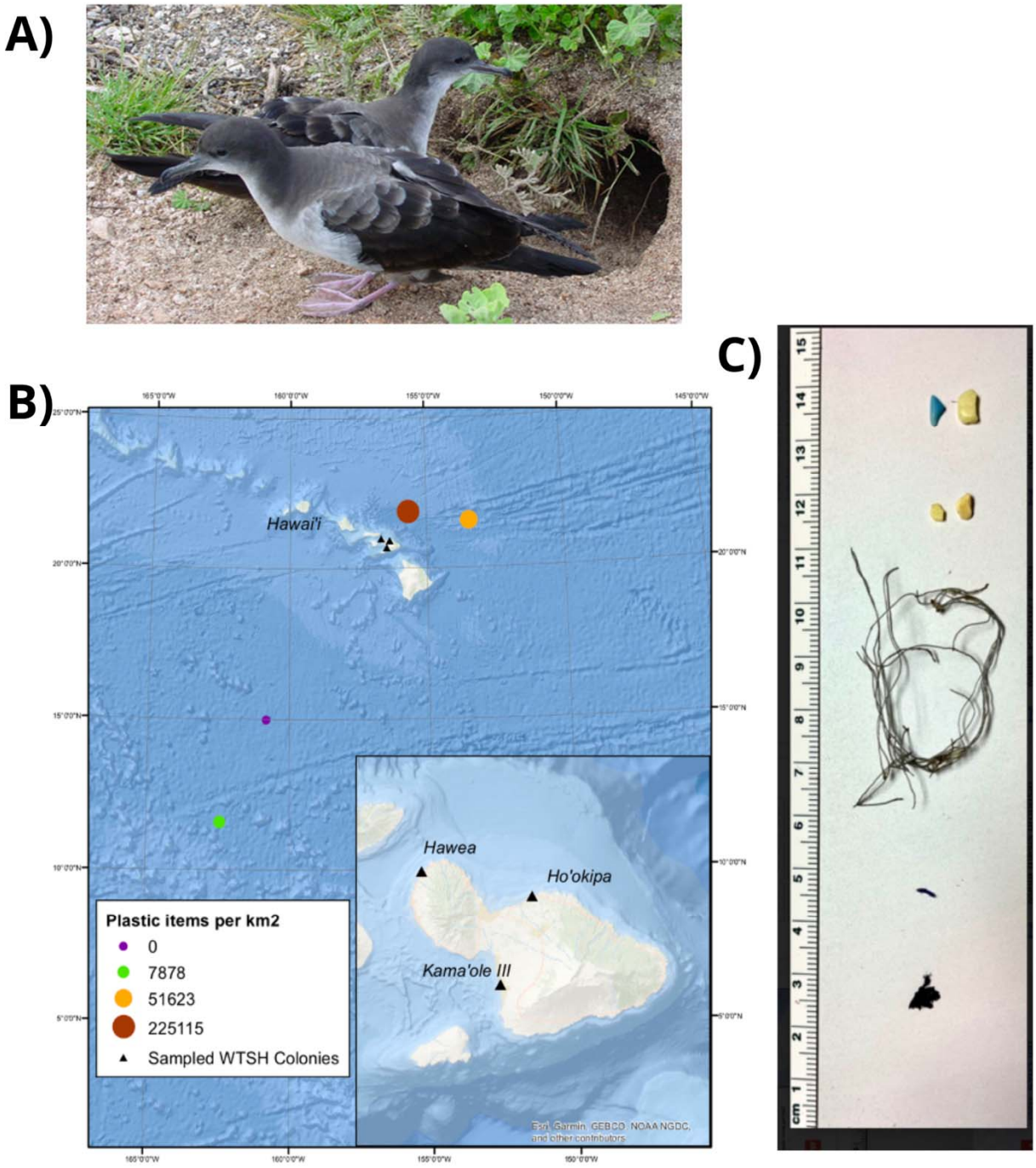


Fig. 1

Bird ID	Sex	Plastic	Blood Sample
N001	F	NA	Gene only
N002	F	(+)	Both
N003	F	(-)	Gene only
N004	NA	(+)	Chem only
N005	M	(+)	Both
N006	F	(-)	Both
N007	F	(+)	Both
N008	F	(-)	Both
N009	F	(-)	Both
N010	M	(+)	Both
N011	M	(+)	Both
N012	F	(-)	Both
N013	F	(-)	Both
N014	M	(-)	Both
N015	F	(+)	Both
N015	M	(-)	Both
N017	M	(+)	Both
N018	F	(-)	Both
N019	M	(-)	Both
N020	F	(+)	Both
N021	M	(-)	Both
N022	F	(-)	Both
N023	F	(-)	Both
N024	F	(+)	Gene only
N025	F	(+)	Both
N026	M	(-)	Both
N027	M	(-)	Both
N028	F	(+)	Both
N029	F	(-)	Gene only

