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ABSTRACT

The Baltic/Wadden Sea Flyway of common eiders has declined over the past three decades. Multiple factors such as contaminant exposure, global warming, hunting, white-tailed eagle predation, decreased agricultural eutrophication and infectious diseases have been suggested to explain the decline. We collected information on body mass, mercury (Hg) concentration, biochemistry and untargeted metabolomics of incubating birds in two colonies in the Danish Straits (Hov Røn, n = 100; Agersø, n = 29) and in one colony in the Baltic proper (Christiansø, n = 23) to look into their metabolisms and energy balance. Body mass was available from early and late incubation for Hov Røn and Christiansø, showing a significant decline (25–30%) in both colonies with late body mass at Christiansø being the lowest. Whole blood concentrations of total mercury Hg were significantly higher in birds at Christiansø in the east compared to Hov Røn in the west. All birds in the three colonies had Hg concentrations in the range of ≤1.0 μg/g ww, which indicates that the risk of effects on reproduction is in the no to low risk category for wild birds. Among the biochemical measures, glucose, fructosamine, amylase, albumin and protein decreased significantly from early to late incubation at Hov Røn and Christiansø, reflecting long-term fastening as supported by the decline in body mass. Untargeted metabolomics performed on Christiansø eiders revealed presence of 8,433 plasma metabolites. Of these, 3,179 metabolites changed significantly (log_2-fold change ≥1, $p \leq 0.05$) from the early to late incubation. For example, smaller peptides and vitamin B_2 (riboflavin) were significantly down-regulated while 11-deoxycorticosterone and palmitoylcarnitine were significantly upregulated. These results show that cumulative stress including fasting during incubation affect the eiders' biochemical profile and energy metabolism and that this may be most pronounced for the Christiansø colony in the Baltic proper. This amplify the events of temperature increases and food web changes caused by global warming that eventually accelerate the loss in body weight. Future studies

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should examine the relationship between body condition, temperature and reproductive outcomes and include mapping of food web contaminant, energy and nutrient content to better understand, manage and conserve the populations.

1. Introduction

The common eider is the largest and heaviest sea duck in the Northern Hemisphere. The Danish eiders is a biological subunit of the Baltic/Wadden Sea Flyway population, which consists of an estimated 900,000 birds (Christensen et al. 2013; Helcom 2013; Waltho and Coulson 2015). The flyway comprises breeding populations from Finland, Sweden, Denmark, southern Norway and Germany. Between 1990 and 2000 the number of wintering eiders in Danish waters has decreased from 800,000 to 380,000 birds and overall, the flyway population has decreased from 1.2 million to around 760,000 individuals (Delaney and Scott 2002, 2006).

The colony of eider ducks (*Somateria mollissima*) located in the southern part of the Baltic Proper at the island of Christiansø is the second largest in Denmark (Christensen and Bregnballe 2011). The breeding population comprised 1,445 and 1,750 nesting females in 2007 and 2015, respectively. The eiders on Christiansø migrate between wintering grounds at the western part of the Baltic Sea south to the Dutch part of the Wadden Sea (Noer 1991). They return to the summer breeding grounds from late February to early April (Lyngs 2014). Reflecting the flyway population, the Christiansø colony has also experienced population decline in the past decades (Lyngs 2014). Long-term monitoring has revealed that the population increased from approximately 1,000 to approximately 3,000 breeding eiders during 1970–1990, while from 1990 until today, the population has declined by approximately 50% for unknown reasons (Lyngs 2009, 2014).

Despite that the reasons for these fluctuations are unknown, several factors have been suggested. These include changed access to food in winter areas and breeding location, agriculture eutrophication, infectious diseases such as fowl cholera, parasitic burdens and blooming toxic algae among other together with predation from the Baltic population of white-tailed eagle (*Haliaeetus albicilla*) which has increased significantly over the past decades (Buchman 2010; Camphuysen et al. 2002; Christensen et al. 2008; Larsson et al. 2014; Laursen and Møller 2014; Öst 2018). Starvation due to fishery activities and food web changes from global warming and increasing winter temperatures affecting blue mussel (*Mytilus edulis*) stocks have also been proposed as the potential causes of the population decline although these factors cannot fully account for the mortality of birds in good body condition (Cramp and Simmons 1977; Laursen and Møller 2014; Madin 2009; Waldeck and Larsson 2013). In addition, high prevalence of acanthocephalan parasites has also been associated with mortality in eiders (Camphuysen et al. 2002; Garbus et al. 2018a, 2019). Furthermore, candling of eggs has shown that at least 14% of eggs failed to fertilize at Christiansø (Ernst et al. 2004; Garbus et al. 2018a). In addition, the colony at Christiansø experienced mortality events with a total of 235 birds found dead during May-June of 2007, 2015 and 2016, affecting 5–10% of the total colony (Garbus et al. 2018b, 2019). The mortality was due to starvation and organ failure while high burdens of endoparasites acanthocephalan parasite *Polymorphus* and *Echinostoma* spp. (intestinal flukes) causing severe damage to the intestinal mucosa. It has been proposed that Swedish eiders may suffer from thiamine (vitamin B_1) deficiency that reduces their reproduction rates and increases the mortality of chicks (Balk et al. 2009, 2016; Mörner et al. 2017). Exposure to persistent organic pollutants has been investigated previously and suggested to increase DNA lesions (Fenstad et al., 2016, 2017). It also remains unknown if the high concentration of mercury in the Baltic Sea is affecting the reproduction of eiders (Ackerman et al. 2016; Helcom 2018).

As reflected above, there is limited knowledge on the physiology and metabolism of incubating fasting eiders in the Baltic/Watten Sea area. To investigate this, we analysed body mass, plasma biochemistry and untargeted metabolomics in three different colonies spanning from the western Danish straits to the Baltic proper in the East. In addition, we analysed mercury as it is known to affect reproduction in birds and is found in high concentrations in the eastern Baltic Sea food webs. We hypothesize that fasting from early to late incubation affects body mass, blood biochemistry, metabolism and the metabolome and that this may differ among the three colonies due to differences in food availability. We also hypothesize that there is a difference in mercury exposure as the concentrations in the lower food web is known to be high in the Baltic proper towards the east, and it may therefore pose a risk to the reproduction of the birds.

2. Materials and methods

2.1. Study area

The study was performed in 2018 at three different Baltic colonies: Hov Røn (55°54′N 10°15′E) in the western Danish straits, Agersø (55°13′N 11°07′E) in inner Danish waters, and Christiansø towards the Baltic proper in the east (55°19′N 15°11′E) (Fig. 1). The three colonies were visited during early incubation on 18-19th of April (Hov Røn and Christiansø), mid-incubation on 5th of May (Agersø) and again during late incubation on 14-15th of May (Hov Røn and Christiansø). At Christiansø, the same birds (n = 23) were taken directly from their nest and sampled for their blood at the stage of both early and late incubation. At Hov Røn, hand-held nets were used for capture in the early (n = 33) and late (n = 67) stage of incubation and none of the birds were resampled. Hand-held nets were also used at Agersø which, for logistical reasons, was only visited once during mid-incubation (n = 29). At Hov Røn and Agersø, eiders were sampled haphazardly throughout the islands while three study plots at Christiansø were solely defined by the on-going monitoring program. For blood sampling of nesting females, the study plots were inspected daily to locate new nests containing 1–2 freshly laid eggs.

