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Decoding sex-specific metabolomic biomarkers in the loggerhead sea turtle (*Caretta caretta*)

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There is need for advancing minimally invasive diagnostic techniques for sex differentiation at early life-stage classes in sea turtles. The objectives of this study were to determine whether there are sex-specific effects on the metabolome of the loggerhead sea turtle (*Caretta caretta*) and to identify potential biomarkers for sex classification at early life-stage classes (50 post-hatchling and 50 juvenile). Comprehensive analyses including plasma chemistry (n = 100) and untargeted (n = 48) metabolomic analyses were performed. Unlike with plasma chemistry analytes, there were significant differences in the metabolomic profiles between sexes. Nine metabolites differed significantly between sexes, with several metabolites deserving greater attention as potential biomarkers: $C_{22}H_{32}O_6$ and pantothenic acid was higher in females than in males, and carboxylic acid ester ($C_{10}H_{16}O_5$), 1-methylhistidine and $C_7H_{14}N_2O_7$ was higher in males than females. These differences remained constant both across and within the same life-stage class. This work identified a discernible effect on the metabolic profile based on sex of loggerhead sea turtles and it suggests that these metabolites and/or their ratios could serve as potential biomarker candidates for sex classification in this species.

The loggerhead sea turtle (*Caretta caretta*) is distributed in oceans around the globe and is listed in nine distinct population segments under the United States Endangered Species Act. Additionally, it is listed by the Convention on International Trade in Endangered Species Appendix I and as vulnerable on the red list of the International Union for the Conservation of Nature[1,2]. Several factors pose a threat to loggerhead sea turtles, including bycatch in fisheries, climate change, interactions with watercraft, illegal hunting, and loss or degradation of nesting beaches, among others[3–11]. Major threats to hatchlings include pollution (including artificial lighting), beach degradation, hurricanes, increasing nest temperatures, and predation, while for juveniles (2.5–33 kg, 20–60 cm, 19–48 cm, 18–59 cm, and 16–65 cm for the range of weight, long straight carapace, wide straight carapace, long curved carapace, and wide curved carapace, respectively), fisheries interactions represent a significant concern[4].

Due to their long life cycle and the emergence of sex-based phenotypic differentiation later in life, developing techniques for sex differentiation at early life stages in reptiles is particularly intriguing for several reasons[12,13]. These include a better understanding of the physiological mechanisms regulating sexual differentiation in reptiles[14,15], the assessment of the impacts of climate change on potential increased number of females relative to males[16], and understanding its relation to population survival are particularly noteworthy[17–19].

Because of the temperature-dependent sex c in sea turtles, the utilization of genetic differences for testing is not feasible[20]. Consequently, sex classification in sea turtles is achieved by evaluation of external features in adult life-stage classes, endoscopy, or blood analysis (e.g., plasma chemistry analysis, hormones). Given the absence of external morphologic characteristics in immature life-stage classes of sea turtles, evaluation based on secondary sex characteristics is impossible. Endoscopy has become a well-established tool for diagnosis and for surgical procedures in managed care settings[21]. Coelioscopy, with or without biopsy sampling of the gonads, has been employed for sex classification in sea turtles in various life-stage classes,

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including loggerhead sea turtles post-hatchlings (0.0–2.5 kg, 9.0–20 cm, 8.0–19 cm, 14–18 cm, and 12–16 cm for the range of weight, long straight carapace, wide straight carapace, long curved carapace, and wide curved carapace, respectively)[14,20,22,23]. In field settings, endoscopy can pose potential risks to turtles because of the need for sterility and the lack of anesthesia, pain management, and post-procedural monitoring. These considerations are also important in laboratory settings due to potential mortality, although it is rarely reported[14,24].

Blood sampling is a minimally invasive sampling technique that is integral for assessing the health of sea turtles in various settings, including studies of wild sea turtles, which focused on population health in different species, geographical locations, and life-stage classes, studies focused on questions related to physiology or stressors on free-ranging populations, and in individual turtles admitted to rehabilitation facilities for diagnosis, monitoring, and treatment[21,25–31]. Routine plasma chemistry analysis associated with vitellogenesis in mature females includes total protein, calcium, phosphorus, triglycerides, and cholesterol[26,32]. However, these analytes are considered less sensitive than hormone assays, several of which have been validated for sea turtle plasma. Testosterone, estrogen, and their ratios have been utilized to assess sex differences in wild sea turtles. However, these assays require the use of considerable sample volume, and the target hormones may be present in insufficient or variable quantities in hatchlings and immature turtles[20,22,23,33], resulting in low sensitivity for sex classification. Given the inherent methodological challenges associated with these assays, standardized best practice recommendations have been recently published for validation and utilization of enzyme immunoassays for testosterone (T5) quantification in sea turtles[34]. Additionally, the quantification of Anti-Mullerian hormone in plasma samples has the potential to facilitate sex differentiation of males already at the neonatal stage[15].