Fig. 2

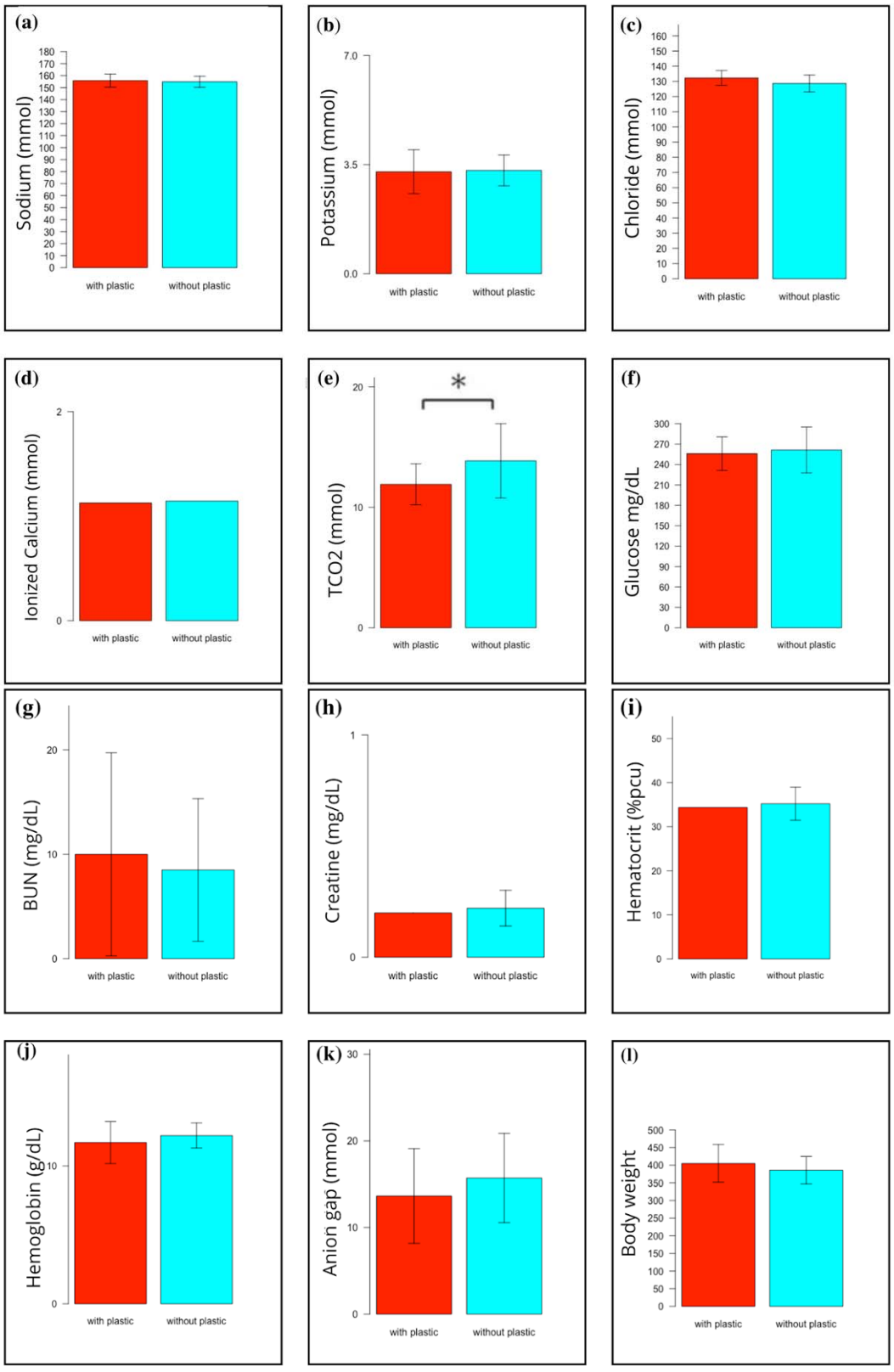


Fig. 3

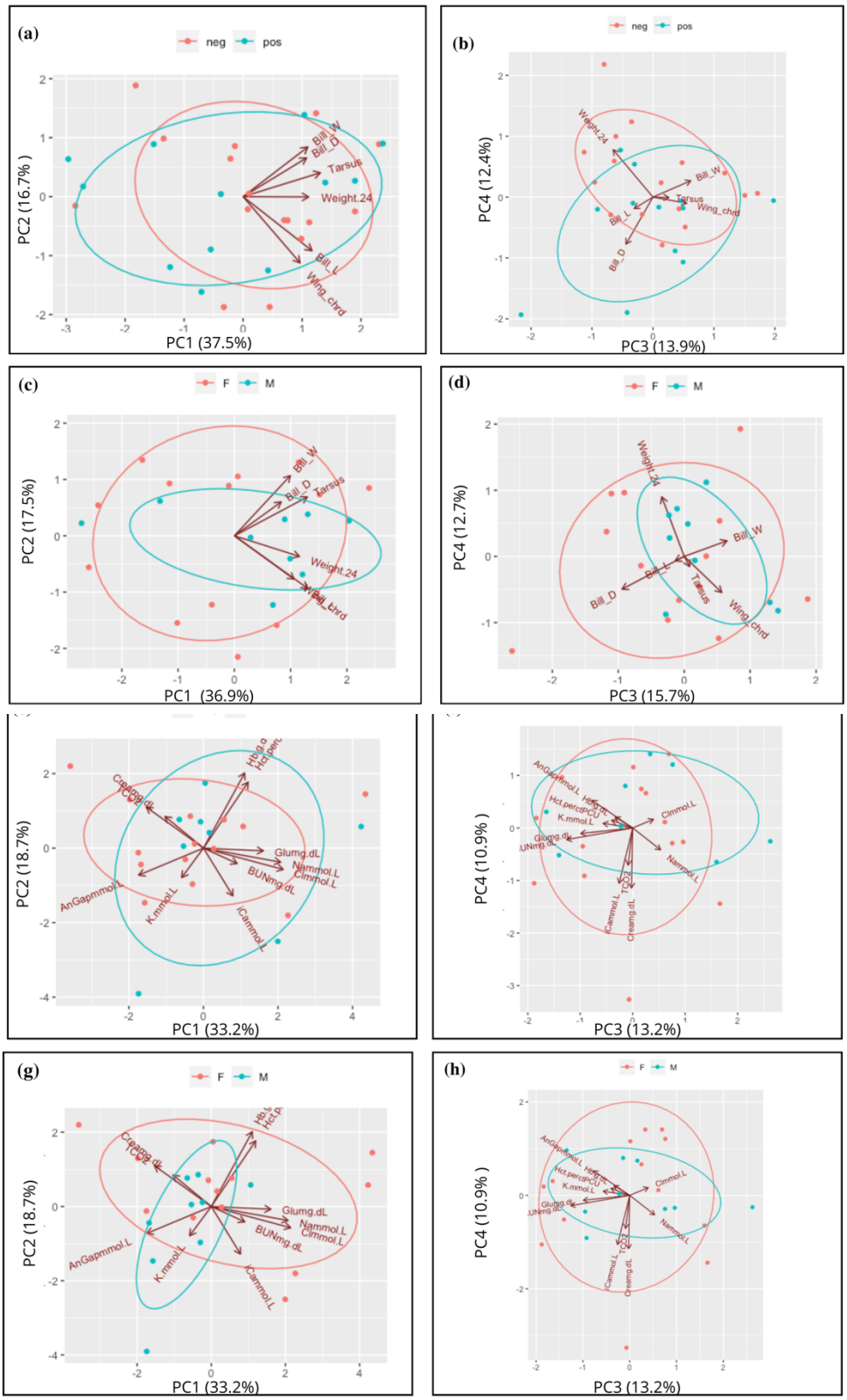
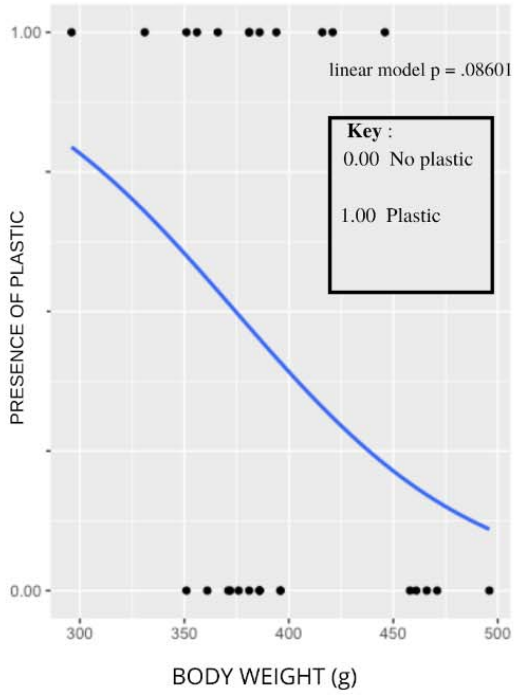


Fig. 4

a.



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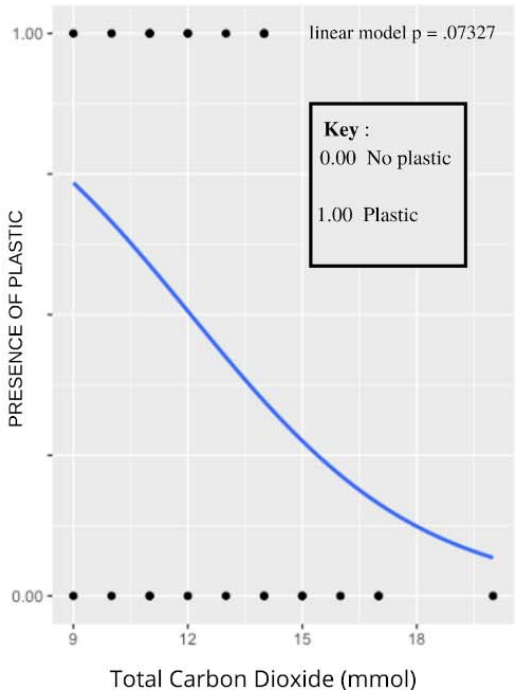