Nests, adults, eggs and chicks of eiders are protected according to Danish law (Wildlife Management and Hunting Act; present LBK no. 265 of 31/03/2019) and a permission to handle eggs, nest material and female eiders was granted by the Nature Agency and the Danish Ministry of Environment and Food (NST-304–0008). Blood samples and handling of incubating females were conducted under permit no. 2017–15-0201–01205 (case no. 2017–15-0201–01205/MABJE) granted by The National Committee for the Protection of Animals used for Scientific Purposes.

2.2. Blood sampling and body mass

Body mass was recorded with a Pesola Spring balance with 10 g accuracy. Blood was sampled from the brachial vein and transferred to a 4 mL BD Vacutainer® Lithium Heparin tube. All tubes were centrifuged within 8 h at 2,500 rpm for 10 min (~839 *g*) and the supernatant plasma was transferred to a sterile Eppendorf® tube. The ca. 0.5 mL of blood pellets was kept in the post-centrifuged vacutainers for mercury analyses. All material was frozen at −20 °C until biochemical and elemental analyses.

2.3. Mercury analyses

Blood pellets were analysed for total Hg (THg) using a Milestone

Fig. 1. Map of the study areas showing Hov Røn (left, blue), Agersø (middle, green) and Christiansø (right, red) eider breeding colonies in the Danish Baltic proper. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

DMA-80 Direct Mercury Analyzer at the Trace Metal Laboratory at the Department of Bioscience, Aarhus University, Roskilde, Denmark. Samples were thawed and homogenized prior to analyses. A 0.060–0.080 g of subsample was pipetted off, weighted into sample boats and analysed. Blank samples and aqueous control standards (10 ng and 100 ng Hg prepared from 1,000 ± 4 mg/l stock solution, Sigma-Aldrich, Switzerland) were analysed for every 10–15 samples. Blank samples were either empty sample boats or boats with Milli-Q water (instead of blood) filled into similar vials as the samples. Subsequently, sample concentrations were calculated using corrections for background Hg (based on blank samples results) and instrument drift (based on control standards results). The weighted sample contained 3–65 ng of Hg, which is well above the instrumental detection limit (approximately 0.05 ng of Hg). For QA/QC, duplicates and Certified Reference Materials (CRMs; Seronorm Whole Blood L-2 and DORM-4) were analysed along with the samples. The relative standard deviation of duplicate samples were 0.1–2.3% from the mean (n = 16). Measured recovery percentages (mean ± SD) of the CRMs were 96 ± 6% for Seronorm Whole Blood L-2 (n = 27; Certified THg value = 0.0170 ± 0.0034 μg/g ww) and 100 ± 4% for DORM-4 (n = 30; Certified THg value = 0.416 ± 0.053 μg/g dw). Previous analyses of THg μg/g ww in RBC:whole blood show a ratio of 1.8:1 and this ratio was used to convert RBC THg values into THg whole blood values as $THg_{blood} = THg_{RBC}/1.8$ in order to make a risk assessment of mercury exposure and reproduction in birds. The risk category was defined according to Ackerman et al. (2016), categorizing these on 225 wild birds species including 19,998 individuals as: 1) no risk (< 0.2 μg/g ww), 2) low risk (0.2 to < 1.0 μg/g ww), 3) moderate risk (1.0 to < 3.0 μg/g ww), 4) high risk (3 to < 4.0 μg/g ww), and 5) severe risk (≥4.0 μg/g ww).

2.4. Blood biochemical parameters

Blood biochemical analyses were performed on heparinized plasma and conducted at the Veterinary Diagnostic Laboratory, Department of Veterinary Clinical and Animal Sciences, University of Copenhagen, and included 20 biochemical parameters (Table 1) composing three liver enzymes, i.e. alkaline phosphatase (ALKP; U L^{-1}), alanine aminotransferase (ALAT; U L^{-1}) and gamma-glutamyltransferase (GGT; U L^{-1}), one digestive enzyme, i.e. amylase (Amy; U L^{-1}), three protein groups, i.e. total protein (TP; g L^{-1}), globulin (Glo; g L^{-1}) and albumin (Alb; g L^{-1}), two hepatic/erythrocyte metabolic products, i.e. total bilirubin (TB; μmol L^{-1}) and bile acids (BA; μmol L^{-1}), two carbohydrates, i.e. glucose (Glu; mmol L^{-1}) and fructosamine (Fru; μmol L^{-1}), two muscle and protein metabolic products, i.e. creatinine (Cre; μmol L^{-1}) and urea (Urea; mmol L^{-1}), cholesterol (Cho; mmol L^{-1}), and finally six electrolytes, i.e. inorganic phosphate (IP; mmol L^{-1}), calcium (Ca; mmol L^{-1}), magnesium (Mg; mmol L^{-1}), sodium (Na; mmol L^{-1}), potassium (K; mmol L^{-1}) and chloride (Cl; mmol L^{1}). All analyses was routinely conducted at the laboratory using an automated spectrophotometric analyser containing ion-selective electrodes (ADVIA 1800, Siemens). All assays was subjected to daily internal and quarterly external quality control. Information on the methods and their interpretations in clinical and wildlife studies can be found in for example Harr (2006), Lumeij

Table 1
Basic statistics (Mean ± SD, Min-Max) for body mass, mercury in whole blood, and biochemistry in incubating common eiders in three colonies in the Inner Danish Straits and the Danish Baltic proper collected April and May 2018.