Metabolomic technologies are employed to quantify metabolites in a variety of sample matrices, including blood samples. As omics-based analyses have become more available for wildlife research in recent years, several studies on sea turtle blood have been published on metabolomic changes during rehabilitation[35], the effects of extrinsic factors[36], oil exposure[37], cold-stunning[38], and on efforts for optimization of sample analyses[39]. Small-sized molecules can be detected with the potential to identify proteins and metabolites for assay development for specific use such as sex differentiation. This approach has been utilized in humans[40,41], mammals[42], and invertebrates[43]. However, sex differences in the metabolome of reptiles and specifically sea turtles have not been investigated to date. The objectives of this study were to determine sex-specific effects on plasma chemistry analytes and the metabolome of the loggerhead sea turtles, and to identify potential biomarkers for sex classification at early life-stage classes.

Results

Plasma samples from 100 loggerhead sea turtles ($n = 50$ females and $n = 50$ males) from two different life-stage classes ($n = 50$ post-hatchlings, $n = 50$ juveniles) were analyzed. The six experimental groups included: females ($n = 50$), males ($n = 50$), and within them: female post-hatchlings ($n = 25$), male post-hatchlings ($n = 25$), female juveniles ($n = 25$) and male juveniles ($n = 25$). No samples exhibited hemolysis or other plasma discolorations.

Tables 1, 2 and supplementary Tables 1, 2 present summaries of the sex-specific effects on plasma chemistry analytes in all animals, including post-hatchlings and juvenile turtles, respectively. The results indicate that sex did not significantly affect any of the studied plasma analytes.

Supplementary Fig. 1 shows the results derived from untargeted metabolomics, regardless of post-hatchling and juvenile loggerhead sea turtles. Supplementary Fig. 1a, c shows the first two principal component analyses obtained through partial least-squares discriminant analysis (PLS-DA) of untargeted metabolomics data in negative and positive modes, respectively. The variability associated with these principal components, which account for 30% and 28% of the total variance, effectively differentiates between sexes, as minimal overlap between the sexes is observed. The volcano plots (Supplementary Fig. 1b for negative mode and d for positive mode) highlight the metabolites responsible for the discrimination between sexes. In summary, a sex-specific effect on the metabolome of loggerhead sea turtles independently of life-stage classes is evident. After selecting the most important features from the volcano plots, Table 3 summarizes the tentatively identified metabolites that explain the differences observed.

Table 1 | Plasma chemistry analytes (least-square means ± standard error) according to sex in post-hatchling loggerhead sea turtles (*Caretta caretta*) ($n = 50$)

Metabolites[a]	Females ($n = 25$)	Males ($n = 25$)	*p*-value
NEFA (μ eqv./L)	455 ± 43.0	529 ± 42.1	0.6021
Albumin (g/L)	14.0 ± 0.75	14.8 ± 0.73	0.8921
Total Protein (g/L)	25.4 ± 1.69	26.5 ± 1.66	0.9675
Glucose (mM)	6.50 ± 0.66	7.20 ± 0.66	0.8920
Urea (mM)	47.1 ± 2.89	51.0 ± 2.83	0.7658
Triglycerides (mM)	1.39 ± 0.13	1.34 ± 0.13	0.9901
Uric acid (μM)	18.3 ± 17.0	10.7 ± 16.7	0.9887
Cholesterol (mM)	3.78 ± 0.25	4.63 ± 0.25	0.0807
Phosphorus (mM)	2.21 ± 0.16	2.03 ± 0.16	0.8588
Calcium (mM)	1.18 ± 0.12	1.24 ± 0.12	0.9819

[a]*NEFA* Non-esterified fatty acid.

Table 2 | Plasma chemistry analytes (least-square means ± standard error) according to sex in juvenile loggerhead sea turtles (*Caretta caretta*) ($n = 50$)

Metabolites[a]	Females ($n = 25$)	Males ($n = 25$)	*p*-value
NEFA (μ eqv./L)	466 ± 42.1	398 ± 43.0	0.6752
Albumin (g/L)	13.1 ± 0.73	14.0 ± 0.74	0.8324
Total Protein (g/L)	38.9 ± 1.66	39.2 ± 1.69	0.9990
Glucose (mM)	9.70 ± 0.65	9.10 ± 0.65	0.9388
Urea (mM)	35.3 ± 2.83	37.5 ± 2.89	0.9476
Triglycerides (mM)	0.84 ± 0.13	0.95 ± 0.13	0.9384
Uric acid (μM)	98.2 ± 16.7	86.2 ± 10.7	0.9582
Cholesterol (mM)	2.70 ± 0.25	2.72 ± 0.25	1.0000
Phosphorus (mM)	2.50 ± 0.16	2.50 ± 0.16	1.0000
Calcium (mM)	1.38 ± 0.12	1.53 ± 0.12	0.7851

[a]*NEFA* Non-esterified fatty acid.