Fig. 5

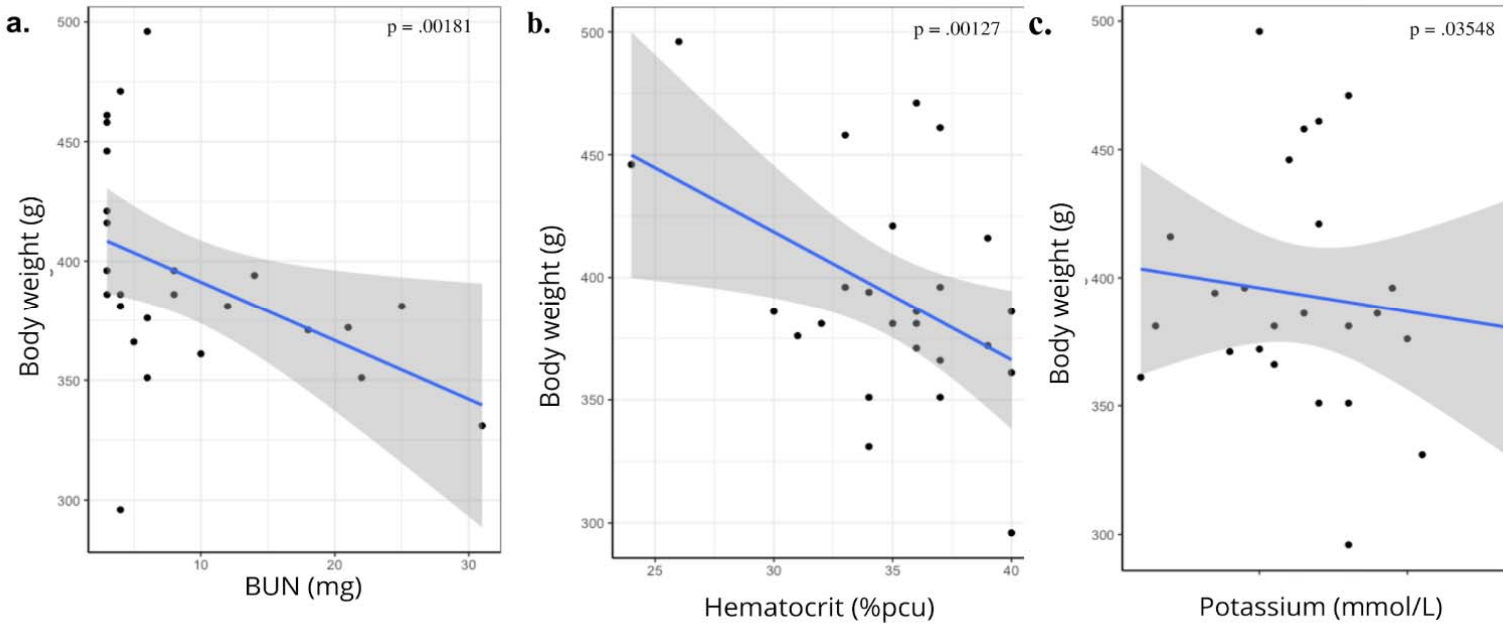

Fig. 6

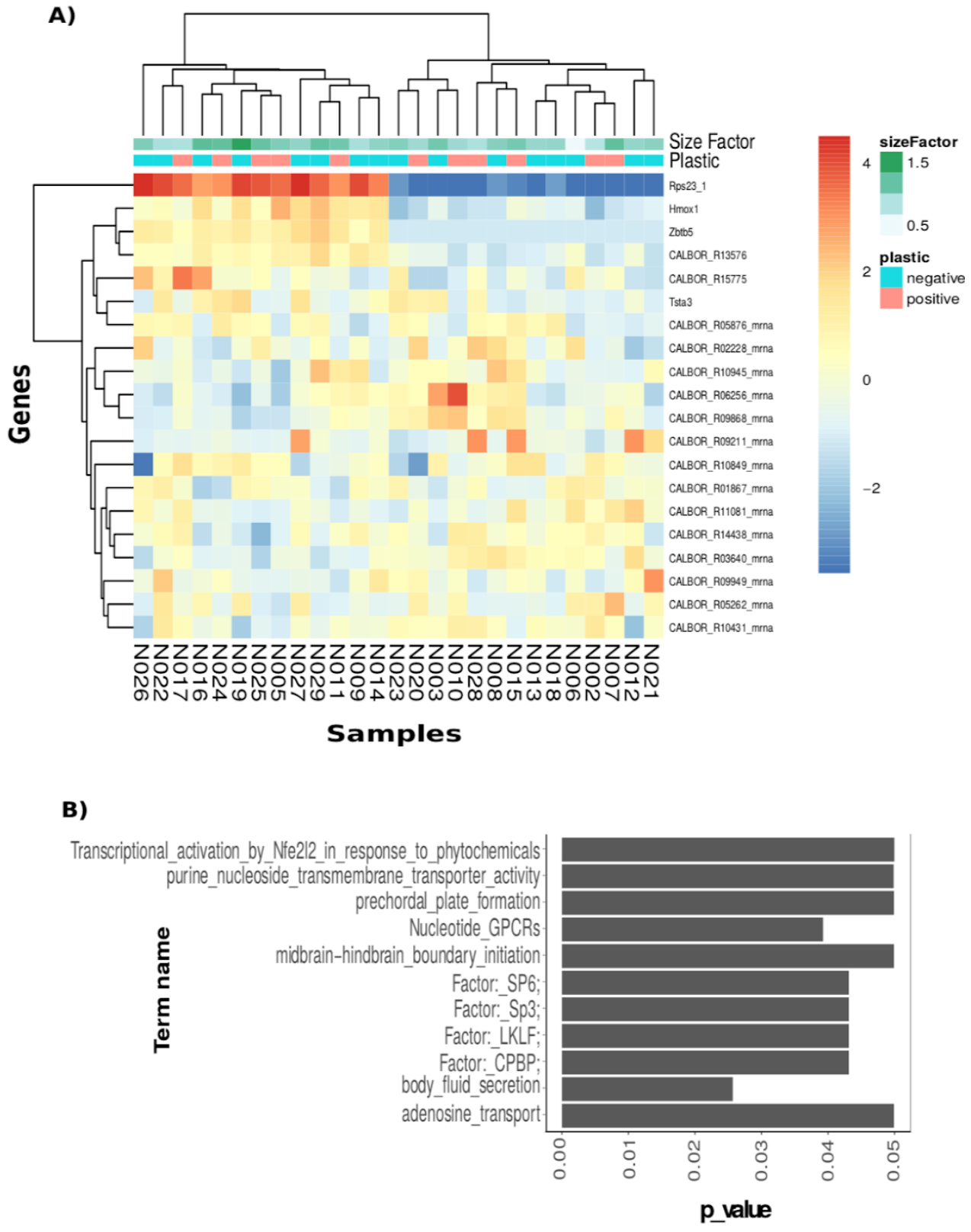

Fig. 7

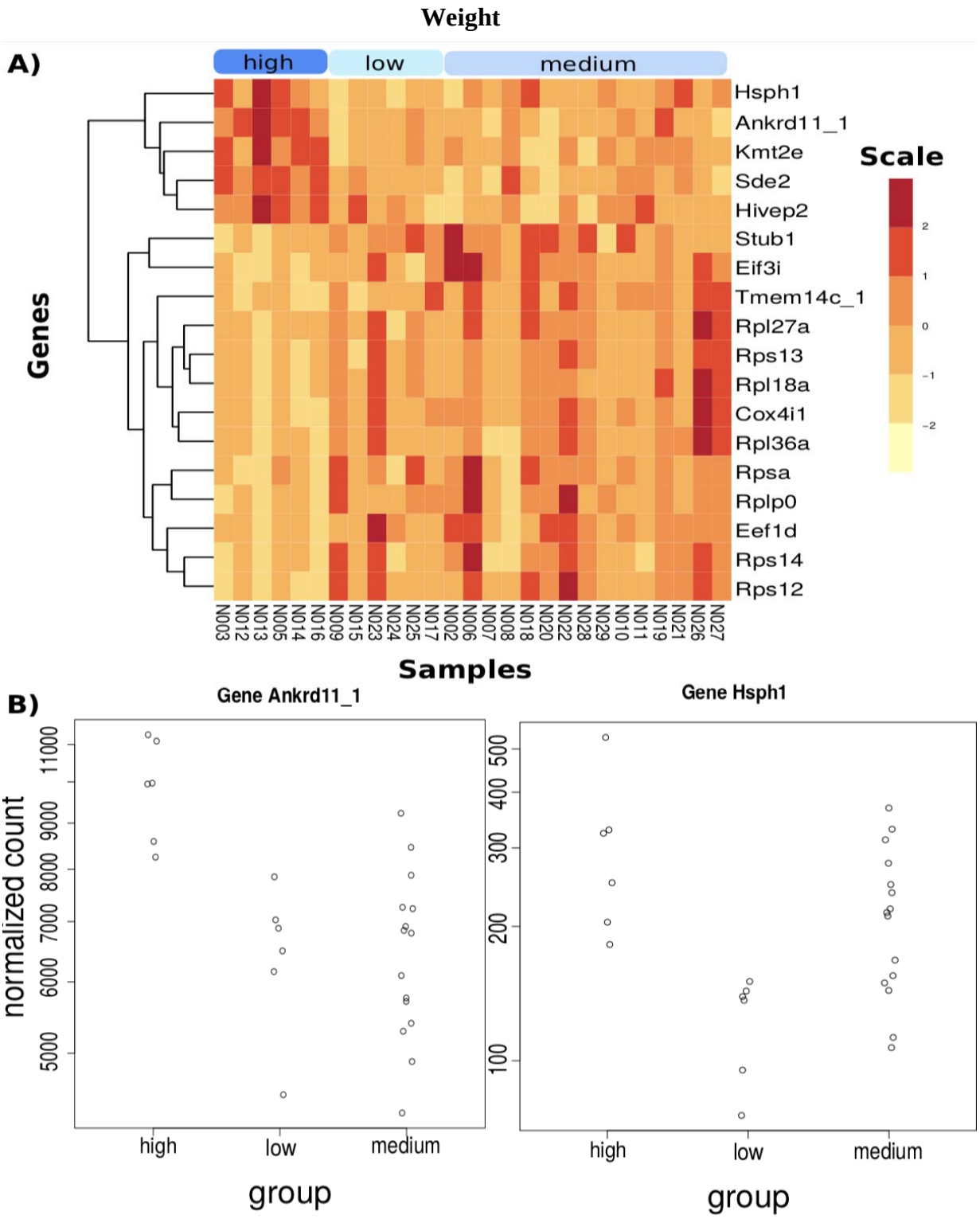

Fig. 8

Supporting Information

Effects of Plastic Ingestion on Blood Chemistry, Gene Expression and Body Condition in Wedge-Tailed Shearwaters (*Ardenna Pacifica*)