Variable	Hov Røn		Agersø	Christiansø		Significant colony and Incubation differences*	Reference values # (mean ± SD, Min-Max)	Physiological endpoints ¥
	Early (n = 33)	Late (n = 67)	Intermediate (n = 29)	Early (n = 23)	Late (n = 23)			
Date	18–19 April	14–15 May	5 May	18–19 April	14–15 May			
Body mass (g)	2262 ± 186(1990–2650)	1694 ± 148(1365–2010)	1887 ± 242 (1230–2430)	2183 ± 140 (1960–2410)	1561 ± 154 (1225–1865)	Christiansø < Hov Røn Late < Early	–	Fasting and energetic stress are most pronounced in Late incubation and at Christiansø
Mercury, whole blood (μg/g ww)	0.12 ± 0.07 (0.05–0.38)	0.13 ± 0.07 (0.04–0.48)	0.21 ± 0.08 (0.1–0.5)	0.41 ± 0.19 (0.14–0.82)	0.40 ± 0.19 (0.14–0.91)	Christiansø < Hov Røn	–	Levels are too low to affect reproduction
Haematocrit	58 ± 4 (48–66)	55 ± 4 (41–62)	58 ± 4 (50–69)	45 ± 13 (0.5–53)	47 ± 4 (38–55)	Christiansø < Hov Røn	–	Birds at Christiansø may be best hydrated
Alkaline phosphatase (ALKP; U L^{-1})	266 ± 294 (32–1279)	91 ± 107 (28–892)	119 ± 159 (29–766)	148 ± 118 (25–554)	55 ± 35 (18–218)	Christiansø < Hov Røn Late < Early	84 ± 59 £ (32–265)	Egg laying, liver metabolism and cell activity are decreasing from early to late incubation
Alanine aminotransferase (ALAT; U L^{-1})	11 ± 7 (4–36)	6 ± 2 (3–12)	10 ± 12 (3–53)	12 ± 10 (2–55)	5 ± 2 (1–10)	Late < Early	12 ± 9 (4–29)	Hepatic metabolism is decreasing from early to late incubation due to energy conservation
Gamma glutamyltransferase (GGT; U L^{-1})	6 ± 7 (0–30)	3 ± 3 (0–19)	6 ± 12 (0–45)	7 ± 10 (0–61)	2 ± 1 (0–5)	Late < Early	3 ± 2 £ (1–6)	Hepatic metabolism is decreasing and entero-hepatic circulation is increasing from early to late incubation due to energy conservation
Amylase (U L^{-1})	1434 ± 796 (544–3494)	545 ± 156 (218–922)	884 ± 439 (416–2279)	1675 ± 600 (700–3753)	673 ± 191 (390–1372)	Hov Røn < Christiansø Late < Early	553 ± 101 £ (436–692)	Pancreas activity is decreasing from early to late incubation due to decreased food intake. Food intake may be higher at Christiansø prior to incubation
Albumin (g L^{-1})	19 ± 3 (14–27)	17 ± 2 (13–21)	17 ± 3 (13–24)	17 ± 3 (11–25)	13 ± 2 (10–18)	Christiansø < Hov Røn Late < Early	24 ± 9 (14–42)	Albumin concentration is decreasing from early to late incubation due to egg laying and starvation. Birds at Christiansø may starve the most
Total protein (g L^{-1})	45 ± 4 (34–53)	40 ± 4 (32–51)	41 ± 5 (30–61)	48 ± 7 (35–65)	41 ± 6 (30–61)	Hov Røn < Christiansø Late < Early	54 ± 12 (27–81)	Albumin concentration is decreasing from Early to Late incubation due to egg laying and starvation. Birds at Christiansø may starve most
Bile acids (μmol L^{-1})	15 ± 11 (3–48)	20 ± 15 (4–83)	13 ± 8 (2–31)	8 ± 6 (1–38)	14 ± 13 (2–105)	Christiansø < Hov Røn Early < Late	–	Increased entero-hepatic circulation for energy conservation
Total bilirubin (μmol L^{-1})	7 ± 4 (0–19)	7 ± 3 (2–14)	6 ± 3 (1–12)	5 ± 4 (2–15)	3 ± 1 (2–7)	Christiansø < Hov Røn	3 ± 3 £ (0–9)	Increased entero-hepatic circulation for energy conservation may be higher at Christiansø
Urea (mmol L^{-1})	0.4 ± 0.5 (0–2.2)	1 ± 0.6 (0–2.8)	1 ± 1 (0–2)	0.5 ± 0.4 (0–3)	0.5 ± 0.2 (0.2–2)	Christiansø < Hov Røn Early < Late	1.1 ± 0.7 (0.36–2.86)	The birds dehydrate from early to late incubation. Birds at Christiansø may be best hydrated

(*continued on next page*)

Table 1 (*continued*)

Variable	Hov Røn		Agersø	Christiansø		Significant colony and Incubation differences*	Reference values # (mean ± SD, Min-Max)	Physiological endpoints ¥
	Early (n = 33)	Late (n = 67)	Intermediate (n = 29)	Early (n = 23)	Late (n = 23)			
Cholesterol (mmol L^{-1})	7 ± 3 (1–11)	9 ± 2 (6–16)	8 ± 3 (2–13)	7 ± 2 (2–12)	8 ± 2 (5–12)	Early < Late	8.2 ± 2.4 (4.6–13.8)	Precursor for bile acids related to increased entero-hepatic circulation for energy conservation
Glucose (mmol L^{-1})	16 ± 2 (11–19)	16 ± 2 (12–22)	15 ± 1.5 (11–18)	11 ± 1.4 (7–14)	11 ± 1.2 (9–14)	Christiansø < Hov Røn	13.2 ± 2.4 (7.8–21.2)	Birds at Christiansø may be fasting the most
Fructosamine (µmol L^{-1})	350 ± 249 (177–1610)	313 ± 116 (208–1008)	342 ± 188 (204–1012)	177 ± 59 (101–389)	165 ± 33 (119–333)	Christiansø < Hov Røn	–	Birds at Christiansø undergoing fasting the most
Creatinine (µmol L^{-1})	17 ± 6 (9–31)	14 ± 5 (10–46)	15 ± 5 (10–26)	8 ± 6 (0–24)	7 ± 4 (2–17)	Christiansø < Hov Røn Late < Early	53 ± 18 (0–97)	Metabolism of muscle creatinine decreases from early to late incubation and is lower at Christiansø.
Inorganic phosphate (mmol L^{-1})	2 ± 1 (1–4)	1 ± 0.4 (0.2–2)	2 ± 1 (1–4)	2 ± 0.4 (1–3)	1 ± 0.2 (1–2)	Late < Early	1.2 ± 0.6 (0.5–2.2)	Fasting is increasing and metabolism is decreasing from early to late incubation
Chloride (mmol L^{-1})	116 ± 5 (99–126)	124 ± 6 (112–139)	121 ± 6 (99–135)	114 ± 4 (105–124)	110 ± 4 (98–119)	Christiansø < Hov Røn	118 ± 5 (108–128)	Dehydration may be the lowest at Christiansø as indicated by haematocrit and urea
Calcium(mmol L^{-1})	4 ± 1 (3–7)	3 ± 0.2 (2–4)	4 ± 1 (2–7)	4 ± 1 (3–9)	2 ± 0.2 (2–3)	Late < Early	2.7 ± 0.2 (2.3–3.1)	Calcium release from bones for egg production is decreasing from early to late incubation
Magnesium (mmol L^{-1})	1 ± 0.4 (1–2)	1 ± 0.2 (1–2)	1 ± 0.3 (1–2)	1 ± 0.2 (1–2)	1 ± 9.1 (1–2)	Late < Early	–	The electrolyte balance is changing from early to late incubation
Sodium (mmol L^{-1})	157 ± 5 (138–173)	165 ± 5 (157–182)	161 ± 6 (134–168)	155 ± 4 (143–166)	154 ± 4 (144–165)	Christiansø < Hov Røn Early < Late	163 ± 6 (148–178)	Fasting is increasing and metabolism is decreasing from early to late incubation
Potassium (mmol L^{-1})	2 ± 1 (1–5)	3 ± 1 (1–2)	4 ± 1 (2–7)	2 ± 0.3 (2–3)	2 ± 0.4 (2–4)	Christiansø < Hov Røn	2.1 ± 0.4 (1.6–3.2)	The electrolyte balance is changing from early to late incubation

*: Statistically significant difference (ANOVA Tukey's Post Hoc: $p < 0.05$).
#: reference values according to Species360 (2015) and Garbus et al. (2020).
£: values outside reference interval.
¥: Physiologically endpoints according to Harr (2002, 2006), Hochleithner (1994), Lumeij (2008), Samour (2006).