Table 3 | List of plasma metabolites differentiating among sexes in loggerhead sea turtles (*Caretta caretta*)

m/z-RT	ION	Metabolite[a]
544.3371RT9.423	$[M+H]^+$	$C_{28}H_{50}NO_7P$ - LysoPC(0:0/20:4)
568.33643RT9.411	$[M+H]^+$	$C_{30}H_{50}NO_7P$ - LysoPC(22:6/0:0)
526.28967RT9.367	$[M+H]^+$	$C_{27}H_{44}NO_7P$ - LysoPE(22:6/0:0)
415.20901RT9.084	$[M+Na]^+$	$C_{22}H_{32}O_6$
500.27371RT8.816	$[M+H]^+$	$C_{27}H_{44}NO_7P$ - LysoPE(22:6/0:0)
217.1049RT4.974	$[M+H]^+$	$C_{10}H_{16}O_5$ - Carboxylic acid ester
239.08701RT4.972	$[M+H]^+$	$C_7H_{14}N_2O_7$
522.35211RT10.295	$[M+H]^+$	$C_{26}H_{52}NO_7P$ - 1-acyl-sn-glycero-3-phosphocholines
170.0905RT0.669	$[M+H]^+$	1-Methylhistidine
343.2258RT10.837	$[M-H]^-$	$C_{22}H_{32}O_3$ - 7-HDoHE
218.1019RT2.792	$[M-H]^-$	Pantothenic acid
191.0182RT1.305	$[M-H]^-$	Citric/Isocitric acid

[a]Identification was performed tentatively using MS/MS and METLIN/HMDB databases.

