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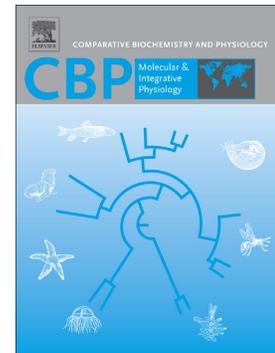
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**Beyond body condition: Experimental evidence that plasma metabolites improve nutritional state measurements in a free-living seabird**

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**ABSTRACT**

The ability to efficiently measure the health and nutritional status of wild populations in situ is a valuable tool, as many methods of evaluating animal physiology do not occur in real-time, limiting the possibilities for direct intervention. This study investigates the use of blood plasma metabolite concentrations, measured via point-of-care devices or a simple plate reader assay, as indicators of nutritional state in free-living seabirds. We experimentally manipulated the energy expenditure of wild black-legged kittiwakes (Gill & Hatch) on Middleton Island, Alaska, and measured the plasma concentrations of glucose, cholesterol, B-hydroxybutyrate, and triglycerides throughout the breeding season, along with measures of body condition (size-corrected mass [SCM] and muscle depth). Supplemental feeding improved the nutritional state of kittiwakes by increasing feeding rate (higher glucose and triglycerides, lower cholesterol), and flight-handicapping caused a slight nutritional decline (lower glucose and triglycerides, higher cholesterol and B-hydroxybutyrate). Glucose and triglycerides were the best indicators of nutritional state when used alongside SCM, and improved upon commonly used metrics for

measuring individual condition (i.e. SCM or mass alone). Metabolite concentrations varied across the breeding period, suggesting that the pre-laying stage, when feeding rates tend to be lower, was the most nutritionally challenging period for kittiwakes (low glucose, high cholesterol). Muscle depth also varied by treatment and breeding stage, but differed from other nutritional indices, suggesting that muscle depth is an indicator of exercise and activity level rather than nutrition. Here we demonstrate potential for the use of blood plasma metabolites measured via point-of-care devices as proxies for evaluating individual health, population health, and environmental food availability.

**KEYWORDS**

Blood metabolites; conservation physiology; food-supplementation; point-of-care devices; seabird

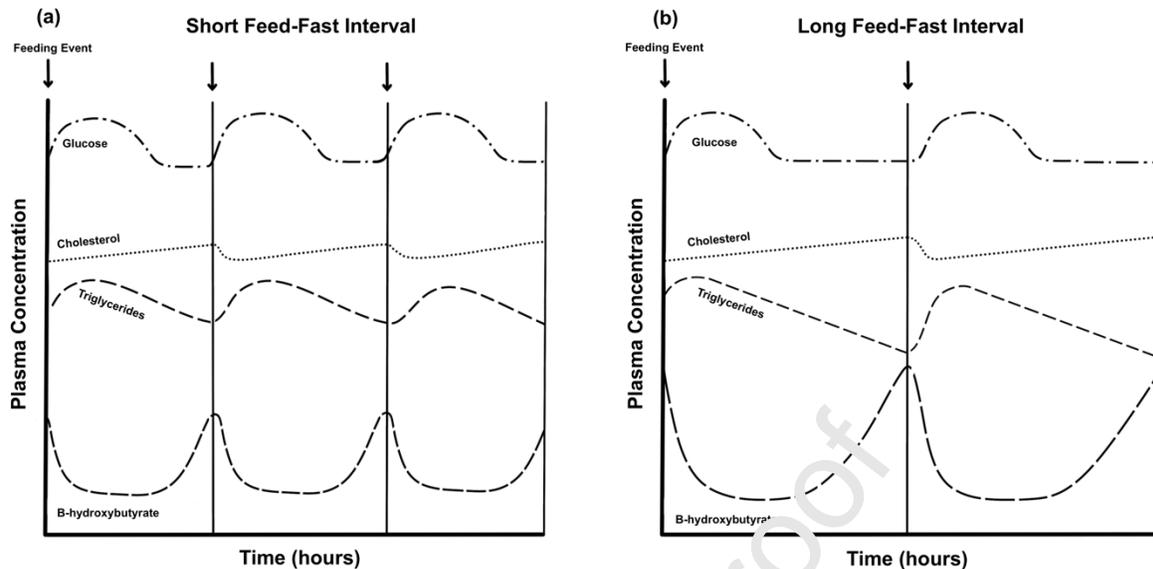
## 1. INTRODUCTION

Conservation physiology is a rapidly developing field, and altered physiology can be an early warning sign of population stress, allowing conservationists to rapidly respond to stressors long before they cause population declines (Wikelski & Cooke, 2006). Because energy is a fundamental currency in nature (Brown et al., 2004; Brown et al., 2012; Yodzis & Innes, 1992), physiological systems closely linked to energy metabolism are likely particularly sensitive indicators. Energy demands fluctuate throughout the annual cycle of an individual, and reproduction is one particularly costly period, during which energy requirements increase as individuals transition from self-maintenance to reproductive investment (Brown et al., 2004; Costa & Sinervo, 2004; Yodzis & Innes, 1992). Breeding events have significant trade-offs (Stearns, 1992; Welcker et al., 2015; Williams, 1966), and maintaining the balance between the cost of living and the cost of reproducing is critical for individual fitness, with consequences for population dynamics (Brown et al., 2004; Morales et al., 2020; Wikelski & Cooke, 2006). It is important to understand how energetic costs vary over distinct phases of the annual cycle, but measuring energy intake and expenditure in free-ranging animals is notoriously difficult (Elliott, 2016; Halsey et al., 2011). Moreover, many physiological measures of energy intake or expenditure (e.g. hormone levels, oxidative stress) do not occur in real time and require lab assays, making them challenging to apply rapidly to conservation. However, techniques for the real time measurement of energy expenditure continue to grow, including methods such as accelerometry, doubly-labelled water, and time-energy budgets.

Body condition is commonly used to evaluate the physical condition or nutritional state of wild animals (Chastel et al., 1995; Drent & Daan, 1980; Jacobs et al., 2012) and is typically determined by scaling body mass by body size, also referred to as “size-corrected mass” (Jacobs et al., 2012; Jakob et al., 1996). Yet, the specific biology of any wild animal is complex, and indices of body condition may occasionally fail to reflect nutritional state in birds. For instance, mass gain from fat deposition can indeed indicate energy storage and nutritional improvement (Cerasale & Guglielmo, 2006; Jenni-Eiermann & Jenni, 1994). However, birds exhibit high rates of phenotypic flexibility for body mass and can adaptively regulate their mass through a variety of mechanisms. Mass may be gained or lost from muscle or fat, and changes in mass are facilitated through processes including food intake, activity level, digestive efficiency, and

metabolic rate (Mathot et al., 2020). For example, gains in muscle mass have been associated with periods of extensive physical effort, such as hypertrophy of pectoral muscles in preparation for migration (Lindstrom et al., 2000; Marsh, 1984; Swanson & Merkord, 2013). Thus, in years when food availability is poor, muscle mass may increase due to increased flight required to find food. Therefore, drawing inference from multiple metrics is generally more informative than relying on any one metric alone, and measures of body condition index may be more reliable when paired with other methods of quantifying nutritional state.