(2008), Marteinson and Verreault (2020) and Sonne et al. (2008, 2010, 2012a, 2013) (Table 1).

2.5. Untargeted metabolomics on paired blood samples

Paired plasma samples, from Christiansø (n = 23 × 2, at early and late incubation stage), were purified by solid-phase extraction prior to injection into the nano ultra-high-performance chromatography system (Ultimate 3000 RSLCnano, ThermoFisher Scientific), consisting of a temperature controlled autosampler and column oven, and a combined nano-flow and loading pump. The chromatographic system is hyphenated with an ultra-high resolution high field Q Exactive Orbitrap tandem mass spectrometer (ThermoFisher Scientific). In short, 100 µL of plasma were enriched on a solid-phase cartridge (ThermoFisher Scientific, HRP 2 mg), preconditioned with acetonitrile (200 µL) and 0.1% trifluoroacetic acid (200 µL), and extracted twice with 50 µL acetonitrile. The extracts were further diluted with 150 µL of 0.1% trifluoroacetic acid and vortexed. Moreover, a quality control sample of all extracts was obtained by combining 25 µL of each sample extract. Twenty microliters of each sample extract were injected into the LC-HRMS system and loaded at 50 µL min^{-1} with 2% acetonitrile and 0.1% trifluoroacetic acid onto an online solid-phase extraction cartridge (C_{18}, 0.3 × 5 mm, 5 µm, 100 Å, ThermoFisher Scientific). After 3 min of loading, the cartridge was back-flushed onto an analytical column (C_{18}, 75 µm × 150 mm, 2 µm, 100 Å, ThermoFisher Scientific) at 300 nL min^{-1} delivered by a nano-flow pump. A biphasic-gradient was applied to elute the metabolites from the analytical column. Mobile phase A and B both consisted of 0.1% formic acid, and in addition 2% and 98% acetonitrile, respectively. The high-field orbitrap mass analyser was operated in data dependent acquisition mode with stepped collision energy. The Top 5 ion peak picking mode for MS-fragmentation was based on a full scan between *m/z* 100 and 1500 at a mass resolving power of 240,000. The metabolome data set was post-processed using in-house optimized untargeted metabolomics pipelines in Compound Discoverer 3.1.0.305 (ThermoFisher Scientific). In short, the pipelines contained peak picking and retention time alignment nodes, as well as elemental composition prediction and HRMS fragment library searching nodes (mzCloud) for metabolite annotation, and finally, multivariate statistics, hierarchal cluster analysis and a differential analysis node were applied (Miller et al. 2015; Nash and Dunn 2019). The metabolites were annotated based on high quality hits in spectral libraries, i.e. mzCloud.

2.6. Statistical analyses

All analyses was performed after testing for normality distribution, correcting by log-transformation and using R for all analyses (R Core Team, 2018). Only at Christiansø, the same birds were measured twice, which was not possible at Hov Røn. The body weight loss was considerably larger in the population from Christiansø than in the population from Hov Røn and a one-way analysis of variance (ANOVA) was therefore applied to test for a difference in the body weight between early and late incubation, separately for the two populations. For the blood parameters that were measured in both Christiansø and Hov Røn, a two-way ANOVA including time and location as explanatory variables was applied for analyzing the different blood parameters. A Parson's correlation analyses was run for body weight and blood biochemistry to look for any correlations. The Hg concentration values were skewed to the right, and these were log_e-transformed prior to analysis by two-way ANOVA with location and time as explanatory factors.

3. Results

3.1. Body mass

Body mass for the three colonies are shown in Table 1. Analyses of body mass (mean ± SD) from early to late incubation showed a significant decrease of 25% at Hov Røn (2,262 ± 186 g vs. 1,694 ± 148 g) and 30% at Christiansø (2,183 ± 140 g vs. 1,561 ± 154 g), while the mid-incubation body mass at Agersø was 1,887 ± 242 g (Fig. 2). The late incubation mass was significantly higher at Hov Røn compared to Christiansø ($p < 0.001$). No significant difference was found between these two locations ($p = 0.21$) during the early incubation, while the late incubation weight was significantly higher at Hov Røn compared to Christiansø ($p < 0.001$).

3.2. Mercury

Concentrations of THg (µg/g ww) in the three colonies are shown in Table 1 and Fig. 3. The concentrations in the three colonies increased in the following order: Hov Røn (mean ± SD: 0.12 ± 0.07 µg/g ww, n = 100) < Agersø (mean ± SD: 0.21 ± 0.08 µg/g ww, n = 29) < Christiansø (mean ± SD: 0.40 ± 0.16 µg/g ww, n = 23). Tukey's *post hoc* test showed that Hov Røn differed significantly from both Agersø and Christiansø ($p < 0.05$) but no significant difference was found between Agersø and Christiansø ($p = 0.58$). It can also be seen that mercury concentrations did not change from early to late incubation for eiders at Hov Røn and Christiansø ($p > 0.05$). The concentrations in eiders from Hov Røn had 92% in the no risk category (0–0.2 µg/g ww) and 8% in the low risk category (0.2–1.0 µg/g ww Hg). At Agersø, the corresponding concentrations were 52% in the no risk category and 48% in the low risk category. At Christiansø, the corresponding concentrations were higher with 96% in the low risk and 4% in the no risk category. In none of the populations Hg were in the moderate (1.0 to < 3.0 µg/g ww), high (3 to < 4.0 µg/g ww) or severe risk (≥4.0 µg/g ww).

3.3. Blood biochemistry

A panel of 20 biochemical profiles was obtained from the three colonies (Table 1). Again, Agersø could not be included in the statistical analysis. However, birds from Agersø were more similar to the birds from Christiansø. Birds from both Christiansø and Hov Røn showed a significant decline in plasma concentration from early to late incubation for ALKP, ALAT, amylase, albumin, total protein, creatinine, inorganic phosphate, calcium and magnesium (all $p < 0.05$). A

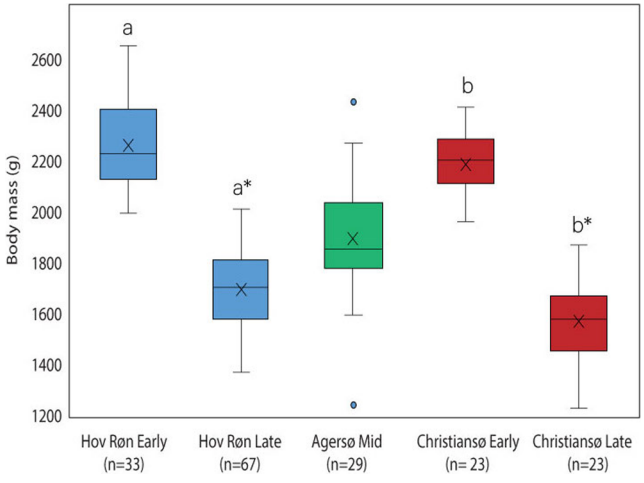

Fig. 2. Box and whisker showing from below minimum, Q1, median (-), mean (×), Q3 and maximum of body mass from common eiders sampled at early, mid and late incubation from three different colonies in the Danish Baltic proper. The small letters indicate significant differences among colonies and * indicate significant differences between early and late incubation (ANOVA Tukey's Post Hoc, $p < 0.05$). Please also consult Table 1.