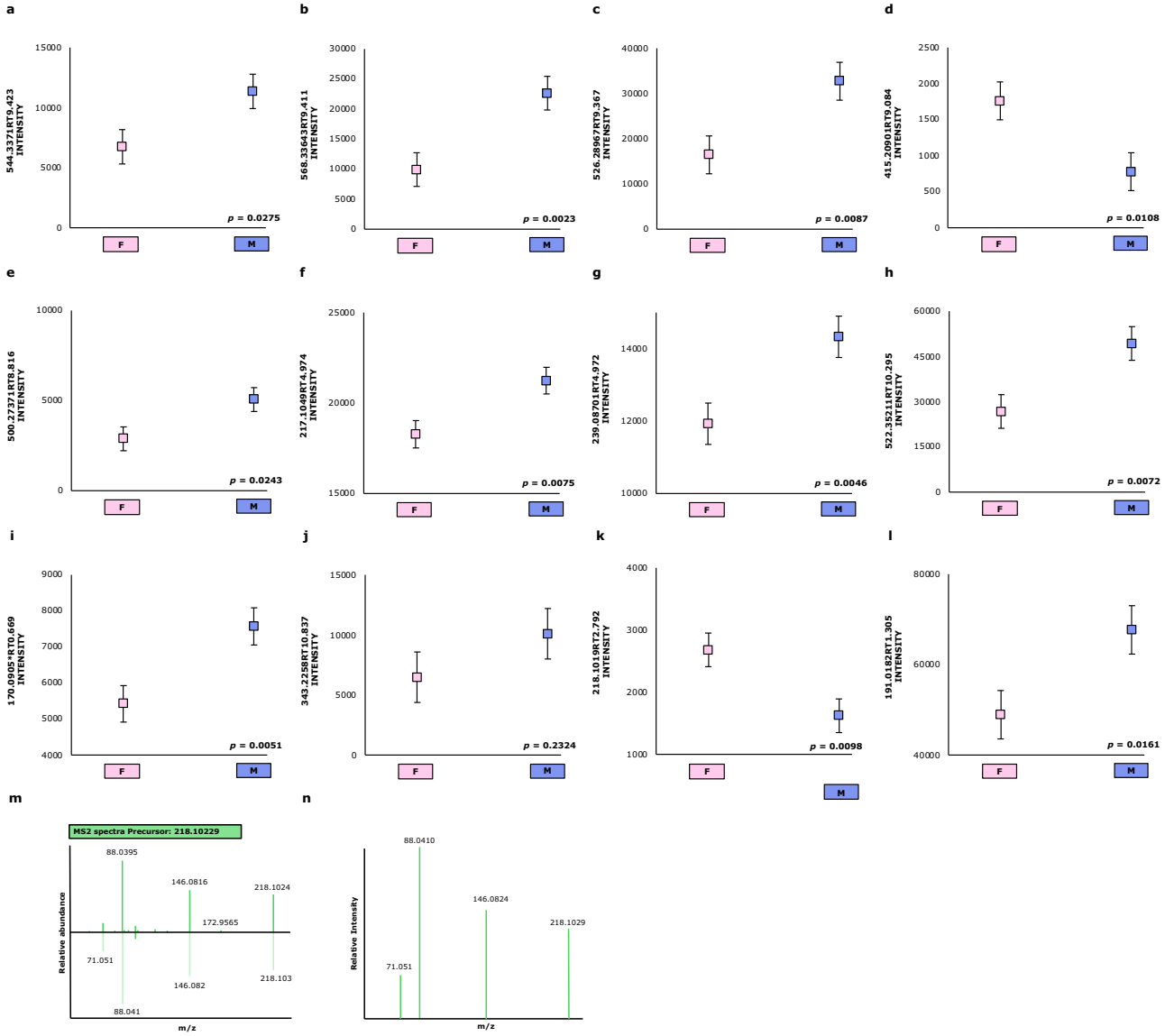


Fig. 1 | Effect of the sex on main discriminant metabolites in loggerhead sea turtles (*Caretta caretta*). a–l Top 12 metabolites according to sex, regardless of life-stage class. Colors represent different sexes ■: females and ■: males. **m** Example of chromatogram obtained for feature "218.1019RT2.792" provided from MS-DIAL. The upper part shows the spectrum obtained from the experimental samples, while the lower part shows the spectrum of the suggested metabolite (in this case, pantothenic acid). **n** Adaptation obtained from a search for the fragmentation of pantothenic acid, in this case obtained from HMDB. Necessary for the identification of metabolites. ($n = 48$). Obtained by GLM analysis. LS means and standard errors (error bars).

Figure 1 provides a summary of the most distinctly different metabolites explaining the variation between sexes, regardless of their physiological stage. Two metabolites ($C_{22}H_32O_6$ and pantothenic acid) were higher in females than males (+169%; $p = 0.0108$ and +65%: $p = 0.0098$, respectively). Several metabolites were higher in males than compared to females: LysoPC(0:0/20:4), LysoPC(22:6/0:0), LysoPE(22:6/0:0), LysoPE(22:6/0:0), carboxylic acid ester, $C_7H_{14}N_2O_7$, 1-acyl-sn-glycero-3-phosphocholines, 1-Methylhistidine, and citric/isocitric acid (+68%; $p = 0.0275$, +129%; $p = 0.0023$; +99%; $p = 0.0087$; +75%; $p = 0.0243$; +16%; $p = 0.0075$; +20%; $p = 0.0046$; +85%; $p = 0.0072$; +40%; $p = 0.0051$; and +38%; $p = 0.0161$), respectively. Finally, Fig. 1m presents an example chromatogram generated by the MS-DIAL software, suggesting the presence of a metabolite (e.g., pantothenic acid). The identity of this metabolite was confirmed by comparing it to the chromatogram obtained from the Human Metabolome Database (HMDB) for the same compound (Fig. 1n).

Figure 2 includes the pathway analysis plot. It aligns with the graphical summary of the pathway analysis. In this plot, all matched pathways are represented as circles. This plot shows that histidine and citrate metabolism were the most enriched pathways.

With the primary intention of observing whether there are effects on the metabolome at different life-stage classes and with the aim of identifying potential biomarkers for both life-stage classes, the main results of the untargeted metabolomics analyzed for each life-stage class are presented below.

Figure 3 presents a summary of the results derived from untargeted metabolomics. Figure 3a, b illustrates the first two principal components obtained through PLS-DA of untargeted metabolomics data in negative and positive modes, respectively, for post-hatchlings. Conversely, Fig. 3f, g represents these identical modes for juveniles. As demonstrated, the variability linked to these principal components extracted from the metabolic profile (32%, 27%, 27%, and 30%, respectively, of the total) can effectively distinguish between sexes in both life-stage classes, as there is no overlap between the two sexes. The volcano plots (Fig. 3c, d for negative and positive mode in post-hatchlings and Fig. 3h, i for negative and positive mode in juveniles) allow observation of the metabolites responsible for the