Blood plasma metabolites can offer clues about an individual's nutrition, diet quality, body condition, and other factors intrinsically linked with energy intake and metabolism (Alonso-Alvarez & Ferrer, 2001; Lerma et al., 2022; Lindholm & Almiras, 2016). Energy from food is used in cellular processes, for self-maintenance or the production of new biomass—either for growth or reproduction (Brown et al., 2004). Metabolites including glucose, cholesterol, BUTY, and triglycerides vary with nutritional condition (Alonso-Alvarez & Ferrer, 2001; Castellini & Rea, 1992; Morales et al., 2020). Glucose is a simple carbohydrate that is often considered the most important energy source for living organisms, and a spike in glucose is associated with recent feeding events. Glucose concentrations peak shortly after feeding, before dropping to a slightly lower level, where they remain at a consistent concentration in the body as an animal enters a fasting period (Alonso-Alvarez & Ferrer, 2001; Braun & Sweazea, 2008; Morales et al., 2020). Circulating cholesterol levels are representative of long-term dietary intake, and during fasting cholesterol concentrations are expected to increase over time, with higher cholesterol levels reflecting longer periods of fasting and lower nutritional status (Morales et al., 2020). Beta-hydroxybutyrate is a ketone body produced when an animal is in a fasting state (Lerma et al., 2022; Morales et al., 2020). High levels of B-hydroxybutyrate (hereafter “BUTY”) represent the mobilization of lipids from energy reserves, and the conversion of fatty acids to BUTY spares proteins and carbohydrates from being used as glucose precursors during fasting (Alonso-Alvarez & Ferrer, 2001; Castellini & Rea, 1992; Morales et al., 2020). Triglycerides are fats that constitute the largest stock of fuel in the body. They are used as a source of energy for muscles and for storage of energy reserves in adipose tissues (Alonso-Alvarez & Ferrer, 2001; Buyse & Decuyper, 2015; Morales et al., 2020). During fasting, triglyceride levels decline as fat reserves are used for energy.



**Fig. 1.** Predicted changes of plasma metabolite composition following a feeding event and subsequent fasting in birds, under (a) short feed-fast intervals (higher feeding rates) and (b) long feed-fast intervals (lower feeding rates). A bird with lower feeding rates when sampled at a random time, would on average have lower glucose and triglycerides, and higher cholesterol and BUTY than a bird with higher feeding rates. Modified from Alonso-Alvarez and Ferrer (2001) and Morales et al. (2020). The timescale on the x-axis is for illustrative purposes only.

Glucose, cholesterol, BUTY, and triglycerides have been characterized through experiments where plasma metabolites are measured under fasting conditions. The metabolite concentrations in the blood stream change throughout the duration of a food-restricted period (Fig. 1), revealing predictable trends that range from an animal's last meal until complete starvation (Alonso-Alvarez & Ferrer, 2001; Castellini & Rea, 1992; Morales et al., 2020). For instance, high glucose and triglyceride concentrations indicate recent feeding events, and are typically indicators of good nutritional state (Fig 1). Meanwhile, high cholesterol and BUTY levels have been associated with fasting conditions and poorer nutritional status in individuals (Morales et al., 2020). Evaluating the relative levels of these four blood metabolites can give a holistic evaluation of recent feeding intensity and nutritional state in wild animals.

Body condition index (i.e. size corrected mass) is another metric commonly used to assess the nutritional state of animals, and is typically representative of more seasonal changes and longer-term nutritional status. An animal's body condition is influenced by its ability to

forage effectively, and animals that consistently demonstrate effective foraging ability will generally have a higher body condition index. Although plasma metabolites reflect recent feeding intensity and nutritional status on a rather short time scale, the use of plasma metabolite panels, when used in conjunction with other measures of condition, may improve upon current indices of nutritional assessment beyond body condition index.

Recently, the use of point-of-care (POC) devices—such as simple glucose and cholesterol meters readily available at most pharmacies—has made the quantification of many metabolites more accessible (Lindholm & Altimiras, 2016; Morales et al., 2020; Stoot et al., 2014). These devices enable the user to analyze metabolite panels quickly in small samples of whole blood or plasma, opening the door to rapid *in situ* assessment of nutritional state and condition of wild animals.

Here we investigate how metabolites vary with the nutritional state of free-ranging animals, using a wild population of black-legged kittiwakes (*Rissa tridactyla*, hereafter “kittiwake”). We experimentally manipulated the energy intake or expenditure of individuals and assessed changes in metabolites levels over three consecutive breeding stages: pre-laying, incubation, and chick-rearing. We hypothesized that increased food availability would shorten the feed-fast interval, and thereby improve the nutritional state of individuals as reflected in plasma metabolite levels. Furthermore, we predicted that a flight-handicap would lengthen the feed-fast interval, and thereby cause nutritional decline. We also hypothesized that plasma metabolite levels would differ between breeding stages due to changing energy demands. We predicted that fed birds would have higher glucose and triglyceride levels, and unfed birds would have higher cholesterol and BUTY levels (Morales et al., 2020) across all breeding stages. We also predicted that handicapped birds would have higher cholesterol and BUTY levels, but lower glucose and triglycerides than fed or unfed birds (Morales et al., 2020). Because energy expenditure of seabirds peaks during incubation and chick-rearing, we predicted that the nutritional state of individuals would be better in pre-laying compared to incubation and chick-rearing, but similar between incubation and chick-rearing (Tremblay et al., 2022). We also tested if metabolites vary according to size-corrected mass (SCM) and pectoral muscle depth, as these measures are commonly used to assess the nutritional state or body condition of individuals (Jacobs et al., 2012; Jenni-Eiermann & Jenni, 1994; Marsh, 1984). We expected muscle depth to be a good metric of nutritional condition because kittiwakes have low percent lipids, and most of

the variation in body mass from variable environmental conditions is due to lean mass changes (Jacobs et al., 2011; Jacobs et al., 2012). Moreover, Jacobs et al. (2012) found that none of 56 measures of SCM, including scaled mass index and residual of mass on size, improved prediction of total lipids compared to mass alone, and so we expected that including size in models would not improve models for nutritional state.

## 2. MATERIALS AND METHODS

### 2.1 Study System

We conducted the study across the 2022 breeding season (late April to late July) on a population of kittiwakes nesting on an abandoned radar tower on Middleton Island, Alaska. We observed artificial nest sites built on upper sections of the tower from within the tower through sliding one-way windows (Gill & Hatch, 2002), which facilitated capture and monitoring of breeding adults. We determined the sex of breeding individuals by repeated behavioral observations (e.g., courtship feeding, copulations) or through morphometric measurements (for two individuals only).