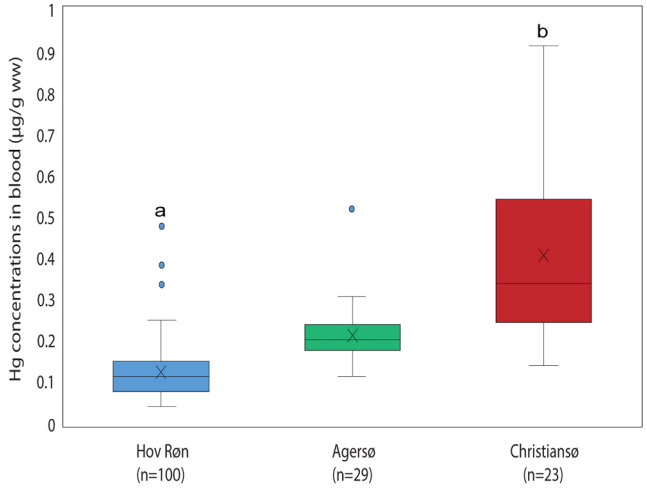

Fig. 3. Box and whisker showing from below minimum, Q1, median (-), mean (×), Q3 and maximum of total mercury concentrations in whole blood of common eiders samples from the three different colonies in the Danish Baltic proper. The small letters indicate significant differences among colonies (ANOVA Tukey's Post Hoc, $p < 0.05$). Please also consult Table 1.

significant increase was found for plasma concentrations of GGT, bile acid, urea, cholesterol and sodium (all $p < 0.05$).

The results for blood biochemistry also showed a west-east difference as fourteen of twenty biochemical profiles were significantly lower at Christiansø compared to Hov Røn (all $p < 0.05$) (Table 1). Of these twelve profiles, albumin, creatinine, glucose and fructosamine were the compounds showing the largest differences (Fig. 4). These changes reflect that long-term calcium mobilisation, fasting and energy depletion are most pronounced at Christiansø leading to catabolism of protein as well as dehydration as reflected in the decline in body mass (all $p < 0.05$) (Table 1).

Correlation analyses showed that albumin, total protein, amylase, ALKP, ALAT, total bilirubin, GGT, calcium, Mg and creatinine increased significantly with body mass while cholesterol, bile acid, urea and sodium decreased significantly with body mass (all $p < 0.05$) (Table S1). Comparing with reference intervals in Table 1, it can be seen that ALKP, GGT, amylase and total bilirubin were outside the reference intervals.

3.4. Untargeted metabolomics

Metabolomics were conducted on the paired blood samples from Christiansø. The untargeted metabolomics analysis revealed 8,433 unique chemical entities in the entire dataset. Of these, 852 compounds were also present in procedural blank quality control samples and

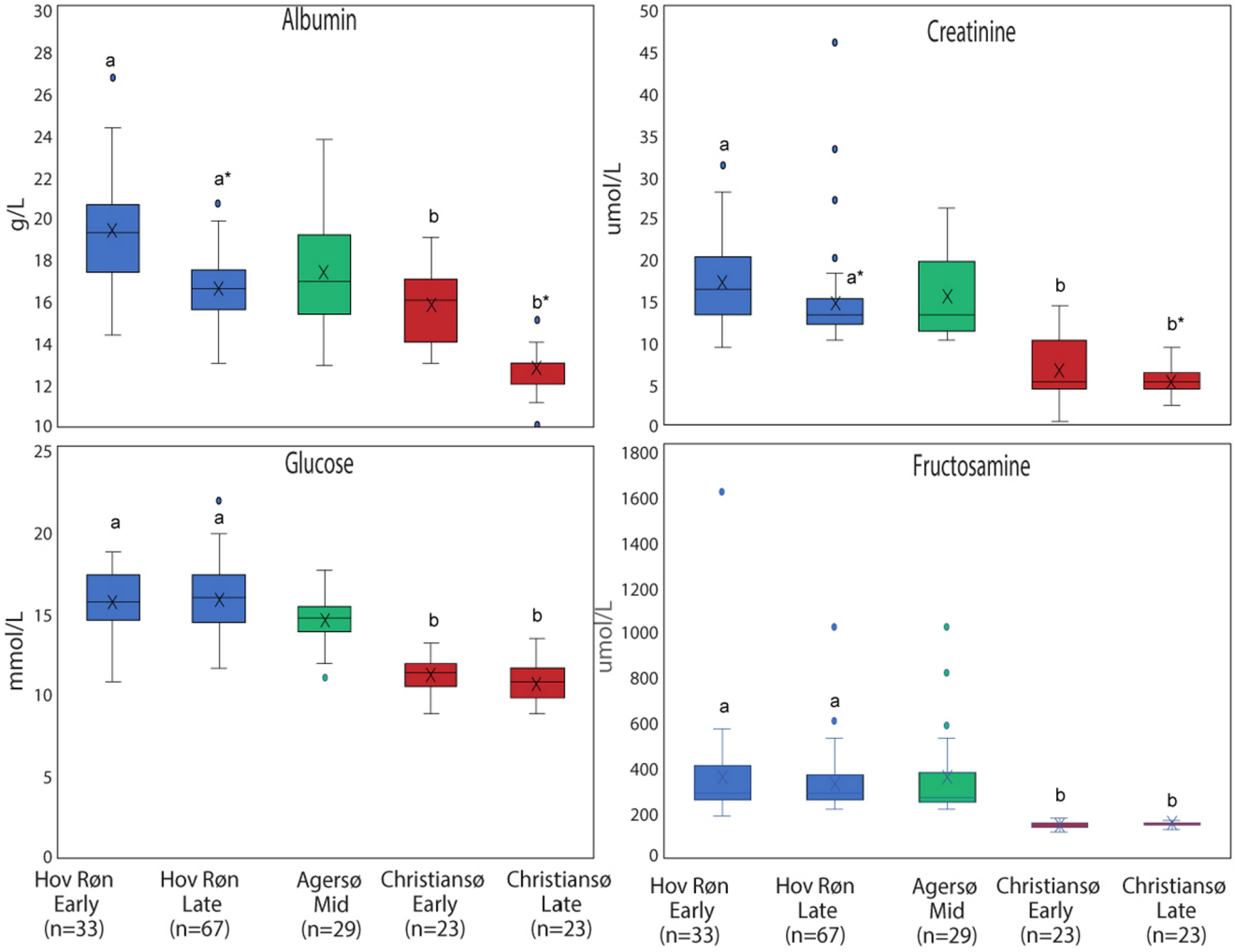

Fig. 4. Box and whisker showing from below minimum, Q1, median (-), mean (×), Q3 and maximum of albumin, creatinine, glucose and fructosamine of common eiders sampled at early, mid and late incubation from three different colonies in the Danish Baltic proper. The small letters indicate significant differences among colonies and * indicate significant differences between early and late incubation (ANOVA Tukey's Post Hoc, $p < 0.05$). Please also consult Table 1.