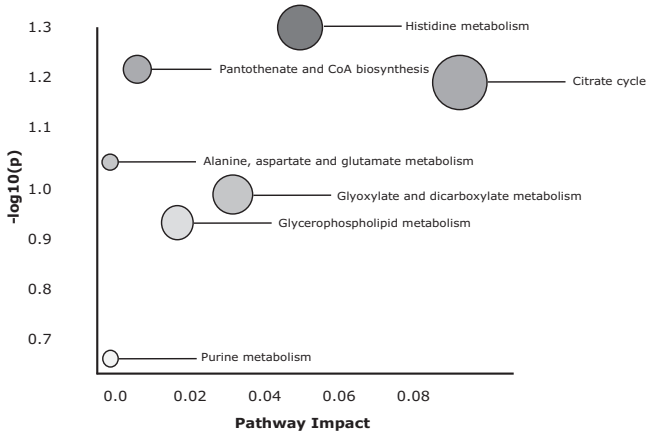


Fig. 2 | Overview of the pathway analysis plot. The screen provides a graphical summary of the pathway analysis in loggerhead sea turtles (*Caretta caretta*). In the plot, all matched pathways are represented as circles. The color and size of each circle correspond to its *p*-value and pathway impact value, respectively.

discrimination between sexes. Finally, the dendrograms demonstrate the classification of samples based on their metabolome. These figures confirm that there are indeed sex effects on the metabolome of this species.

Figure 4 presents variable importance in projection (VIP) scores for the top eight metabolites that contribute to the differentiation of sexes in post-hatchlings (a) and juvenile animals (b). It should be noted that all metabolites align with the observed differences when comparing sexes, regardless of life-stage class.

Figure 5a provides a summary of the identified metabolites that explains the differences between sexes in post-hatchlings. Notably, females exhibited higher $C_{22}H_32O_6$ ($p = 0.0008$) and pantothenic acid ($p = 0.0115$) than males. Conversely, males showed higher LysoPC (O:O/20:4) ($p < 0.001$), LysoPC (22:6(0:0)) ($p < 0.001$), LysoPE (20:5/0:0) ($p < 0.001$), carboxylic acid ester ($p < 0.0001$), $C_7H_{14}N_2O_7$ ($p = 0.0006$), LysoPC (18:1/0:0) ($p = 0.0001$), 1-methylhistidine ($p = 0.0350$), 7-HDoHE ($p = 0.0199$), pantothenic acid ($p = 0.0115$), and citric/isocitric acid ($p = 0.0052$) than females. In the same figure, the violin plots illustrate the intensity of $C_{22}H_32O_6$ (b), carboxylic acid ester (c) and the ratio between both (d).

Figure 6a illustrates the identified metabolites that contribute to the observed differences between sexes in juvenile specimens. Females exhibited