Nicole Mejia[1,2], Flavia Termignoni Garcia[1,2], Jennifer Learned[3], Jay Penniman[3], Scott V. Edwards[1,2]

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Supporting Text

Heavy metals and organic pollutants. Heavy metals, metals with density greater than 5 g/cm3, find their way into the environment both through natural means and as cause of human activity (Briffa et al., 2020). Weathering of earth's crust, urban runoff, industrial waste, pesticides, sewage runoff and many other anthropogenic sources introduce heavy metals into the environment. Heavy metals are found in significant concentrations near areas of anthropogenic activity such as harbors and marinas (Bighiu, 2017). Conversely, these are also areas where there are significant amounts of microplastics (Claessens et al.2011). Heavy metals then attach to the surface of microplastics due to the strong physical interactions. In excess quantities, heavy metals are toxic to organisms (Furness & Monaghan, 1987).

Organic chemicals, pollutants containing carbon bonded with other compounds, include persistent organic pollutants (or POPs) (Liu et al., 2021). POP's are resistant to degradation and can bioaccumulate to toxin levels. Bioaccumulation refers to the accumulation of a contaminant in an organism. Similar to heavy metals, POPs can be traced back to both natural and anthropogenic activities. These activities include volcanic eruptions or synthesis of chemicals. POPs are known to be easily transported from the source and easily absorbed in a new environment (Ashraf, 2017). Due to the low solubility in water, they are also easily absorbed by microplastics (Verla et al., 2019). Some well-known examples of POPs include the insecticide DDT, PCBs (Polychlorinated biphenyls) and BPAs (Bisphenol A) (Verla et al., 2019).

Wedge-tailed Shearwater (A. pacifica) Biology. Wedge-tailed Shearwater are pelagic seabirds that are monogamous and are known to be natal philopatric. Shearwater pairs often form a long-term pair bond which lasts several years. They have extensive feeding ranges, with a mean maximum range of 615 km (Adams et al., 2020). Although not endangered, global

numbers are in decline. When it is not breeding season, Wedge-tailed Shearwaters take long migrations and use specific migratory routes that take advantage of the oceanic wind patterns (Schaffer et al., 2006) It has been documented that the birds sometimes make long dispersive movements (Weimerskirch et al. 2020).

Science of plastic accumulation in the ocean. Due to ocean circulation patterns, there are certain regions in the open ocean where there is a greater concern for ocean pollution, such as the Great Pacific Garbage Patch in the Northern Pacific that stretches from the west coast of North America (Cózar et al., 2014). These garbage patches form because of gyres, large systems of circulating water in the ocean. Five gyres in particular play an important role in circulating water around the globe: North Atlantic, South Atlantic, North Pacific, South Pacific and Indian (NOAA). Plastic congregates around these slow-moving whirlpools, forming massive areas of circulating plastic (NOAA).

Gut sampling from the proventriculus. The flushing technique empties out gut contents from the proventriculus of a bird, but we cannot be assured that it empties out gut contents from the ventriculus in Procellariids (Duffy & Jackson, 1986). Procellariids' stomachs can be divided into two sections: the proventriculus and the gizzard. A lack of plastic content in the proventriculus often means that it is either regurgitated or emptied quicker than the gizzard (Nania & Shugart, 2021). This creates the possibility that the proventriculus of birds we sampled had been already cleared of plastic and we did not fully capture the plastic load.

Challenges of gene expression with blood. Using whole blood to create a genetic profile is a relatively new approach, especially in non-model organisms and livestock (Désert et al., 2016). Most gene profiles use tissue samples for a particular study because the composition and content of RNA, responsible for genetic activity that we are able to investigate, is specific to the tissue

activity (Jax et al., 2018). Target tissues also provide information on specific adverse effects specially in response to toxic exposure (Lobenhofer et al., 2008).

The use of whole blood for genetic profiling is a rising and useful tool (Désert et al., 2016). It could be used as a new approach in conservation to assess the health status of natural populations of species in threatened status. Studies with whole blood transcriptome have quantified immune response in birds and identifying sex chromosome evolution in two rare species of kiwi birds (Désert et al. 2016, Ekblom et al. 2014, Ramstad et al. 2016, Sandford et al. 2012). It is worthy to note the importance of continuing to advocate for these procedures which may provide a less invasive way of conducting data collection.

Figures

Test-experiment	Total Transcripts	Net Transcripts	Outlier Transcripts	Outlier genes	Pvalue_used
PLASTIC					
-all samples	15799	12842	0	0	0.05,0.1
-males	15799	8824	4,14	4,14	0.05,0.1
-females	15799	12503	1,1	1,1	0.05,0.1
SEX					
-all samples	15799	12853	2,3	2,3	0.05,0.1
-plastic	15799	12177	1,10		0.05,0.1
-no plastic	15799	12477	12,43		0.05,0.1
WEIGHT 3 factor					
-all samples	15799	12842	5,17		0.05,0.1
-males (just 2 factor)	15799	12048	1,2		0.05,0.1
-females	15799	12507	5,14		0.05,0.1

Fig. S1. Table summarizing differential genetic expression analyses. The test/experiment column describes the three main analyses that we conducted and the variables that we used for each test. Transcripts represent the count of transcripts that were aligned with the reference genome. Net transcripts represent the count of transcripts that were represented or quantifiable in all of the samples. Outlier transcripts and outlier genes represent the count of transcripts that differentiated within the respective test. P-value represents the two p-values we used to determine significance of the results. We used both a marginal p-value (0.1) and a standard value (0.05).

	Na	K	Cl	iCa	TCO2	Glucose	BUN	Crea	Hct	Hb	AnGap	Weight
MN+	155.8182	3.272727	132.2727	1.127273	11.90909	256	9.998182	0.199	34.36364	11.7	13.63636	405.25
MN-	154.8571	3.314286	128.6429	1.145	13.85714	261.3571	8.497857	0.2205	35.21429	1.524467	15.71429	3386
SD+	5.473905	0.7044017	4.900835	0.09360458	13.85714	24.62113	9.727693	0	4.500505	1.524467	5.463931	53.36176
SD-	4.588567	0.4943638	5.583039	0.09920841	3.08488	33.69685	6.82659	0.08044563	3.745327	0.9110132	5.150483	39.0299
T-test (p-value)	0.6454	0.87	0.0975	0.6516	0.05789*	0.6506	0.6694	0.3356	0.6196	0.3446	0.3441	0.6516

Fig. S2. Summary of values from blood analytes and weight. This table provides the mean, standard deviation and p-values from the t-tests for each of the blood chemistry analytes and weight. The plus sign (+) indicates the values for individuals with plastic. The minus sign (-) indicates the values for individuals without plastic. The blood analytes measured were sodium (Na mmol/L), potassium (K mmol/L), chloride (Cl mmol/L), ionized calcium (iCa mmol/L), total carbon dioxide (TCO2), glucose (Glu mg/dL), Urea nitrogen/urea (BUN mg/dL), creatinine (Crea mg/dL), hematocrit (Hct %PCU), hemoglobin (Hb g/dL), anion gap (AnGap mmol/L) and are ordered respectively in the table. Differences were considered statistically significant when $p < 0.07$.