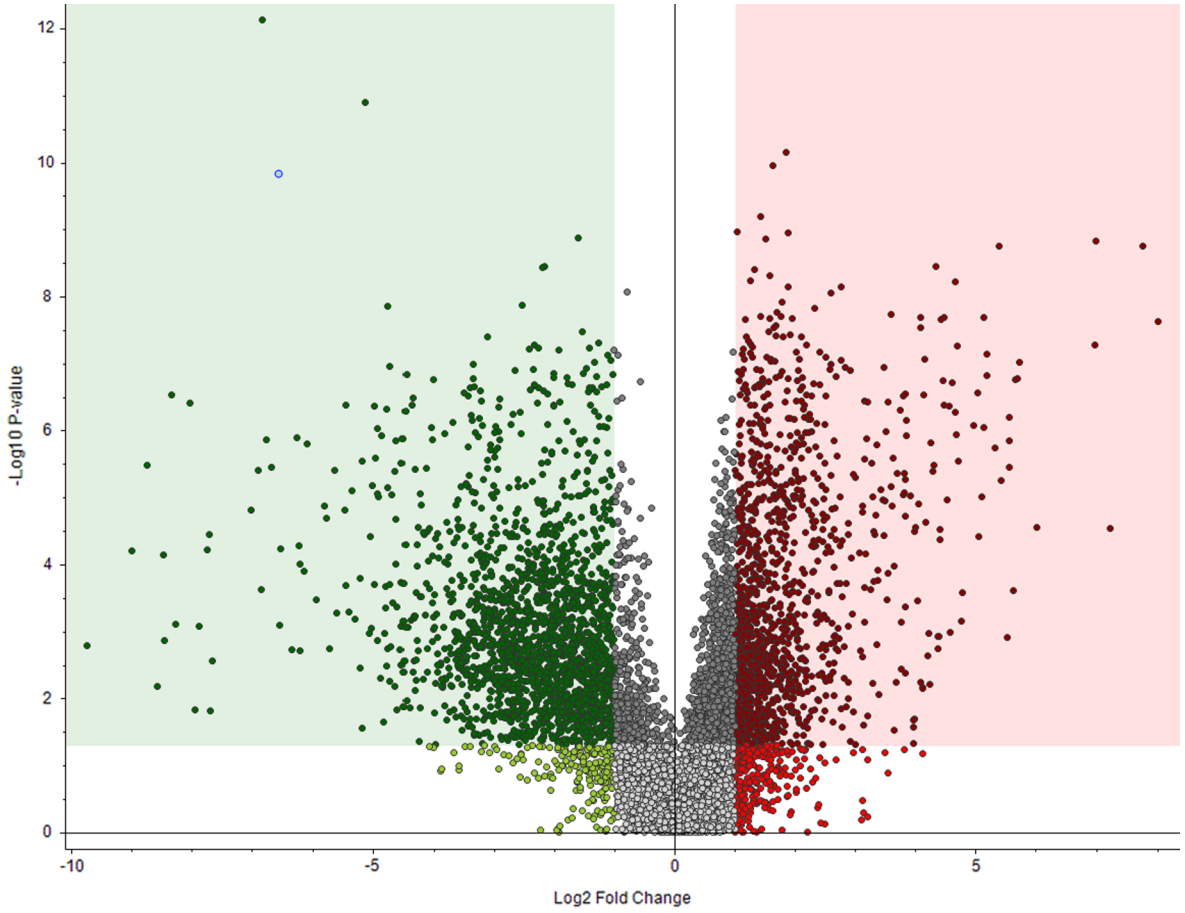

Fig. 5. Volcano plot summarizing differential analysis of metabolites differing between the two sample groups from Christiansø by comparing early versus late incubation. 1,861 metabolites are significantly decreased (log2-fold change < -1) late incubation (green area). 1,318 metabolites are significantly increased (log2-fold change > 1) at late stage of incubation (red area). The marked bright blue dot in green area is vitamin B_2 (riboflavin; log2-fold change −6.56; p = $1.5x10^{-10}$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hence they were subtracted from the dataset. The remaining 7,581 compounds were subjected to a differential analysis to highlight up- or down-regulated metabolites in a nested design between samples taken at early and late late stage of incubation, respectively. The differential analysis revealed that 1,861 and 1,318 unique metabolites were significantly down-regulated or up-regulated (log_2-fold change $\geq|1|$; $p \leq 0.05$), respectively at at late incubation when compared to samples taken at at early stage of incubation (Fig. 5). Of these, 3,179 metabolites showing a significant change in titres, we were able to putatively annotate 1,941 metabolites from HRMS/MS fragmentation spectral libraries (mzCloud) and suspect screening searches in ChemSpider, BioCyc and mass lists containing endogenous metabolites following the recommendations from Metabolomics Standards Initiative (Viant et al. 2019). Additionally, we performed hierarchal cluster analysis for visualizing the correlation between samples and detected metabolites (Fig. 6). When omitting a single outlier sample (sample ID F22, bird 23 after incubation), samples were clustered into the two main sample groups and correlated with up- or -down regulated metabolites using semi-quantitative *z*-scores. Of these, many identified metabolites were significant down-regulated at late incubation. This included for example several small peptides, e.g. leucyl-leucyl-norleucine, methionyl-leucine and leucine-glutamine as well as vitamin B_2 (riboflavin) (log_2-fold change of −6.56, adj. p-value $1.5x10^{-10}$, highlighted in Fig. 5). On the other hand, other metabolites were significantly upregulated such as for example palmitoylcarnitine (log_2-fold change 2.37) and jasmonic acid (log_2-fold change 8.01). We also observed significant changes, due to the incubation, in lipids, e.g. sphingolipids and fatty acids, carnitines, e.g. *trans*-2-dodecenoylcarnitine and 2-hexenoylcarnitine and bile acids, e.g. glycolithocholate. In addition, we observed many other metabolites, such as thyroxine, corticosterone, cortisol and taurocholic acid with < 1 log_2-fold change between the two groups. The principal component analysis made from the 1,941 identified metabolites showed significantly differences between early and late incubation (Figure S1). By superimposing the PCA loadings plot of the 1,941 metabolites, the group clustering is evident and e.g. riboflavin is found in the 'early incubation' cluster (at loading 0.0192, −0.03873 in Figure S2).