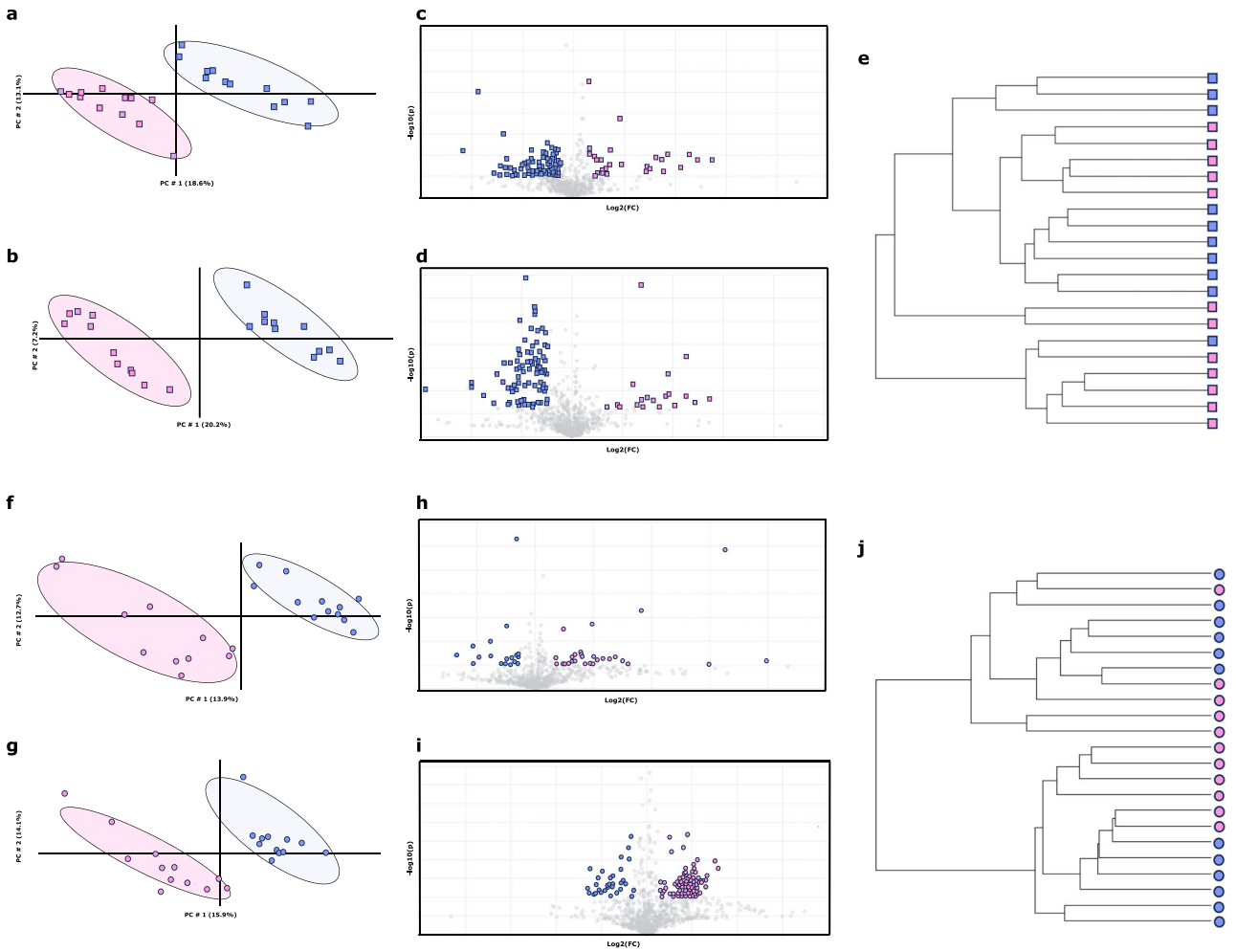


Fig. 3 | Summary of the results of the untargeted metabolomics assay according to life-stage class in loggerhead sea turtles (*Caretta caretta*). **a**, **b**, **f**, **g** PLS-DA score plot of plasma in negative (**a**, **f**) and positive mode (**b**, **g**) in post-hatchlings (**a**, **b**) and juveniles (**f** and **g**), respectively. The colors and shapes correspond to the four experimental groups: : female post-hatchlings, : male post-hatchlings, : female juveniles, and : male juveniles. ($R^2 = 0.998$, $Q^2 = 0.536$; $R^2 = 0.980$, $Q^2 = 0.655$; $R^2 = 0.981$, $Q^2 = 0.593$; $R^2 = 0.998$, $Q^2 = 0.379$, for **a**, **b**, **f**, **g**, respectively). **c**, **d**, **h**, **i** Volcano plots show significantly different abundant metabolites between both life-stage classes (two-sided Wilcoxon rank tests with the value adjusted by false discovery rate, FDR < 0.05) are shown; : fold change > 1.037, : fold change < 0.949, : fold change > 1.003, and : fold change > 1.003 in the volcano plot. Volcano plots are in negative (**c**, **h**) and in positive mode (**d**, **i**). **e**, **j** Dendrogram of the different samples obtained by ultra-high-performance liquid chromatography and mass spectrometer for post-hatchlings and juveniles, respectively.