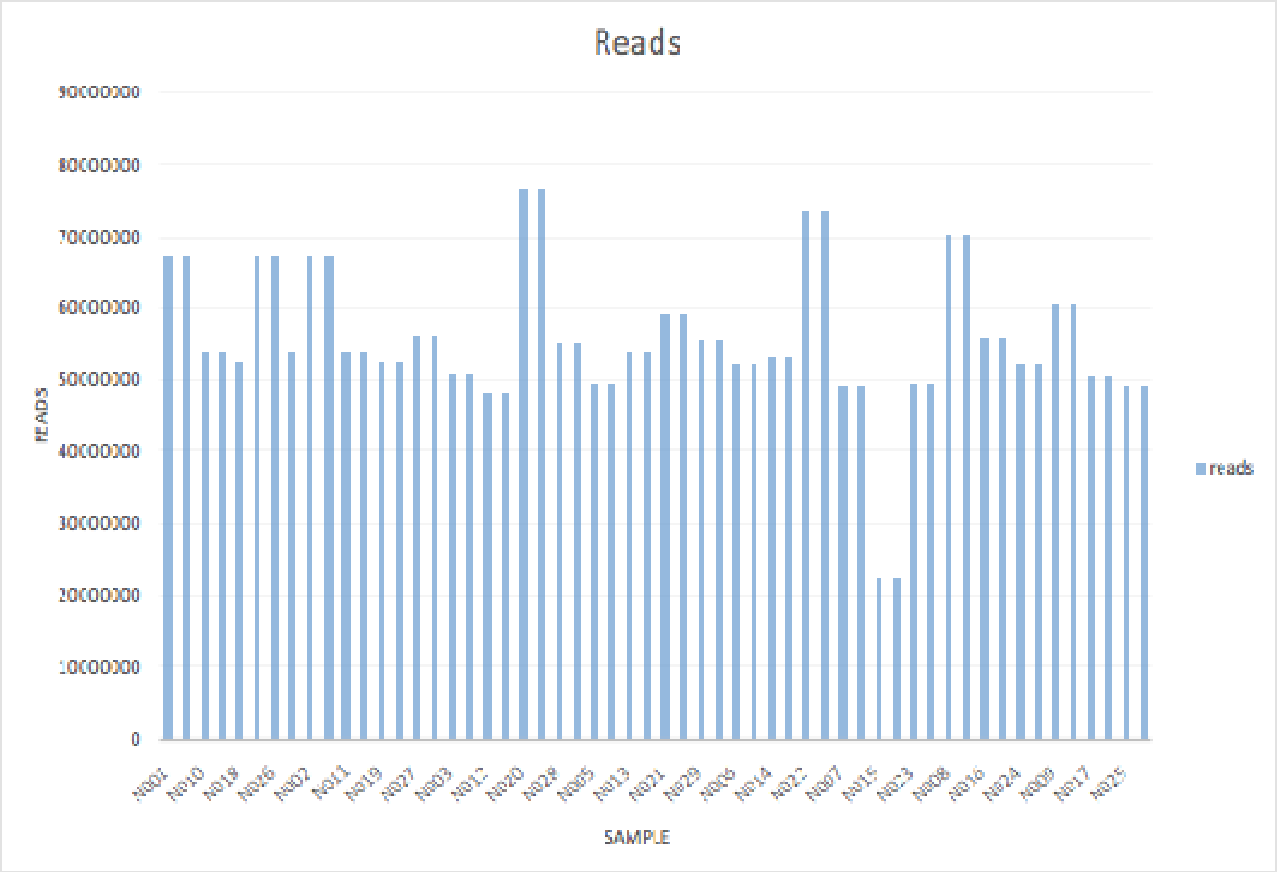

Fig. S3. Reads per sample. This measurement assesses inferred sequence of base pairs that correspond to a single DNA fragment.

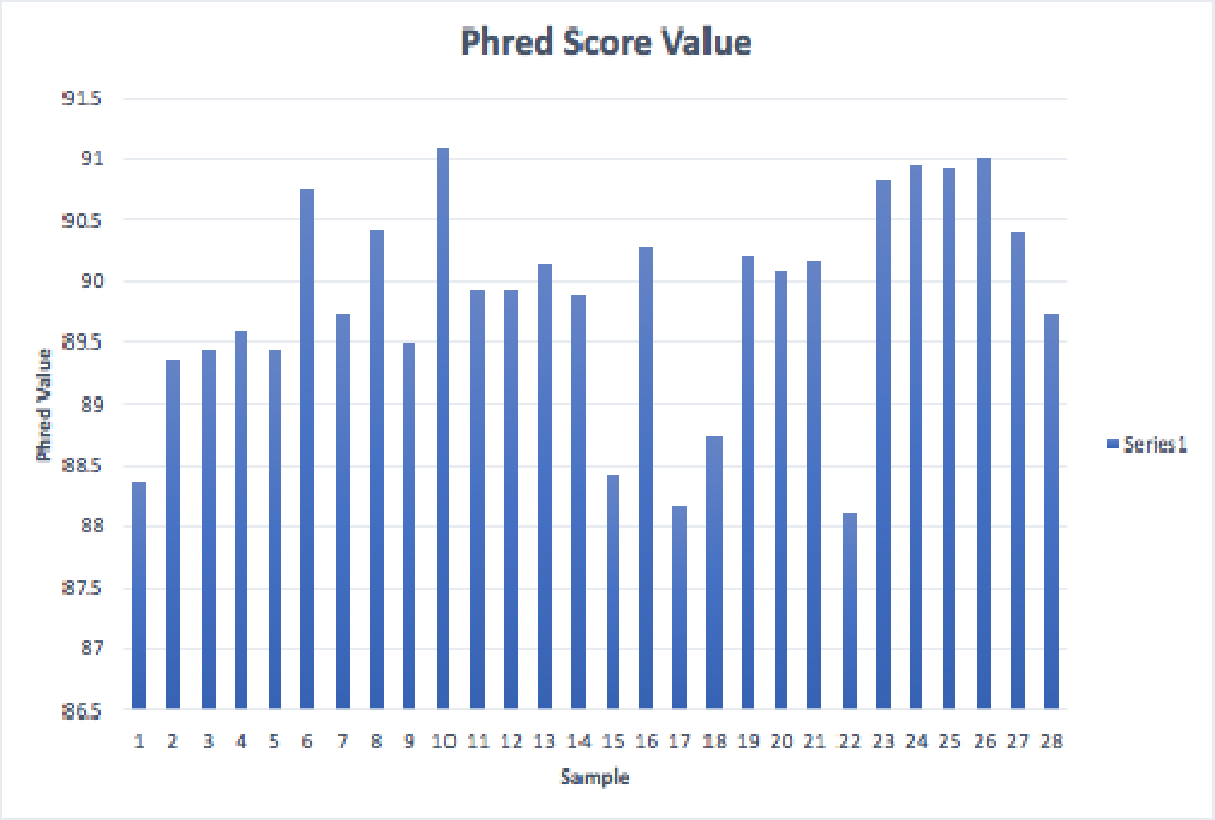

Fig. S4. Phred score of samples. Phred score is used for quality assessment of sequencing