4. Discussion

4.1. Body mass

Eiders fast during incubation while they need to incubate and protect their eggs (Waltho and Coulson 2015). A previous study on incubating eiders at Christiansø has shown that females in the colony left the nest on average 13 times (range: 7–17 times) for 7–70 min during the incubation period of ca. 27 days (Garbus et al. 2018b). The amount of time spent away from nest was surprisingly small, and reflected the loss in body mass of 28–37% during the 4-week period of continuous fastening with only limited time spent foraging. Due to mobilization of energy from adipose tissue and gain of water from metabolisation of fat, eiders do not have to rehydrate very often. Korschgen et al. (1977) suggested 1,100 g as the lowest critical body mass in eiders during incubation resulting in nest abandonment or death. The body mass found in this study were all above this threshold (lowest: 1225 g) which is also reflected the fact that no mortalities were observed during the search for eider nests. This is in contrast to the data shown for the

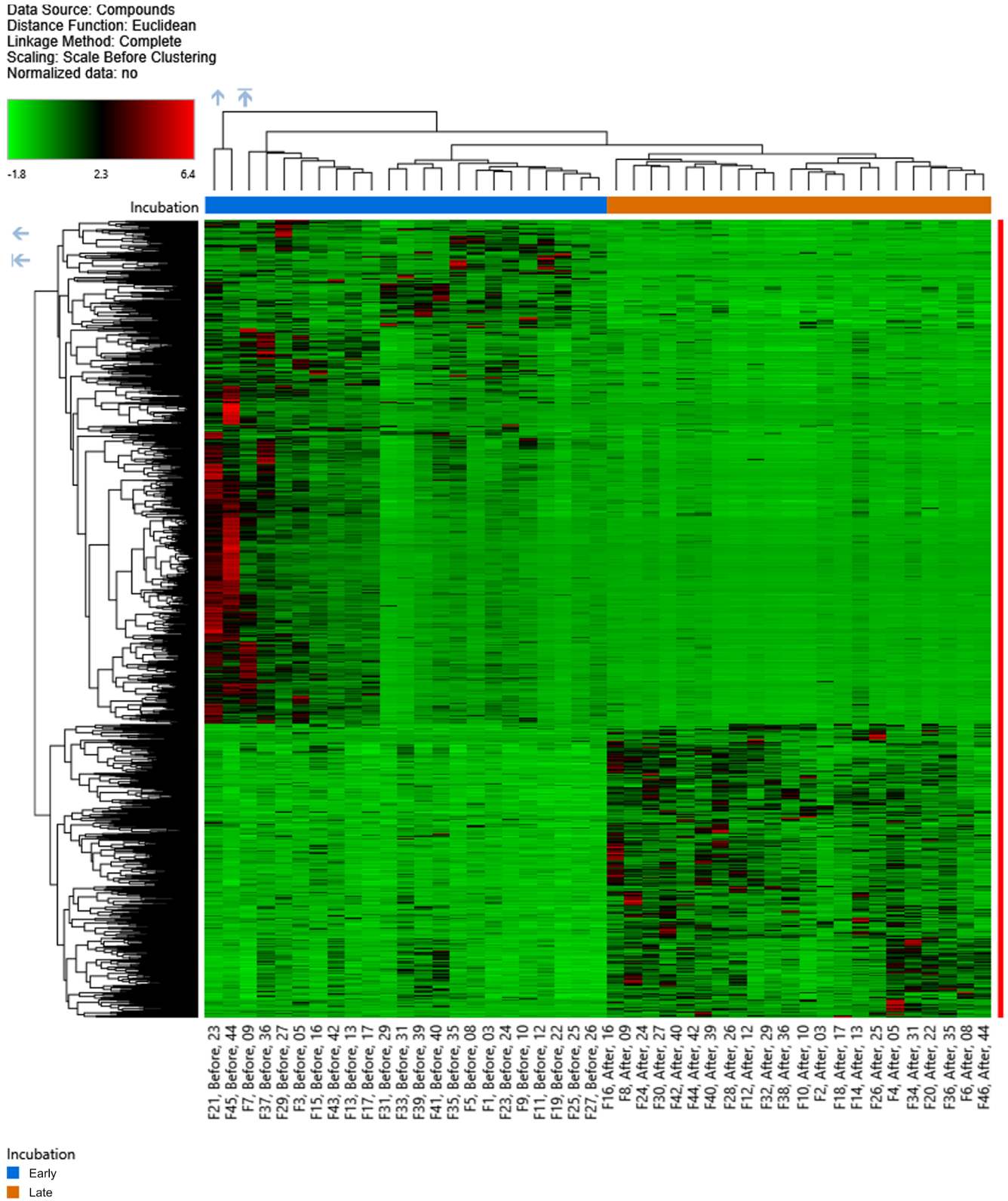

Fig. 6. Hierarchical cluster analysis of the identified 1,941 metabolites significant differing between the two groups and each individual bird in the Christiansø samples. Dendrogram to left and top show similarity between metabolites and samples, respectively. Color bar from green to red represent metabolite abundance (z-score). Individual bird sample identification numbers are shown at bottom row. The blue sample group corresponds to early incubation stage, while the orange sample group corresponds to the late incubation stage. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

previous years 2007, 2015 and 2016, where mortality of hundreds of birds has been recorded in the Christiansø colony during the pre-incubation period, likely due to food deprivation (Garbus et al. 2018c, 2019). Eiders from Hov Røn winter primarily in inner Danish waters, whereas eiders from Christiansø winter in the Wadden Sea (Noer 1991; Bønløkke et al. 2006). Therefore, the differences in body mass among the two colonies could reflect differences in food resources at the overwintering areas but also locally around the colonies (Laursen et al. 2009; Laursen and Möller 2014).

4.2. Mercury

The concentrations of mercury in the three colonies reflect the current geographical differences showing lower levels in the western compared to the eastern part of the Baltic (Helcom 2018). All birds were within the range of 0–1.0 μg/g ww, which according to Ackerman et al. (2016) mean a low risk of effects on reproduction. These concentrations are comparable to concentrations in eiders analysed from West North America and Svalbard during the period from 2000 to 2015 (Ackerman et al. 2016; Dietz et al. 2019; Saunes et al. 2011). Interestingly, mercury concentrations increased from Hov Røn in west to Christiansø in the Baltic Sea, reflecting the known fact that the brackish Baltic ecosystem historically has been among the most contaminated in the world with respect to mercury and also lead and persistent organic pollutants (Cederqvist et al. 2019; Helcom 2010, 2018a, 2018b, 2019). Despite this significant increase towards the east in the Christiansø colony, mercury alone therefore does not pose a risk for reproduction in the Danish eider colonies based on the information provided by Ackerman et al. (2016). However, other environmental contaminants such as polychlorinated biphenyls (PCBs) and lead (Pb) have previously been shown to increase DNA lesions and reduce viability and reproduction in Baltic eiders, which altogether add to the multiple stress in the Baltic/Wadden Sea Flyway population (Fenstad et al. 2016; Lam et al. 2020).