We sampled and divided breeding adult kittiwakes for this study ( $n = 160$ ) into three treatments. Food-supplemented ( $n = 70$ ): we supplementally fed fish (herring, *Clupea* sp and capelin, *Mallotus villosus*) to individuals to increase net energy intake, as food resources were made readily available and flight foraging costs were reduced, thereby improving nutritional state. We provided breeding pairs with fish *ad libitum* via a PVC tube at their nest site, three times daily from early May until mid-August (Gill & Hatch, 2002; Whelan et al., 2020) Food-supplemented individuals reduce, but do not eliminate, natural foraging at sea (Kahane-Rapport et al., 2022). We later excluded four individuals from analyses, as samples were collected prior to the beginning of food supplementation in April. Handicapped ( $n = 20$ ): we gave individuals a flight handicap to decrease net energy intake by experimentally reducing flight efficiency, thereby worsening nutritional state. To reduce flight efficiency, we conducted a feather clipping procedure on ten breeding pairs during the incubation period following methods by Leclaire et al. (2011). We cut selected flight feathers close to the base (primary remiges P8, P6, and P4 on each wing, and rectrices R4 and R2 on either side of the tail). Reductions in wing area are compensated by increased wing flapping, thus increasing the energetic cost of flight (Pennycuik, 1989). The handicap remains for the duration of the breeding season, but

disappears after the post-breeding moult when birds replace their flight feathers (Pyle, 2008), limiting the effect of the handicap to approximately four months. We only sampled nine handicapped birds later in chick-rearing to test the effects of treatment, while the remaining individuals were excluded due to nest failure. Control (n = 70): we sampled individuals that experienced the same blood sampling procedure, but no treatment, which therefore only foraged naturally at sea.

## 2.2 Measuring Plasma Metabolites

We collected blood samples within 3 minutes of capture to determine baseline metabolite concentrations (except for three individuals that slightly exceeded that time limit; 3:02, 3:10, and 3:40). Further, there was no relationship between time sampled and values for any metabolites ( $p > 0.05$ ). Blood samples were collected from the brachial vein (1 ml syringe, 25-gauge heparinized needle) and were tested immediately after collection with POC devices based on methods described in Morales et al. (2020), to quantify levels of glucose, cholesterol, and BUTY using Contour®Next (detection range = 20–500mg/dL, CV = 1.3–2.1%), Precision Xtra® (detection range = 0.1–8.0mmol/L, CV = 3.2%), and CardioChek® (detection range = 100–400mg/dL, CV = 9.1%) devices, respectively (Morales et al., 2020). Following POC testing, we centrifuged the remaining blood samples to separate plasma from red blood cells, and froze plasma at  $-20^{\circ}\text{C}$  until later analysis in the lab. We determined plasma triglyceride concentrations with laboratory analyses, using methods described in Morales et al. (2020), with the Fujifilm Wako LabAssay™ Triglyceride kit. This kit reports total triglycerides, incorporating measures of free glycerol within a single step. Prior to analysis, frozen plasma samples were thawed and vortexed. We performed all assays according to the manufacturer's protocol, and read the plates using a BioTek Epoch 2 Microplate Spectrophotometer. To accommodate samples with exceptionally high triglyceride concentrations, we extended the standard curve range of each plate up to 1480 mg/dL, and performed dilutions on samples that remained beyond the standard curve range.

## 2.4 Morphometric Measurements

We recorded morphometric measurements for each individual, including body mass to the nearest gram and headbill length (back of skull to tip of culmen) to the nearest millimeter. We

used a linear regression between body mass and headbill measurements to determine SCM. We measured pectoral muscle depth during the incubation and chick-rearing stages following methods described in Le Bot et al. (2019), using a TITAN© portable ultrasound system (Sonosite, Inc. USA) with a HST/10–5 MHz broadband transducer. Primary pectoral measurements were recorded by positioning the probe at a 45° angle to the keel, with the probe extending out toward the clavicle. We then recorded secondary pectoral measurements for each individual, in which the probe was positioned perpendicular to the length of the keel. Measurements were recorded in the same positions for every individual, and we found that both measurement techniques produced highly similar results. We used data from only the secondary pectoral measurement for all subsequent analyses.

### *2.5 Statistical Analyses*

We fitted linear models to determine the effects of supplemental feeding treatment and breeding stage on metabolite concentrations within the population. In all metabolite models, we included feeding treatment (control, food-supplemented), breeding stage, sex, and SCM as fixed factors. We normalized the data for BUTY and triglyceride concentrations by natural logarithm transformation in order to meet the assumptions of homoscedasticity and normality of residuals in each linear model.

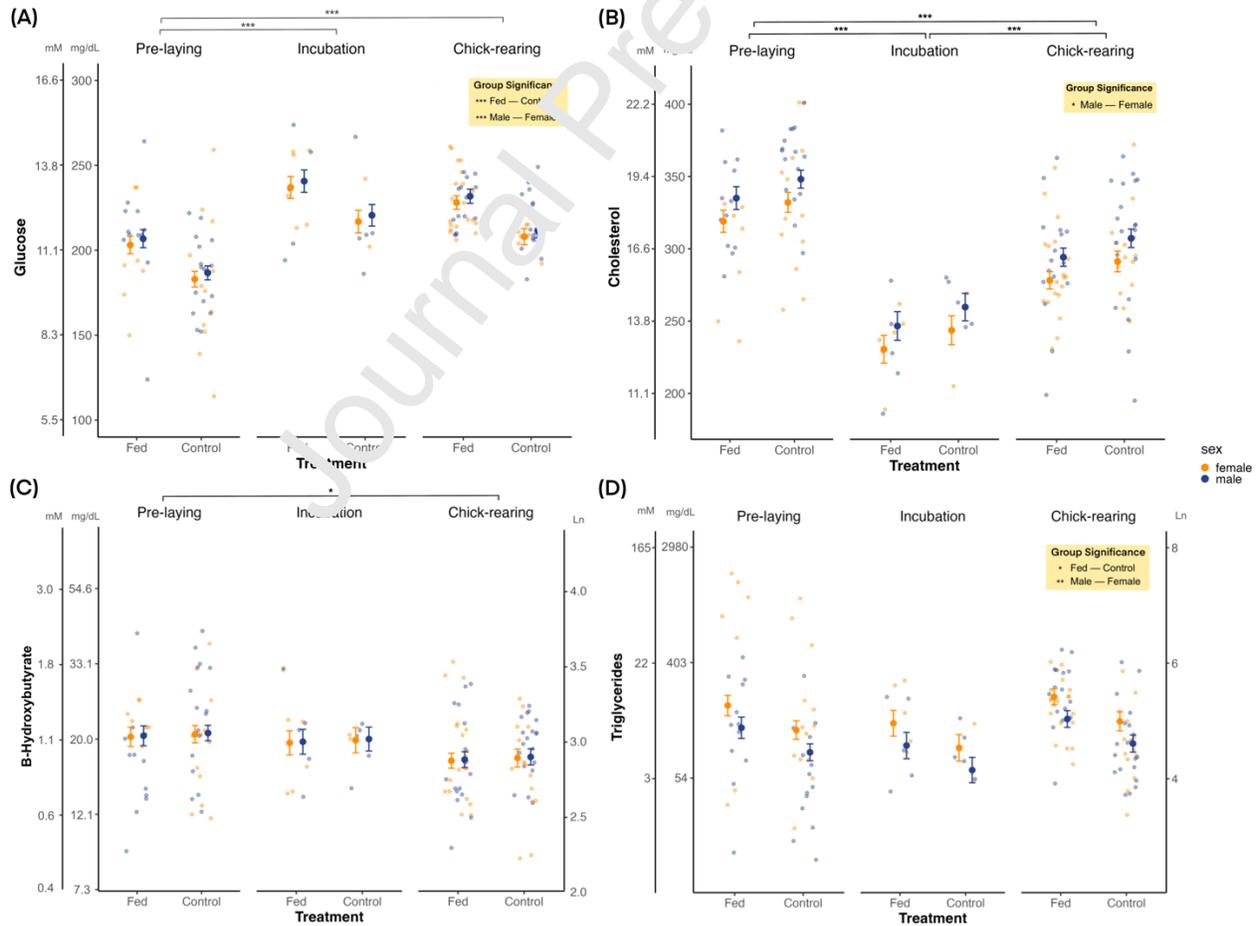
The feather clipping procedure was carried out during the incubation stage, so the effects of the handicap treatment were generally measurable only during the chick-rearing stage. We thus performed separate analyses to determine the effect of the handicap treatment on metabolite concentrations with a second group of linear models that were fitted to incorporate all treatment groups within the chick-rearing stage only. In these models, we included treatment (control, food-supplemented, handicapped), sex, and SCM as fixed factors. We normalized the data for BUTY and triglyceride concentrations by natural logarithm transformation in order to meet the assumptions of homoscedasticity and normality of residuals in each linear model.