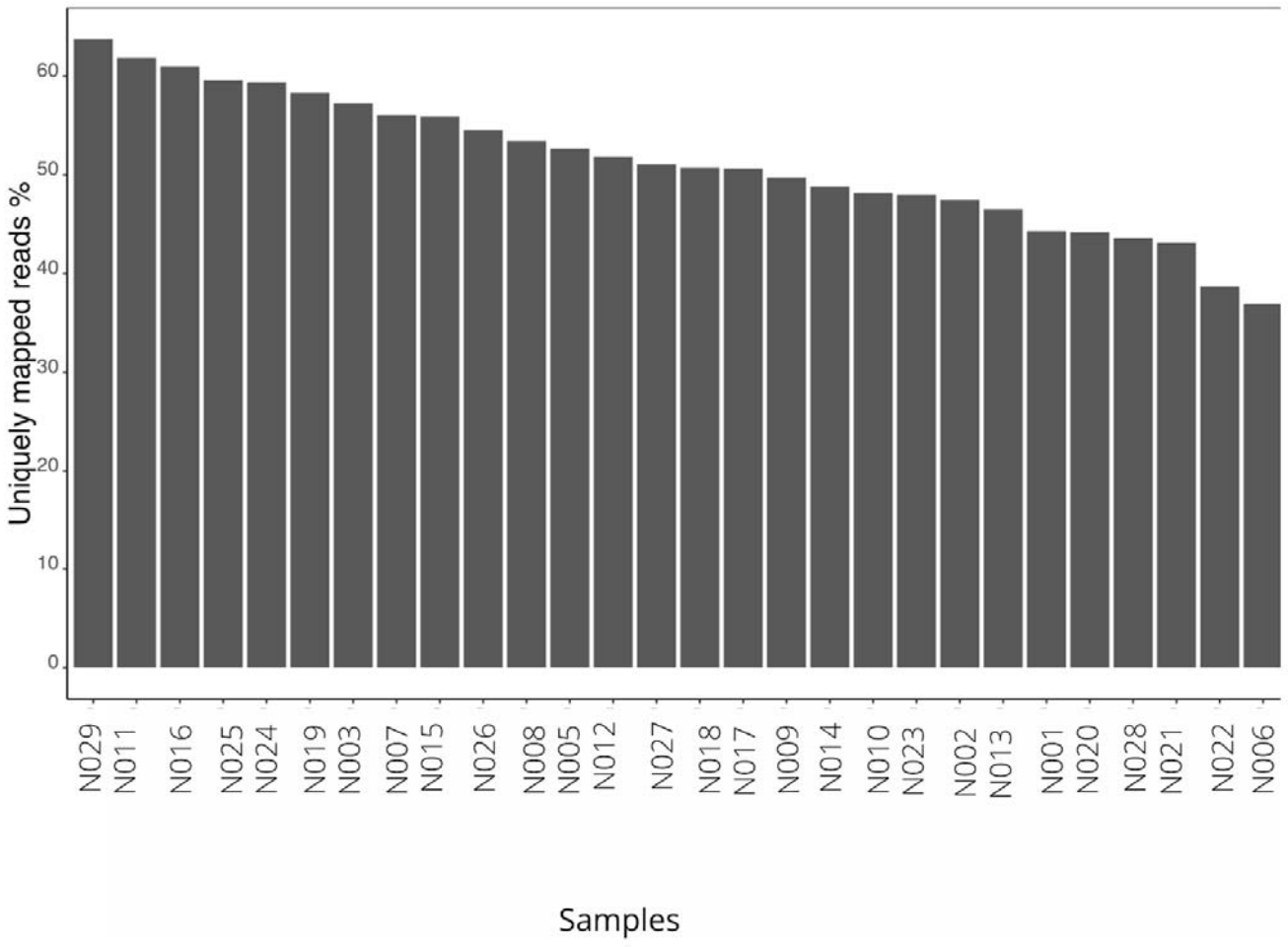

Fig. S5. Uniquely mapped reads. Uniquely mapped reads have one exact location within the reference genome which they map to. This is the number of uniquely mapped reads from the prepared library that are aligned to the Cory Shearwater reference genome.

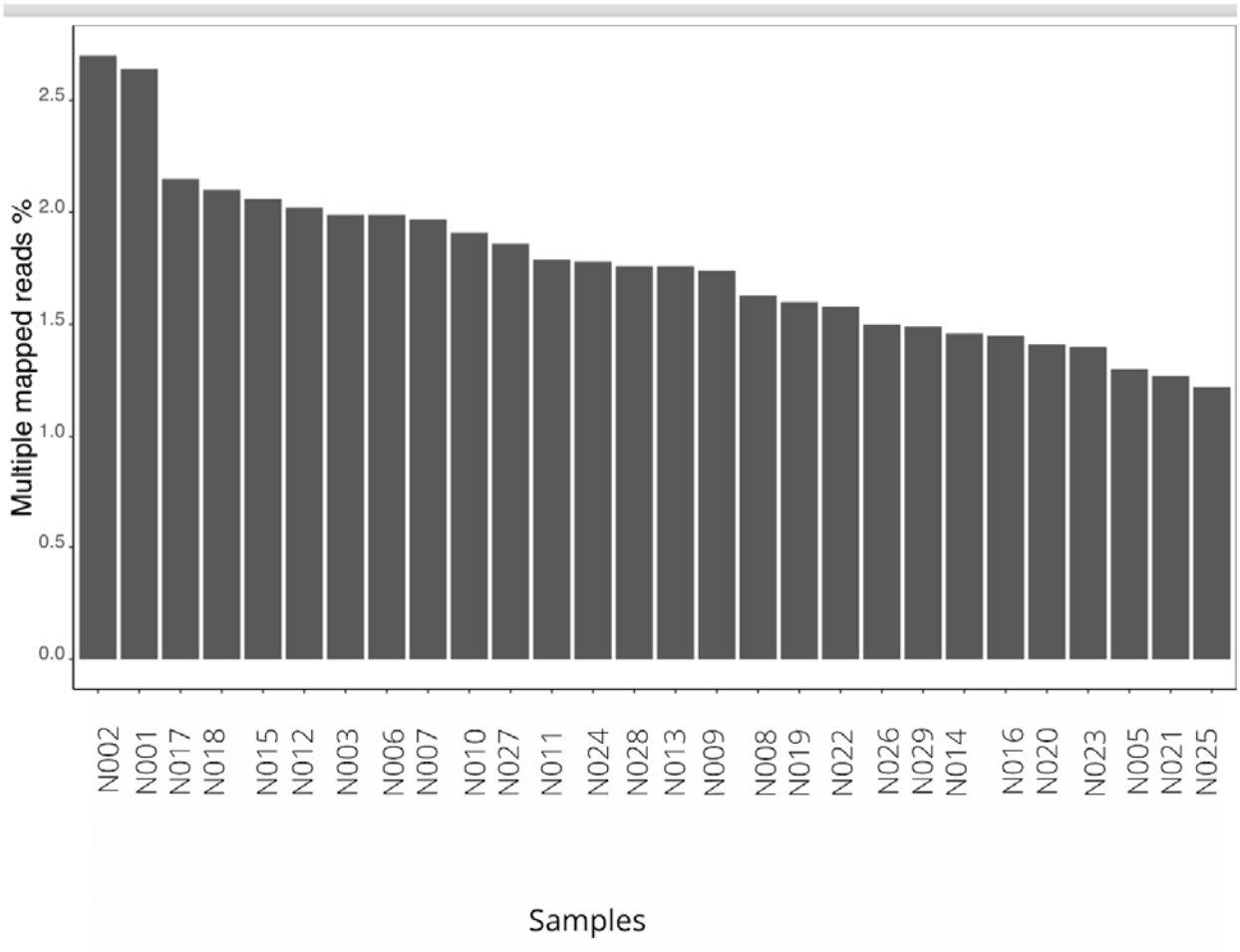

Fig. S6. Multiply mapped reads. Multiple mapped reads are reads that map more than once in the genome. This is the number of multiple mapped reads from the prepared library that are aligned to the Cory Shearwater reference genome.