4.3. Blood biochemistry

As for mammals, blood biochemistry is used in domestic and wild birds to monitor the organ-system physiology and homeostasis, which give an impression of the overall health of the individual (Harr 2002, 2006; Hochleithner 1994; Lumeij 2008; Maceda-Veiga et al. 2015; Samour 2006; Stevens 1996; Thrall et al. 2006). In the current study, the difference in biochemical profiles among the three eider colonies reflect the physiology of the birds which varied from early to late incubation. Table 1 gives the clinical interpretations of our findings including whether the values are outside of the reference intervals. Overall, these changes are due to fasting and egg laying which cause subsequent changes in metabolism and blood biochemistry (Harr 2002, 2006; Hochleithner 1994; Lumeij 2008; Samour 2006). Glucose, fructosamine, amylase, albumin and protein differed between early and late incubation and decreased significantly with body mass reflecting egg production and long-term fastening (catabolism of protein) as well as reduced gluconeogenesis (Langslow 1978). The differences in ALKP, haematocrit, albumin, glucose, fructosamine and creatinine all indicate long-term calcium mobilisation, fasting and energy depletion, being most significant at Christiansø as shown in Table 1 on clinical interpretations and reference intervals. It shows that catabolism of protein as well as dehydration are most pronounced at this colony, which is also reflected in the Christiansø decline in body mass.

A previous study by Garbus et al. (2020), looking at blood biochemistry of the Christiansø incubating females in 2015, showed that plasma concentrations of fructosamine, amylase, albumin and total proteins all decreased significantly from early to late incubation. Except for glucose, this is the same finding across the colonies of Hov Røn and Christiansø and shows that the birds are fasting, i.e. they are low in glucose and muscle protein. The eiders that died in 2007, 2015 and 2016 at Christiansø had terminal values reflecting phase III fast (catabolism of protein) (Garbus et al. 2019; Hollmén et al. 2001). Fructosamine decline and slight glucose decline from early to late incubation indicated low gluconeogenesis from plasma proteins (Langslow 1978). Overall, these changes are also reflected in the multiple significant correlations among body weight and biochemistry.

The significant increase in plasma concentrations of GGT, bile acid, urea, cholesterol and sodium from early to late incubation reflect fasting and long-term calcium metabolism from egg laying and energy conservation, which is also reflected in the multiple significant correlation analyses. This has previously been observed in eiders (Garbus et al. 2020, Hollmén et al. 2001) but also in other bird species including geese around the globe (Alonso-Alvarez et al. 2002; Boismenu et al. 1992; Le Maho 1981; Robin et al. 1987, 1988, Stevens 1996). The reasons for these changes are due to the energy conservation from early to late incubation and also calcium release from bones due to egg production (Harr 2002, 2006; Hochleithner 1994; Lumeij 2008; Samour 2006). In addition, it cannot be excluded that the parasite burdens that we know these colonies possess may also affect blood biochemistry (Garbus et al. 2019).

4.4. Metabolomics and vitamin B

The metabolomics data showed significant physiological changes reflected in up- and down-regulations in the fasting eiders at Christiansø. These were related to energy metabolism, changes in protein, lipid profiles, and also bile acid production from early to late incubation as shown in Table 1 on blood biochemical changes (Harr 2002, 2006; Hochleithner 1994; Lumeij 2008; Samour 2006). Riboflavin (vitamin B_2), for example, was significantly down-regulated at late incubation. This is not surprising as it is a potent antioxidant and central in flavin adenine mono- and dinucleotide formation and energy metabolism. Significant changes in fasting eiders are therefore an expected event together with thousands of other metabolites (Harrison and McDonald 2006). Previously, Balk et al. (2009, 2016) suggested wild Baltic birds including Swedish eiders have low reproduction rates and that both adults and chicks may die from thiamine (vitamin B_1) deficiency. Mörner et al. (2017) also proposed that the decline could be due to thiamine deficiency and high mortality of the ducklings a few days after hatch due to abnormal behaviour resulting in high gull and white-tailed eagle predation. We did not observe changes in thiamine in the present study so our results cannot be used to claim that the eiders suffered from vitamin B_1 deficiency or that there was an overall thiamine deficiency in the Baltic ecosystem. In this case, it would have led to a complete collapse of the seabird populations in the Baltic and there is no such reporting. It should also be kept in mind that thiamine deficiency in for example blue mussels, crustaceans and fish do not tranfer to next trophic level such as eiders and fish-easting gulls (Sonne et al. 2012b).

5. Considerations

These results show that fasting during incubation affect biochemical profile and energy metabolism of female eiders and that this may be most pronounced for the Christiansø colony in the Baltic proper. This adds to the multiple stressor events such as temperature increases and food web changes caused by global warming that eventually accelerate the loss in body weight. Future studies should examine the relationship between body condition, temperature and reproductive outcome including mapping of food web contaminant, energy and nutrient content to better understand, manage and conserve the populations. Such an exercise should also include hypothesis generating data using metabolomics and bioinformatics.

The Baltic Sea population of common eiders has declined dramatically during the last two decades due to multiple factors affecting survival and reproduction in eiders. These multiple stressors include

contaminant exposure, food web changes linked to global warming, hunting, white-tailed eagle and gull predation of eggs, ducklings and eiders as well as decreased agricultural eutrophication, infectious diseases, such as avian cholera, and parasites (Sonne et al. 2012a,b; Garbus et al. 2018a,b,c, 2019). These factors affect the dynamics of the Baltic/Wadden Sea Flyway. This amplify the events of temperature increases and food web changes caused by global warming that eventually accelerate the loss in body weight. In addition, lead concentrations in the Christiansø incubating eiders was very high in 2018, adding further stress to the neuro-endocrine and organ functioning of the birds (Lam et al. 2020; McPartland 2019). This may help explain the Christiansø colony decline over the past 20–30 years. Altogether, Christiansø may be a good colony for the biomonitoring of physiology and population dynamics in the Baltic/Wadden Sea Flyway (Delaney and Scott 2002, 2006; Lyngs 2014).

6. Conclusions

The body mass of incubating eiders was lowest at Christiansø in the east compared to Hov Røn in the west. This was reflected in the blood biochemistry, mercury concentrations and the metabolomics profile showing that eiders to the east fasted more than those to the west. Altogether this indicates that the eiders in the central part of the Baltic Sea are susceptible to global warming and energetic stress, pollution and infectious diseases during incubation. This amplify the events of temperature increases and food web changes caused by global warming that eventually accelerate the loss in body weight. Future studies should examine the relationship between body condition, temperature and reproductive outcomes and include mapping of food web contaminants, energy and nutrient content to better understand, manage and conserve the populations.

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Conflict of interest statements.

We report that there are no conflicts of interests, and that the submitted manuscript has been reviewed and approved by all co-authors, and is not under consideration for publication elsewhere.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.105866.

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