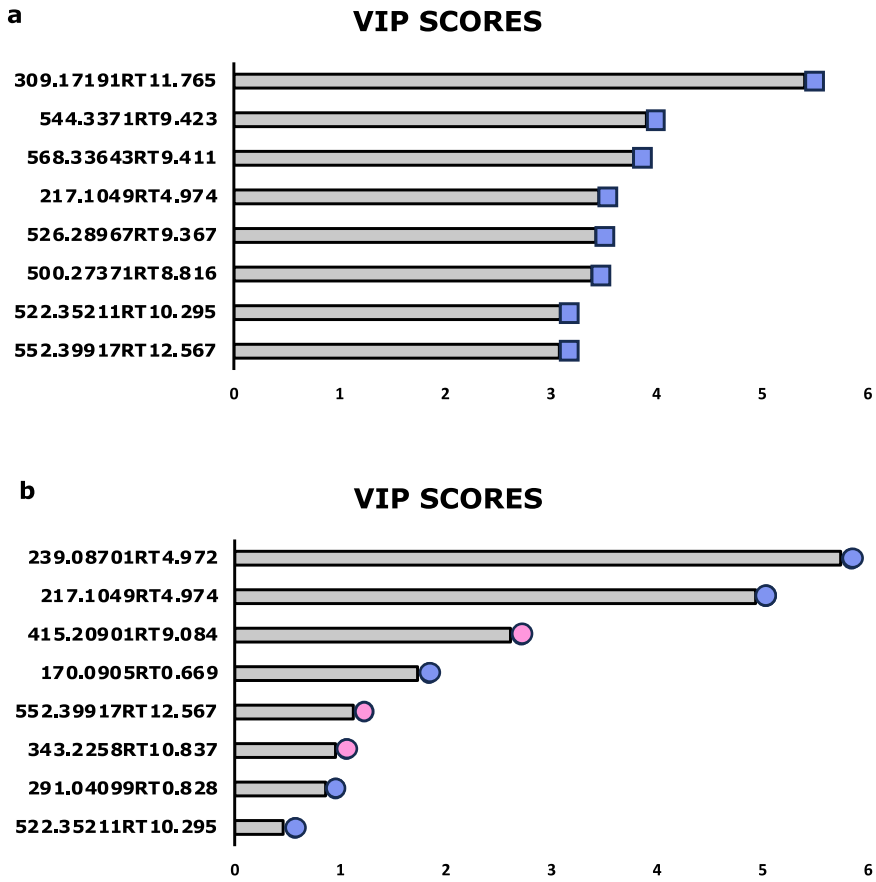


Fig. 4 | Discriminating metabolites according to their variable importance in projection (VIP) scores in each life-stage class in loggerhead sea turtles (*Caretta caretta*). Top 8 metabolites contribute to the separation of the metabolic profiles of females and males in post-hatchlings (**a**) and juveniles (**b**), respectively. Colors and shapes represent higher concentrations in the experimental groups : female post-hatchlings, : male post-hatchlings, : female juveniles, and : male juveniles.

a

m/z-RT	*p*_value	Fold[1]
544.3371RT9.423	<0.001	0.349
568.33643RT9.411	<0.001	0.355
526.28967RT9.367	<0.001	0.384
415.20901RT9.084	0.0008	1.902
500.27371RT8.816	<0.001	0.468
217.1049RT4.974	<0.001	0.837
239.08701RT4.972	0.0006	0.811
522.35211RT10.295	0.0001	0.473
170.0905RT0.669	0.0350	0.751
343.2258RT10.837	0.0199	0.393
218.1019RT2.792	0.0115	1.716
191.0182RT1.305	0.0052	0.636

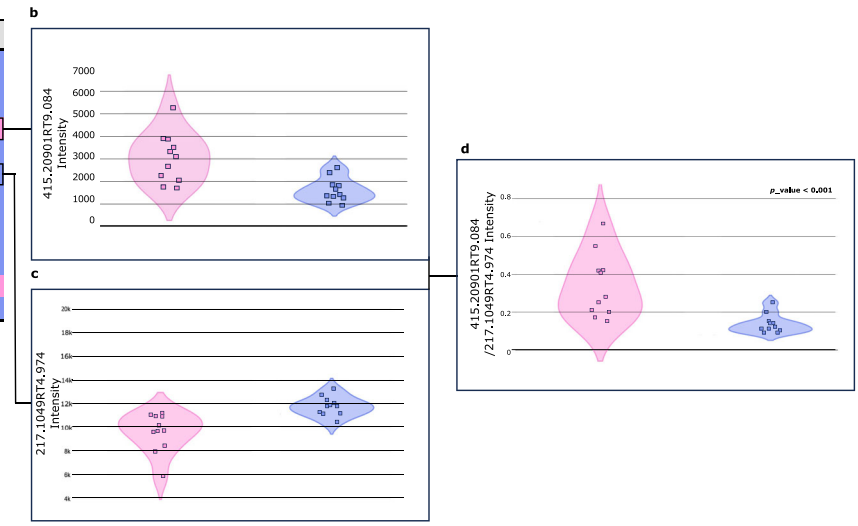
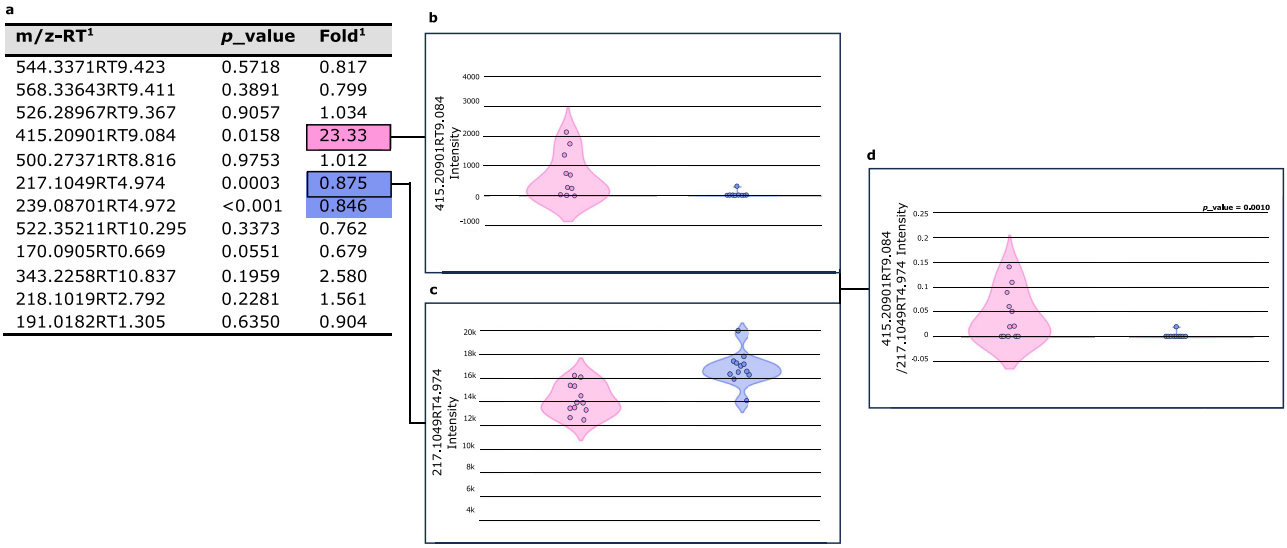