We also fitted a third group of linear models to assess changes in SCM and pectoral muscle depth in response to treatment and breeding stage within the population. We included treatment, breeding stage, and sex as fixed factors for the SCM model. We included treatment, breeding stage, SCM, and sex for the muscle depth models.

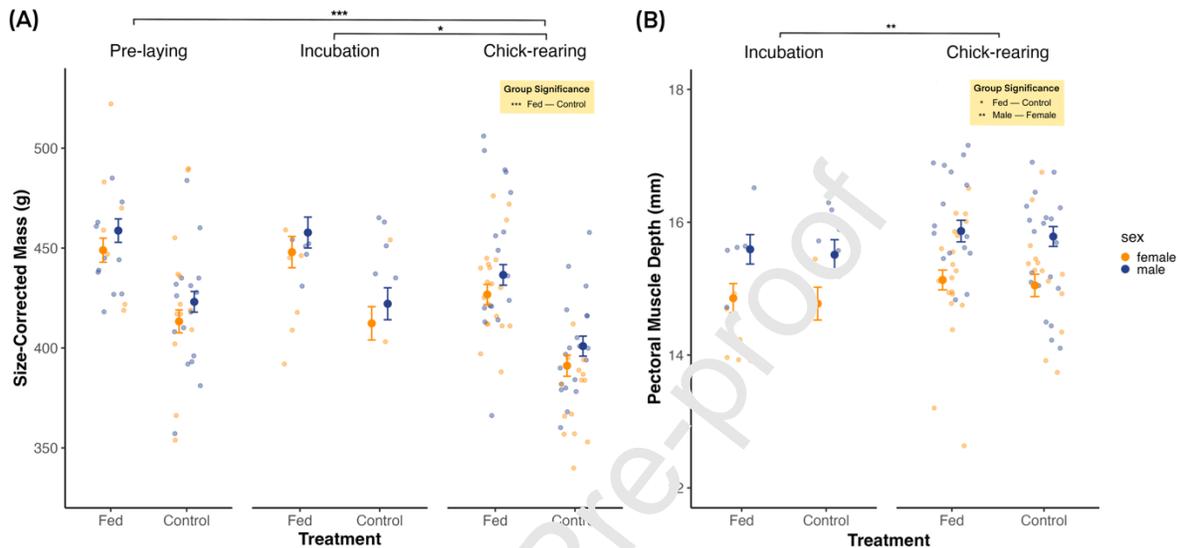


<b>Glucose</b>	240.17	<b>-33.92***</b> CI[-49.93, -17.9]	<b>-25.03***</b> CI[-36.49, 13.6]	8.89 CI[-6.73, 24.5]	<b>-20.1***</b> CI[-29.6, -10.6]	-3.72 CI[-12.0, 4.51]	-0.13 <sup>o</sup> CI[-0.27, 0.001]
<b>Cholesterol</b>	407.96	<b>88.5***</b> CI[64.4, 112.5]	<b>40.9***</b> CI[23.7, 58.1]	<b>-47.6***</b> CI[-71.0, -24.2]	13.1 <sup>o</sup> CI[-1.2, 27.4]	<b>-16.1*</b> CI[-28.4, -3.69]	-0.17 <sup>o</sup> CI[-0.38, 0.02]
<b>Ln (BUTY)</b>	3.57	0.039 CI[-0.16, 0.24]	<b>0.158*</b> CI[0.015, 0.302]	0.118 CI[0.076, 0.314]	0.018 CI[-0.10, 0.13]	-0.007 CI[-0.111, 0.095]	-0.001 CI[-0.002, 0.0004]
<b>Ln (Triglycerides)</b>	2.04	0.308 CI[-0.248, 0.864]	-0.151 CI[-0.552, 0.249]	-0.459 CI[-1.000, 0.081]	<b>-0.425*</b> CI[-0.75, -0.09]	<b>0.385**</b> CI[0.098, 0.672]	<b>0.006**</b> CI[0.001, 0.011]
<b>Size-Corrected Mass (SCM)</b>	413.3	0.968 CI[-19.21, 21.1]	<b>22.15***</b> CI[8.50, 35.8]	<b>21.18*</b> CI[2.02, 40.4]	<b>-5.5***</b> CI[-14.9, -2.3]	-9.79 <sup>o</sup> CI[-20.0, 0.45]	————
<b>Muscle Depth</b>	9.52	————	————	<b>-0.539**</b> CI[-0.942, -0.137]	<b>0.438*</b> CI[0.048, 0.828]	<b>-0.469**</b> CI[-0.805, -0.134]	<b>0.013***</b> CI[0.007, 0.018]

Significance of interactions are indicated by p-value: [0,0.001]\*\*\*, (0,0.01,0.01]\*\*, (0.01,0.05)\*, (0.05,0.1)<sup>o</sup>, (0.1,1).



**Fig. 2.** Plasma metabolite concentrations of fed and control individuals during pre-laying, incubation, and chick-rearing. Significance of interactions for treatment group are averaged over breeding stage and sex. Significance of interactions for sexes are averaged over breeding stage and treatment group.



**Fig. 3.** Size-corrected mass and pectoral muscle depth of fed and control individuals during pre-laying, incubation, and chick-rearing. Mass was corrected using measurements of headbill length. Significance of interactions for treatment group are averaged over breeding stage and sex. Significance of interactions for sexes are averaged over breeding stage and treatment group.