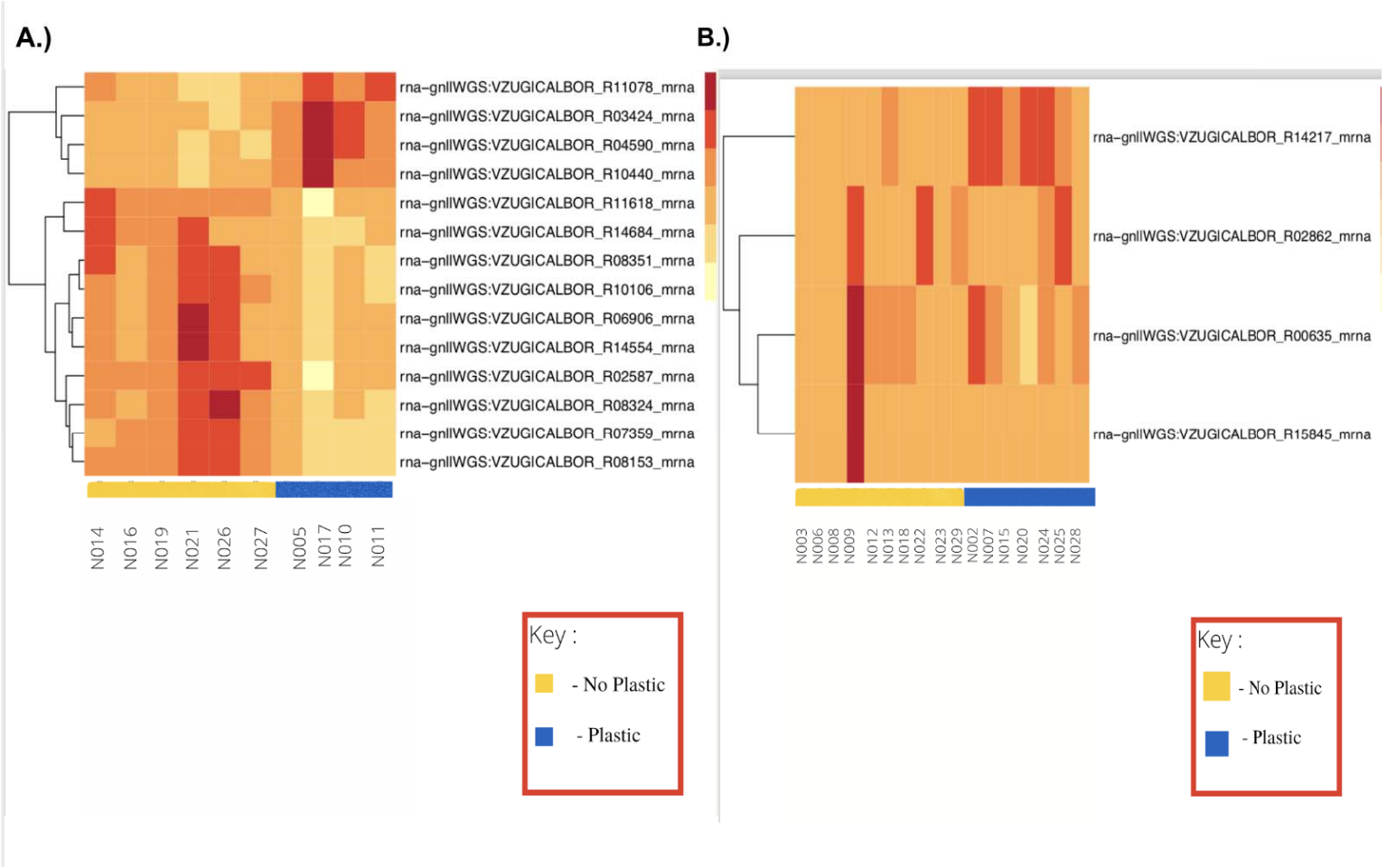

Fig. S7. **Differential gene expression analysis for plastic and sex.** (A) Heatmap showing the 14 significantly differentially expressed genes in males. (B) Heatmap showing the 4 significant differentially expressed genes in females.

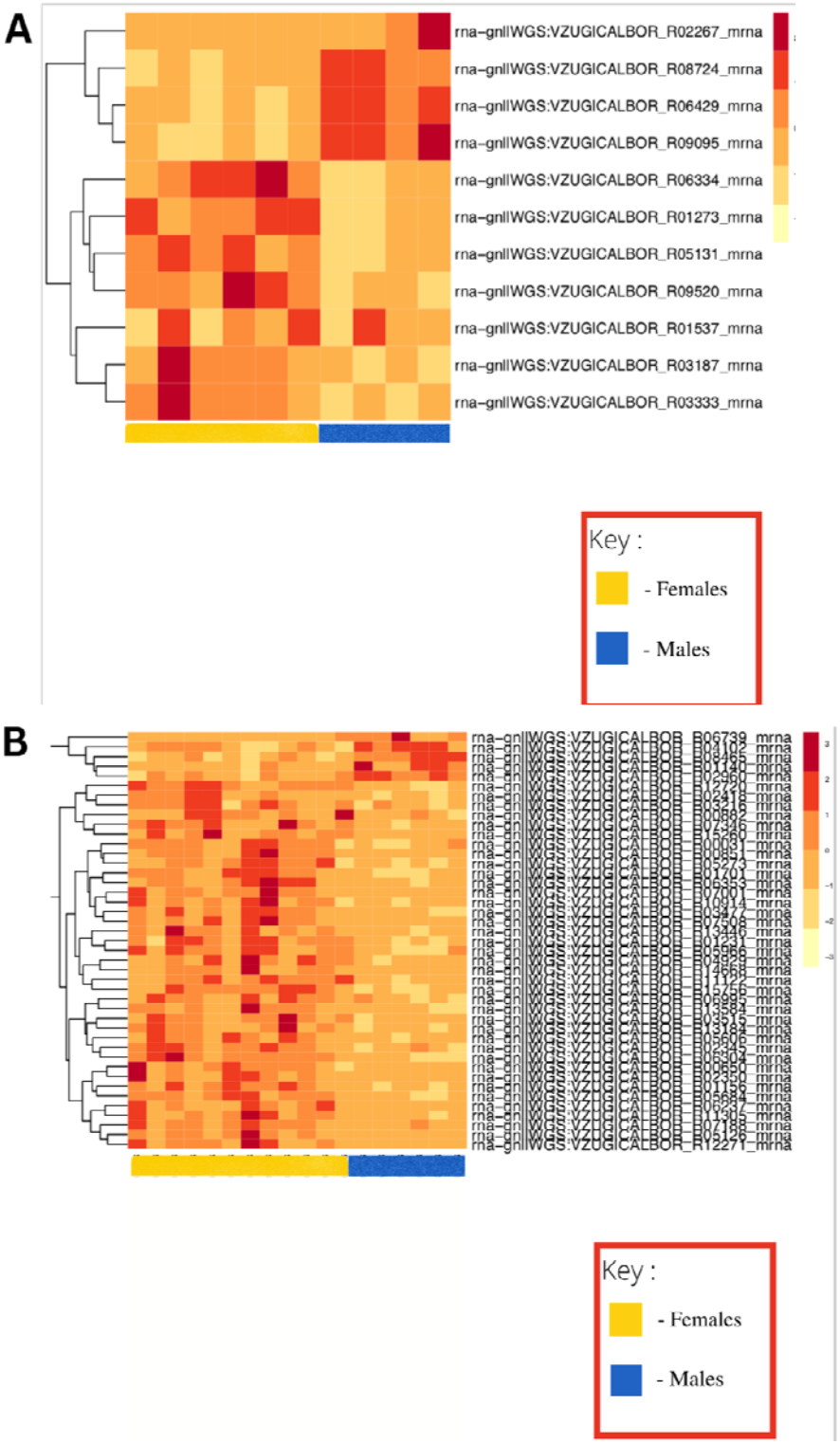

Fig. S8. Differential gene expression analysis divided into samples that had ingested plastic and samples that did not have plastic. (A) Heatmap showing the 11 significant differentially expressed genes in all of the samples containing plastic separated by sex. (B) Heatmap showing the 43 significant differentially expressed genes in all samples that do not contain plastic separated by sex.

Sample ID	RIN Score
N001	7.4
N002	7.6
N003	7.4
N005	4.8
N006	7.7
N007	7.8
N008	7.5
N009	7.1
N010	8.4
N011	7.7
N012	8.4
N013	7.3
N014	6.7
N015	8.3
N016	7.3
N017	8
N018	8
N019	6.4
N020	7.2

N021	**7.3**
N022	**7.3**
N023	**6.6**
N024	**7.6**
N025	**6.1**
N026	**6.7**
N027	**7.4**
N028	**8.3**
N029	**7**

Fig. S9. RIN Values. RIN values assign a numerical value to the quality of the RNA that we worked with. A RIN value of 8 and above indicated higher quality and integrity of RNA and values below 5 indicated some levels of RNA degradations.

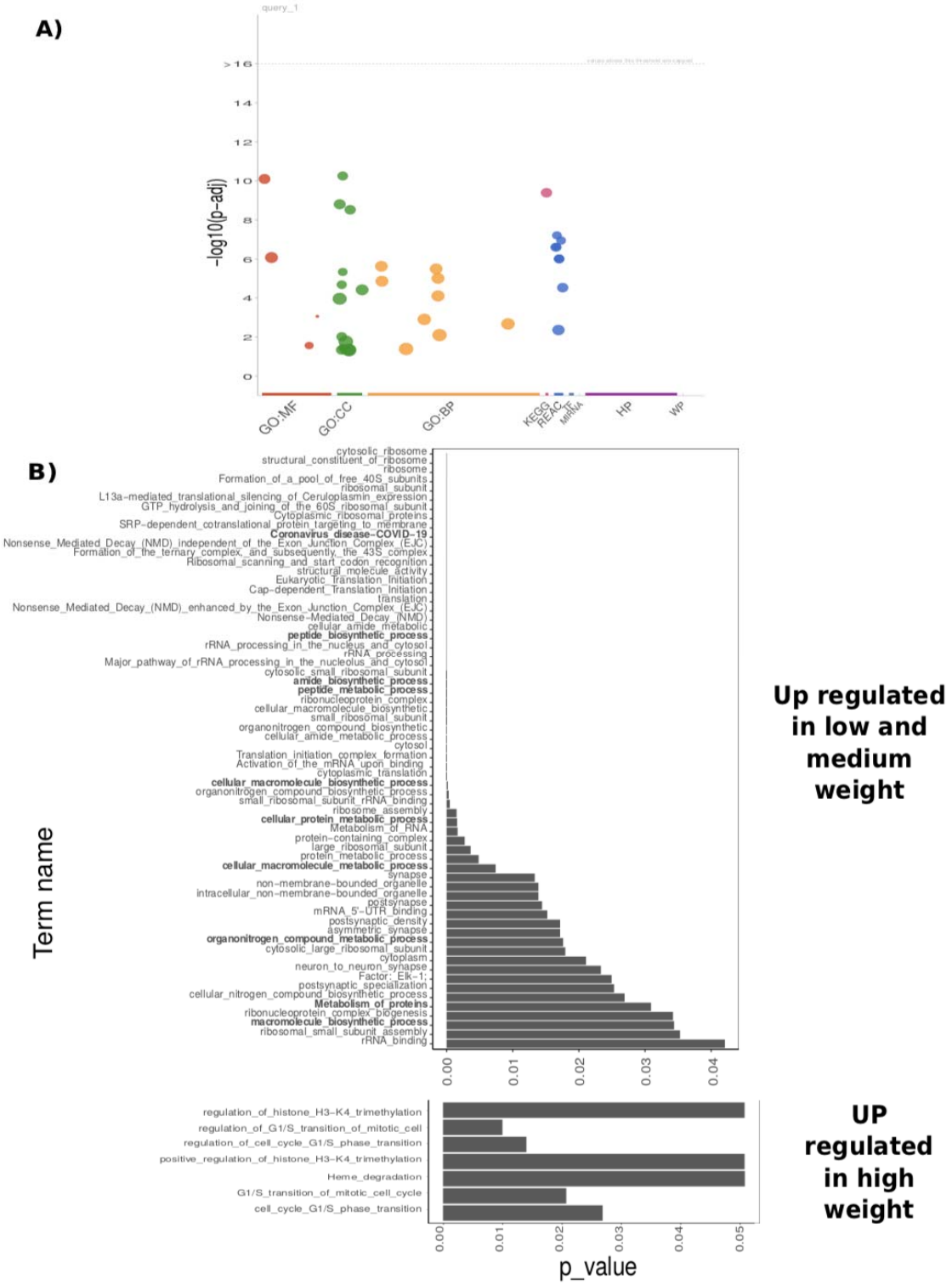

Fig. S10. Gene enrichment analysis results for bird weight. (A) Manhattan plot showing results of enrichment analysis and the databases used. (B) Terms with significant values from the enrichment analysis in the differentially expressed genes between weight categories.

Supporting References

Adams J, Felis J, Czapanskiy M. 2020. "Habitat Affinities and At-Sea Ranging Behaviors among Main Hawaiian Island Seabirds: Breeding Seabird Telemetry, 2013–2016." *BOEM*

Ashraf MA. 2017. Persistent organic pollutants (POPs): a global issue, a global challenge. *Environmental Science and Pollution Research* 24:4223–4227. DOI: 10.1007/s11356-015-5225-9.

Bighiu MA, Gorokhova E, Almroth BC, Wiklund A-KE. 2017. Metal contamination in harbours impacts life-history traits and metallothionein levels in snails. *PLOS ONE* 12:e0180157. DOI: 10.1371/journal.pone.0180157.

Briffa J, Sinagra E, Blundell R. 2020. Heavy metal pollution in the environment and their toxicological effects on humans. *Heliyon* 6:e04691. DOI: 10.1016/j.heliyon.2020.e04691.

Claessens M, Meester SD, Landuyt LV, Clerck KD, Janssen CR. 2011. Occurrence and distribution of microplastics in marine sediments along the Belgian coast. *Marine Pollution Bulletin* 62:2199–2204. DOI: 10.1016/j.marpolbul.2011.06.030.

Cózar A, Echevarría F, González-Gordillo JI, Irigoien X, Úbeda B, Hernández-León S, Palma ÁT, Navarro S, García-de-Lomas J, Ruiz A, Fernández-de-Puelles ML, Duarte CM. 2014. Plastic debris in the open ocean. *Proceedings of the National Academy of Sciences* 111:10239–10244. DOI: 10.1073/pnas.1314705111.

Désert C, Merlot E, Zerjal T, Bed'hom B, Härtle S, Le Cam A, Roux P-F, Baeza E, Gondret F, Duclos MJ, Lagarrigue S. 2016. Transcriptomes of whole blood and PBMC in chickens. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics* 20:1–9. DOI: 10.1016/j.cbd.2016.06.008.

Duffy DC, Jackson S. 1986. Diet Studies of Seabirds: A Review of Methods. *Colonial Waterbirds* 9:1–17. DOI: 10.2307/1521138.

Furness R. W., Monaghan P. 1987. *Seabird Ecology*. Blackie□; Published in the USA by Chapman and Hall.

Jax E, Wink M, Kraus RHS. 2018. Avian transcriptomics: opportunities and challenges. *Journal of Ornithology* 159:599–629. DOI: 10.1007/s10336-018-1532-5.

Liu S, Shi J, Wang J, Dai Y, Li H, Li J, Liu X, Chen X, Wang Z, Zhang P. 2021. Interactions Between Microplastics and Heavy Metals in Aquatic Environments: A Review. *Frontiers in Microbiology* 12:652520. DOI: 10.3389/fmicb.2021.652520.

Liu J, Walter E, Stenger D, Thach D. 2006. Effects of Globin mRNA Reduction Methods on Gene Expression Profiles from Whole Blood. *The Journal of Molecular Diagnostics* 8:551–558. DOI: 10.2353/jmoldx.2006.060021.

Lobenhofer EK, Auman JT, Blackshear PE, Boorman GA, Bushel PR, Cunningham ML, Fostel JM, Gerrish K, Heinloth AN, Irwin RD, Malarkey DE, Merrick BA, Sieber SO, Tucker CJ, Ward

SM, Wilson RE, Hurban P, Tennant RW, Paules RS. 2008. Gene expression response in target organ and whole blood varies as a function of target organ injury phenotype. *Genome Biology* 9:R100. DOI: 10.1186/gb-2008-9-6-r100.

Nania TG, Shugart GW. 2021. Are plastic particles reduced in size in seabirds' stomachs? *Marine Pollution Bulletin* 172:112843. DOI: 10.1016/j.marpolbul.2021.112843.

NOAA. "A Guide to Plastic in the Ocean." NOAA's National Ocean Service, https://oceanservice.noaa.gov/hazards/marinedebris/plastics-in-the-ocean.html.

NOAA. "Ocean Garbage Patches." NOAA's National Ocean Service, https://oceanservice.noaa.gov/podcast/mar18/nop14-ocean-garbage-patches.html.

Plastic debris in the open ocean | PNAS. *Available at https://www.pnas.org/doi/full/10.1073/pnas.1314705111* (accessed November 8, 2022).

Shaffer SA, Tremblay Y, Weimerskirch H, Scott D, Thompson DR, Sagar PM, Moller H, Taylor GA, Foley DG, Block BA, Costa DP. 2006. Migratory shearwaters integrate oceanic resources across the Pacific Ocean in an endless summer. *Proceedings of the National Academy of Sciences* 103:12799–12802. DOI: 10.1073/pnas.0603715103.

Verla AW, Enyoh CE, Verla EN, Nwarnorh KO. 2019. Microplastic–toxic chemical interaction: a review study on quantified levels, mechanism and implication. *SN Applied Sciences* 1:1400. DOI: 10.1007/s42452-019-1352-0.