### 3.2 Handicapped Treatment

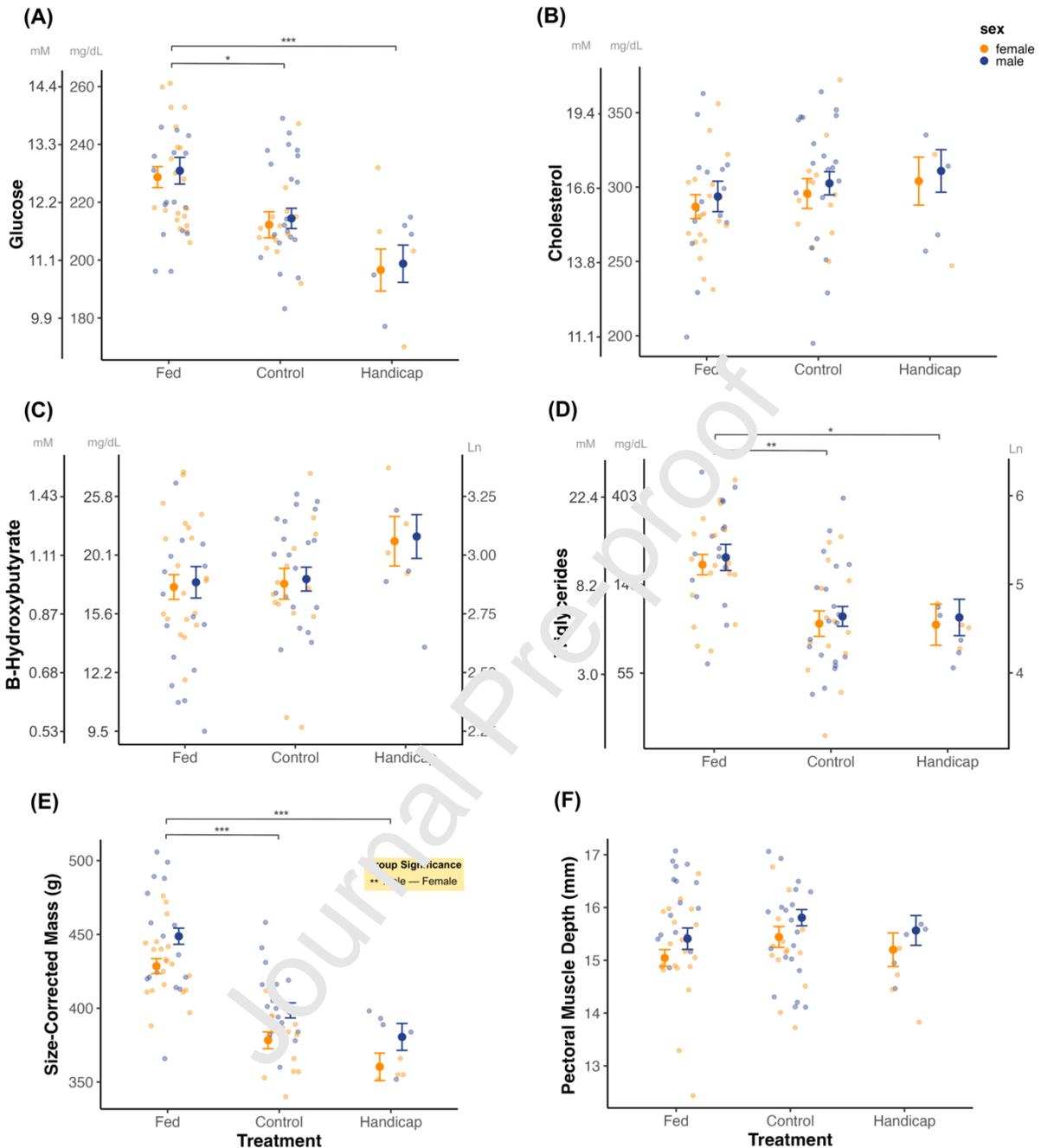
The effects of feather clipping were reflected among individuals by variation in metabolite concentrations between the handicapped, fed, and control treatments (Table 2, Fig 4A–D). There were no significant effects of the handicapped treatment compared to control. However, glucose and triglyceride levels were significantly lower in the handicapped group than the fed group. Handicapped birds (mean = 198 mg/dL  $\pm$  6.48) had 13.9% lower glucose than fed birds (mean = 230 mg/dL  $\pm$  3.63). Similarly, handicapped birds (mean = 4.59  $\pm$  0.21 ln[mg/dL]) had 12.7% lower triglyceride levels than fed birds (mean = 5.26  $\pm$  0.12 ln[mg/dL]). Handicapped individuals also had significantly lower SCM than the fed individuals (Fig. 4E). Handicapped birds (mean = 370 g  $\pm$  8.71) had 15.7% lower SCM than fed birds (mean = 439 g  $\pm$  4.37). Finally, pectoral muscle depth was similar between all treatments. (Fig. 4F).

**Table 2.**

Results of linear model estimates and post hoc analyses for fed, handicapped, and control treatments during chick-rearing. Significant results are shown in bold.

Response Variable	Intercept	Explanatory Variables (Model Estimates, Confidence Interval, and Significance)				
		Control vs. Fed	Control vs. Handicapped	Fed vs. Handicapped	Sex Female vs. Male	Size-Corrected Mass (SCM)
Glucose	261.96	<b>-16.5*</b> CI[-30.29, -2.68]	15.7 <sup>o</sup> CI[-0.479, 31.79]	<b>32.1***</b> CI[11.93, 52.35]	-2.2 CI[-10.6, 6.19]	-0.121 CI[-0.272, 0.030]
Cholesterol	334.98	8.83 CI[-21.9, 39.5]	-8.32 CI[-44.2, 27.6]	-17.15 CI[-62.1, 27.8]	-6.87 CI[-25.5, 11.]	-0.095 CI[-0.433, 0.241]
Ln (BUTY)	3.09	0.013 CI[-0.187, 0.213]	0.18 <sup>o</sup> CI[-0.456, 0.052]	0.195 CI[-0.488, 0.098]	-0.02 CI[-0.142, 0.102]	-0.0005 CI[-0.475, 0.636]
Ln (Triglycerides)	4.16	<b>-0.666**</b> CI[-1.106, -0.225]	0.012 CI[-0.503, 0.527]	<b>0.678*</b> CI[0.032, 1.323]	-0.081 CI[-0.35, 0.186]	0.0009 CI[0.003, 0.005]
Size-Corrected Mass (SCM)	378.26	<b>-50.2***</b> CI[-65.64, -34.8]	18.0 CI[-5.96, 41.9]	<b>68.2***</b> CI[44.36, 92.0]	<b>-20.2**</b> CI[-32.1, -8.41]	—
Muscle Depth	10.20	0.35 <sup>o</sup> CI[-0.20, 0.903]	0.242 CI[-0.466, 0.949]	-0.156 CI[-1.042, 0.730]	-0.365 <sup>o</sup> CI[-0.733, 0.002]	<b>0.012***</b> CI[0.006, 0.019]

Significance of interactions are indicated by p-value: [0,0.001]\*\*\*, (0.001,0.01]\*\*, (0.01,0.05]\*, (0.05,0.1]<sup>o</sup>, (0.1,1].



**Fig. 4.** Measures for fed, control, and handicapped birds during the chick-rearing stage. Plasma metabolite concentrations include glucose (A), cholesterol (B), natural log transformed BUTY (C), and natural log transformed triglycerides (D). Other measures of body condition include size-corrected mass using headbill length (E), and pectoral muscle depth (F). Significance of interactions for sexes are averaged over treatment group.

### 3.3 Breeding Stage

The effects of breeding stage were reflected among individuals by variation in metabolite concentrations throughout the breeding season (Table 1, Fig. 2). Post hoc analyses revealed that glucose levels were significantly different between pre-laying and incubation, and between pre-laying and chick-rearing. Glucose levels in pre-laying (mean = 195 mg/dL  $\pm$  3.56) were 14.8% lower than in incubation (mean = 229 mg/dL  $\pm$  5.67), and 11.4% lower than in chick-rearing (mean = 220 mg/dL  $\pm$  2.93). Cholesterol levels were significantly different between all stages. Cholesterol levels in pre-laying (mean = 333 mg/dL  $\pm$  5.24) were 35.9% higher than incubation (mean = 245 mg/dL  $\pm$  8.5), and 14% higher than chick-rearing (mean = 292 mg/dL  $\pm$  4.4). Chick-rearing cholesterol levels were 19.2% higher than incubation levels. BUTY levels were significantly different between pre-laying and chick-rearing. BUTY levels in pre-laying (mean = 3.03  $\pm$  0.04) were 5.6% higher than chick-rearing levels (mean = 2.87  $\pm$  0.03). SCM was significantly different between incubation and chick-rearing, and between pre-laying and chick-rearing (Fig. 3). Birds in chick-rearing (mean = 414 g  $\pm$  3.58) had 5.0% lower SCM than birds in pre-laying (mean = 436 g  $\pm$  4.37) and birds in incubation (mean = 435 g  $\pm$  7.08). Pectoral muscle depth was 3.3% higher in chick-rearing (mean = 15.5 mm  $\pm$  0.08) than in incubation (mean = 15 mm  $\pm$  0.17, Fig. 3).

### 3.4 AIC Model Selection

AIC model selection revealed that incorporating measures of plasma metabolites, in addition to SCM, improved model fit. The best-fit model for nutritional status using fed and control birds as proxies for nutritional state included breeding stage, sex, SCM, glucose, and triglycerides as predictor variables (AIC weight = 0.48, Table 3). The best-fit model for nutritional status using handicapped and control birds as proxies for nutritional state included SCM, glucose, triglycerides, cholesterol, and BUTY as predictor variables (AIC weight = 0.33, Table 4).

#### **Table 3.**

AIC table of generalized linear models with different fixed effect terms to investigate the relationship between nutritional status (supplementally fed or control treatments, as proxies for nutritional status) and different measures of individual condition (plasma metabolites, SCM,

breeding stage, sex). Only models with AIC < 4.0 are shown, along with the model for SCM alone and the null model.

Model	AIC	$\Delta$ AIC	Weight
<b>Nutritional Status ~ Stage + SCM + Glucose + Triglycerides</b>	<b>136.81</b>	<b>0.00</b>	<b>0.48</b>
Nutritional Status ~ Stage + SCM + Glucose + Triglycerides + Cholesterol	137.83	1.02	0.29
Nutritional Status ~ Stage + SCM + Glucose + Triglycerides + Cholesterol + BUTY	139.46	2.65	0.13
Nutritional Status ~ Stage + SCM + Sex + Glucose + Triglycerides + Cholesterol + BUTY	140.70	3.89	0.07
Nutritional Status ~ SCM	159.62	22.81	0.00
Null model	190.45	53.64	0.00

*Note: the most supported model is shown in bold.*

#### Table 4.

AIC table of generalized linear models with different fixed effect terms to investigate the relationship between nutritional status (handicapped or control treatments, as proxies for nutritional status) and different measures of individual condition (plasma metabolites, SCM, breeding stage, sex). Only models with AIC < 4.0 are shown, along with the model for SCM alone and the null model.

Model	AIC	$\Delta$ AIC	Weight
<b>Nutritional Status ~ SCM + Glucose + Triglycerides + Cholesterol + BUTY</b>	<b>39.38</b>	<b>0.00</b>	<b>0.33</b>
Nutritional Status ~ Sex + SCM + Glucose + Triglycerides + Cholesterol + BUTY	40.13	0.75	0.23
Nutritional Status ~ SCM + Glucose	41.54	2.16	0.11
Nutritional Status ~ BUTY	41.58	2.20	0.11
Nutritional Status ~ SCM + Glucose + Triglycerides + Cholesterol	43.15	3.76	0.05
Nutritional Status ~ SCM	43.87	4.49	0.03
Null model	46.22	6.83	0.01

*Note: the most supported model is shown in bold.*

## 4. DISCUSSION

We investigated how plasma metabolites can reflect variations in the nutritional state of free-ranging animals by manipulating the energetics of kittiwakes across the breeding period, a critical part of the annual cycle of animals in terms of energy demands. As predicted, plasma metabolite concentrations varied according to treatment group and breeding stage. Measures of glucose, triglycerides, and SCM were the best markers of individual nutritional status, reflecting that supplementally fed individuals were in better nutritional state (higher glucose, triglycerides, and SCM) than unfed or handicapped individuals overall. Thus, plasma metabolite concentrations adequately reflected the nutritional state of kittiwakes in response to manipulation

of energy expenditure throughout the breeding season, and in the directions expected from captive studies. Glucose, triglycerides, and SCM were the overall best indicators of nutritional status, and these measures confirmed that supplementally fed individuals were in better nutritional state, with significantly higher levels of glucose and triglycerides. Kittiwakes showed signs of longer feed-fast intervals and worse nutritional state during pre-laying (low glucose, high cholesterol), but later improved nutritional state during incubation and chick-rearing stages (higher glucose, lower cholesterol), as feeding frequency likely increased. In short, we argue that point-of-care devices, especially in conjunction with SCM, can measure nutritional state in free-ranging birds. Our results confirm that measures of plasma metabolites improve upon commonly used indices of individual condition (i.e. SCM or body condition) highlighting the potential for use of plasma metabolites in advancing current metrics for nutritional assessment in wild populations. Overall, this study contributes to the development of diagnostic tools for use in conservation physiology and monitoring of free-ranging animals.

#### *4.1 Experimental Treatments*

Plasma metabolite levels clearly reflected the nutritional state of individuals in each treatment group. Fed birds had significantly elevated levels of glucose (Fig. 2A) and triglycerides (Fig. 2D), while concentrations of cholesterol (Fig. 2B) and BUTY (Fig. 2C) were similar to control. These results provide evidence that the supplemental feeding treatment had a net positive effect on the nutritional state of kittiwakes, and that increased access to food was reflected in the metabolite panel. Moreover, handicapped individuals had significantly lower concentrations of glucose and triglycerides than fed birds, and slightly elevated levels of cholesterol and BUTY (Fig. 4A–D). However, there were no significant effects of the handicapped treatment compared to control. (Fig. 4).

The lack of significant effects between the handicapped and control group may be explained by the underrepresentation of the handicapped treatment as a result of the small sample size included in the study ( $n=9$ ). Alternatively, handicapped kittiwakes may be operating at their physiological limit, with little flexibility to maintain breeding efforts if subjected to further condition decline (Chastel et al., 1995; Drent & Daan, 1980; Erikstad et al., 1998). For example, Erikstad et al. (1998) explores a model in which long-lived birds breeding in stochastic environments face a threshold in environmental conditions, where breeding is profitable and

where breeding efforts sharply increase. Many long-lived species follow the ‘fixed investment hypothesis’, meaning that parents may be restrictive in increasing reproductive investment, rather choosing to prioritize self-survival and future reproduction attempts (Erikstad et al., 1998; Navarro & González-Solís, 2007). This strategy has been clearly demonstrated in Cory’s shearwaters (*Calonectris borealis*), in which individuals with a flight handicap prioritized maintenance of their own physiological condition at the expense of current reproductive output (Navarro & González-Solís, 2007). Abandonment of eggs and chicks has also been observed in the blue petrel (*Halobaena caerulea*), Canada goose (*Branta canadensis*), and common eider (*Somateria mollissima*) when body mass reaches a critical lower threshold (Aldrich & Raveling, 1983; Chaurand & Weimerskirch, 1994; Erikstad et al., 1993). Similarly, Leclaire et al. (2011) found that kittiwakes had a tendency for fixed reproductive investment, and that male birds given a flight handicap generally reduced parental effort. We observed similar trends for individuals in this study, as many pairs experienced nest failure throughout the handicap treatment, suggesting the use of the fixed reproductive investment strategy with individuals redirecting energy intake towards self-maintenance.

Furthermore, these results suggest that, of the metabolites tested, glucose and triglycerides are the most reliable indicators of nutritional state, as they consistently reflected significant differences between treatments. AIC model selection showed that the best-fit model for the relationship between treatment (fed or control, as proxies for nutritional status) and measures of individual condition was the model including glucose, triglycerides, and SCM as fixed factors (Table 3). This model contains 48% of the predictive power of all models assessed (Table 3), clearly demonstrating that assessments of nutritional state are improved by the addition of metabolite panels over SCM alone. Additionally, SCM improved model fit more than mass alone, despite contrasting evidence in previous studies suggesting that the inclusion of structural size in models does not improve measures of nutritional state (Jacobs et al., 2012). Previous studies have found similar supporting evidence for the use of plasma glucose and triglycerides in nutritional assessments, including Morales et al. (2020), which found that food supplemented kittiwakes had significantly higher levels of glucose. Alonso-Alvarez and Ferrer (2001) found that both glucose and triglyceride concentrations decreased overall during a fasting period in yellow-legged gulls (*Larus michahellis*), but later increased during refeeding and

recovery. Similarly, in garden warblers (*Sylvia borin*), triglycerides decreased during fasting but increased during supplemental feeding (Jenni-Eiermann & Jenni, 1994).

Although measurements of triglycerides proved to be important in evaluating individual nutritional state, this study was limited to the use of laboratory assays, rather than POC devices, for triglyceride analyses. Though POC devices for measurement of triglycerides are available, previous studies have found that triglyceride samples from pre-laying female kittiwakes on Middleton frequently tested above the limit of detection for the Cardiochek device used in our study (Morales et al., 2020). Elevated triglyceride concentrations may be attributed to nutrient acquisition for oogenesis, as high levels of triglycerides have been associated with egg formation in female birds (Navarro et al., 2007). For instance, Giudici et al. (2010) found that breeding female Cory's shearwaters (*Calonectris diomedea*) had significantly elevated levels of triglycerides during pre-laying compared to breeding males. Although other POC devices for measurement of triglyceride concentrations are available (Alere Cholestech LDX™, Accutrend® Plus), most have similar ranges of detection to the CardioChek device. A device with higher limits of detection would be necessary to accurately quantify triglyceride levels for kittiwakes. Thus, dilution of blood plasma samples is often necessary to extract actual triglyceride values. The use of methods for dilution of blood and plasma samples *in situ* may improve the feasibility of using POC devices for measurement of triglycerides in the field.

Cholesterol concentrations may be characterized by factors including long-term feeding success, individual diet content, and building of nutritional reserves (Alonso-Alvarez & Ferrer, 2001; Morales et al., 2020; Whiteman et al., 2013). For example, Alonso-Alvarez and Ferrer (2001) reported that plasma cholesterol concentrations were strongly related to total body mass loss in yellow-legged gulls undergoing food restriction, and that cholesterol levels decreased over the duration of a fasting period. However, Morales et al. (2020) found that higher cholesterol levels were associated with nutritional stress in kittiwakes. Overall, it seems that trends in plasma cholesterol concentrations tend to vary based on individual species physiology and behavior.

BUTY concentrations were found to be largely similar between all treatment groups (Figs. 2C, 4C). Previous studies have suggested that increasing BUTY levels represent the mobilization of fat reserves and are negatively correlated with triglyceride levels and body mass (Alonso-Alvarez & Ferrer, 2001; Castellini & Rea, 1992; Morales et al., 2020). Indeed, ketones

are considered reliable predictors of fasting status and body-mass loss in passerines, and B-hydroxybutyrate has been identified as a tool to assess the condition and feeding habits of foraging birds (Jenni-Eiermann & Jenni, 1992, 1994). However, we failed to observe this trend for BUTY in this study, and it is possible that clear differences in BUTY may be more pronounced in food-poor years. Additional years of data would be beneficial to determine the role of BUTY as a nutritional indicator in the study population.

Experimental treatments also had observable effects on SCM (Figs. 3A, 4E), as supplementally fed individuals were significantly heavier than control and handicapped individuals. Similar findings were summarized by Boutin (1990), who concluded that access to additional food leads to increased adult body weight of many terrestrial vertebrates, including species of birds, mammals, amphibians, and reptiles. In birds, this could be a result of higher lipid stores due to feeding or growth of muscle due to exercise (Marsh, 1984; Pennycuick, 1975). It has been found that flight muscles hypertrophy in response to increased weight load, and the supplemental food provided to fed kittiwakes here likely allowed them to mediate the tradeoffs between gaining body mass and increasing costs of flight (Marsh, 1984; Pennycuick, 1975). Additionally, Kahane-Rapport et al. (2022) found that food supplemented kittiwakes spent less time engaged in costly flight behavior, overall reducing energy expenditure and further mediating effects of increased mass. Fed birds could have gained mass due to increased storage of fat reserves (as supported by significantly higher plasma triglyceride concentrations in this group). Handicapped individuals had similar SCM compared to control birds (Fig. 4E), again highlighting the possibility that handicapped birds were operating at their physiological limit, with little flexibility to further decrease body mass (Chastel et al., 1995; Drent & Daan, 1980; Wikelski & Cooke, 2006). However, previous findings by Leclaire et al. (2011) highlighted mass losses in male kittiwakes that were given a flight handicap, as individuals reduced their body mass to compensate for the increased flight costs sustained from the feather clipping procedure (Pennycuick, 1975).

Pectoral muscle depth was significantly different between fed and control birds, reflecting variation in exercise levels between experimental treatments. Control birds had significantly higher muscle depth than fed birds, indicating that control individuals experienced muscular hypertrophy from relatively higher activity and exercise levels, likely due to higher foraging requirements and overall time spent in flight compared to fed birds (Kahane-Rapport et

al., 2022; Marsh, 1984; Tremblay et al., 2022). Fed individuals spend more time at the colony and reduce their natural foraging activity, therefore lowering their exercise and activity requirements (Kahane-Rapport et al., 2022; Tremblay et al., 2022). Pectoral muscle depth of handicapped individuals was not significantly different from any other treatment. Flight was more costly for handicapped individuals due to reductions in wing area, and birds in this group likely reduced their time spent flying to further mediate the effects of the feather clipping treatment (Pennycuik, 1975). Thus, pectoral muscle depth was similar to that of fed birds that also reduced flight and foraging activity. Overall, measurements of pectoral muscle depth do not reflect nutritional state, but rather exercise level.

#### *4.2 Breeding Stage*

Plasma metabolite concentrations varied according to breeding stage as hypothesized, with significant changes in glucose, cholesterol, and BUTY levels (Figs. 2A–C). We predicted that nutritional state would negatively correlate with energetic demands previously established for kittiwakes, in which energetic demands are lowest during the pre-laying stage and increase during incubation and chick-rearing. Despite evidence of decreased energetic demands during pre-laying, as kittiwakes can invest energy in self-maintenance instead of reproduction investment (Tremblay et al., 2022), individuals were most nutritionally stressed during the pre-laying stage (low glucose, elevated cholesterol and BUTY concentrations), suggesting that there is a mismatch between energetic demand and nutrition. In fact, Tremblay et al. (2022) found that kittiwakes spend a higher proportion of time at the colony during pre-laying compared to subsequent stages. Colonial species like kittiwakes must engage in intense agonistic behavior to acquire and maintain nesting sites during early pre-laying, and individuals engaged in site-defense spend more time and energy at the colony defending territory (Bennett et al., 2022; Tremblay et al., 2022). During this period, kittiwakes also spend time interacting with their mate, engaging in courtship behaviors, and building their nests. Such behaviors reduce the amount of time individuals spend foraging (Tremblay et al., 2022) and influence the timing of feeding, resulting in longer feed-fast intervals and overall increased nutritional stress during pre-laying (Fig. 1b).

Additionally, kittiwakes are income breeders that allocate available resources to reproduction directly, relying on both environmental and internal cues to signal the beginning of

the breeding cycle (Whelan et al., 2021). Individuals in this study population are generally short distance or partial migrants that spend an extended period of time at the colony prior to the onset of reproduction (Whelan et al., 2020). Nutritional state may also remain poor until environmental conditions are appropriate for breeding, at which point individuals are prompted to increase energy intake in preparation for egg-laying. Environmental conditions influence the timing of reproduction for income breeders, as demonstrated in the Middleton population of kittiwakes in which increased access to food supply directly advanced reproductive timing (Whelan et al., 2021). Internal physiology linked to reproduction also influences the measured plasma metabolite concentrations of kittiwakes during the breeding season. The significantly higher concentrations of cholesterol observed during pre-laying (Fig. 21') may be partially attributed to the role of cholesterol as a precursor for steroid hormones important for reproduction. Those hormones include progesterone, estradiol, and testosterone, all of which play key roles in the courtship and mating behaviors of individuals during pre-laying (Ottinger & Bakst, 1995).

Although energetic demands increase during the incubation and chick-rearing stages due to reproductive requirements (Tremblay et al., 2022), we found that individuals showed signs of better nutritional state during these periods, including significantly elevated glucose (Fig. 2A) and lower cholesterol (Fig. 2B) concentrations compared to pre-laying. For example, the incubation stage is characterized by significantly higher levels of glucose (Fig. 2A), which likely reflects increased foraging behavior. The higher tendency to forage may also be prompted by rising levels of prolactin, a hormone that stimulates breeding activity and promotes brooding behavior in birds (Angelier et al., 2016). Kittiwakes forage more frequently later in the breeding season, particularly during chick-rearing, to ensure that chicks are fed regularly. Mated birds alternate short shifts at the nest and increase time spent foraging, which produces shorter feed-fast intervals and better nutritional status. (Fig. 1a).

Finally, we found that SCM was highly similar between pre-laying and incubation, but significantly lower in chick-rearing (Fig. 3A). Mass loss during chick-rearing is advantageous for reducing cost of flight associated with increased foraging trips (Golet et al., 1998; Pennycuik, 1975). However, pectoral muscle depth increased during chick-rearing, (Fig. 3B), suggesting that the lower body mass observed during the chick-rearing stage reflected losses from reserves other than pectoral muscle. Increases in muscle depth during chick-rearing likely reflect the muscular hypertrophy that occurs due to increased flight time, as parents must make more frequent

foraging trips to provision their chicks at the nest (Kahane-Rapport et al., 2022; Marsh, 1984; Tremblay et al., 2022). Thus, pectoral muscle depth may not be a good indicator of nutritional state or body condition, but rather a metric of exercise and overall activity level.

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**CRedit author statement**

**Lauren Jackson:** Conceptualization, methodology, formal analysis, investigation, writing (original draft), visualization, funding acquisition

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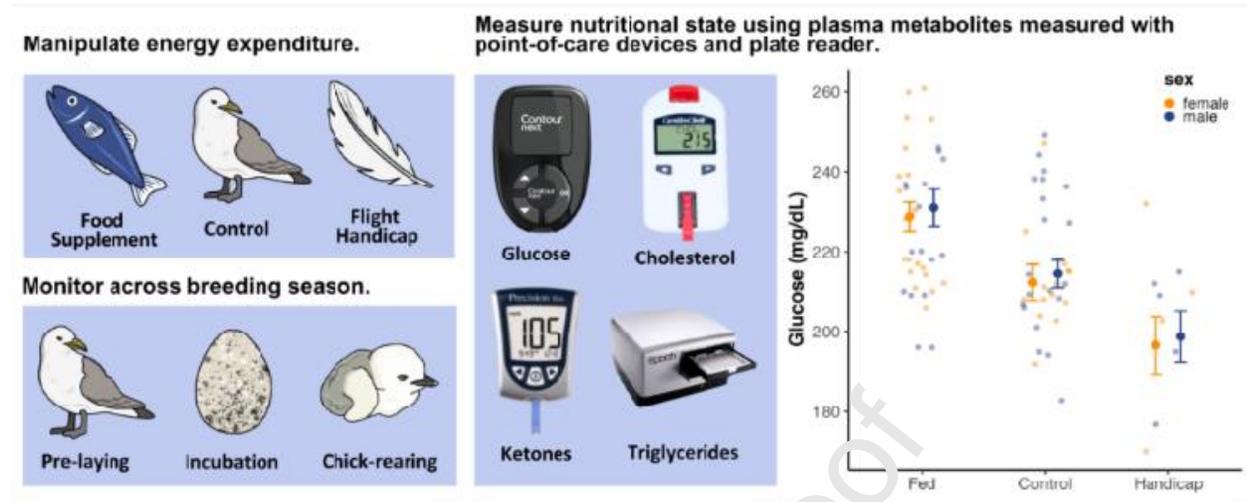
**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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## Graphical abstract



Highlights:

- Plasma metabolites accurately estimate the nutritional status of wild seabirds.
- Glucose and triglycerides are the best measures of nutritional status.
- Metabolite panels improve upon other common measures of body condition.
- Metabolites reflect lower feeding rates among individuals during pre-laying.

Journal Pre-proof