Fig. 5 | Summary of the results of the untargeted metabolomics in post-hatchling loggerhead sea turtles (*Caretta caretta*). **a** List of annotated plasma metabolites that discriminate between post-hatchlings : female post-hatchlings, : male post-hatchlings.[1]Fold change was calculated by dividing the mean intensity of the plasma metabolite in female post-hatchlings by the mean intensity of the corresponding metabolite in male post-hatchlings; pink and blue colors represent a greater amount of that metabolite in females and males, respectively. **b**, **c** The violin plots show the intensity of 415.20901RT9.084—$C_{22}H_{32}O_6$ (**b**), 217.1049RT4.974—Carboxylic acid ester (**c**), and the ratio between both (**d**) determined by ultra-high-performance liquid chromatography and mass spectrometry ($n = 24$).

higher $C_{22}H_{32}O_6$ ($p = 0.0158$) than males, while males displayed higher carboxylic acid ester ($p = 0.0003$) and $C_7H_{14}N_2O_7$ ($p < 0.0001$) than females. The violin plots illustrate the intensity of $C_{22}H_{32}O_6$ (b), carboxylic acid ester (c), and the ratio between both (d). As can be observed in the violin plots, it was possible to discern the maximum and minimum concentrations, which allowed for establishing thresholds at which 100% of males and females were distinguished.

Regarding the identification of biomarkers for sex classification, it can be confirmed that for post-hatchlings, samples with a $C_{22}H_{32}O_6$ metabolite intensity greater than 3000 were 100% female, while those with values below 1500 were 100% male (Fig. 5b). Similarly, for Carboxylic acid ester, animals with intensities above 12,000 were 100% male, and those with values below 10,000 were 100% female (Fig. 5c). Finally, by calculating the ratio between the two metabolites—$C_{22}H_{32}O_6$/Carboxylic acid—a threshold of 0.3 was



m/z-RT[1]	p_value	Fold[1]
544.3371RT9.423	0.5718	0.817
568.33643RT9.411	0.3891	0.799
526.28967RT9.367	0.9057	1.034
415.20901RT9.084	0.0158	23.33
500.27371RT8.816	0.9753	1.012
217.1049RT4.974	0.0003	0.875
239.08701RT4.972	<0.001	0.846
522.35211RT10.295	0.3373	0.762
170.0905RT0.669	0.0551	0.679
343.2258RT10.837	0.1959	2.580
218.1019RT2.792	0.2281	1.561
191.0182RT1.305	0.6350	0.904

Fig. 6 | Summary of the results of the untargeted metabolomics in juvenile loggerhead sea turtles (*Caretta caretta*). a List of annotated plasma metabolites that discriminate between juvenile animals ● : female juveniles, and ● : male juveniles.[1]Fold change was calculated by dividing the mean intensity of the plasma metabolite in female post-hatchlings by the mean intensity of the corresponding metabolite in male post-hatchlings; pink and blue colors represent a greater amount of that metabolite in females or males, respectively. **b**, **c** The violin plots show the intensity of 415.20901RT9.084—$C_{22}H_{32}O_6$ (**b**), 217.1049RT4.974—Carboxylic acid ester (**c**), and the ratio between both (**d**) determined by ultra-high-performance liquid chromatography and mass spectrometry ($n = 24$).

established, with all animals above this value being female and all those below this value being male (Fig. 5d). Similarly, in juveniles, it was observed that samples with $C_{22}H_{32}O_6$ intensity greater than 500 were 100% female, while those with values below 500 were 100% male (Fig. 6b). Similarly, for Carboxylic acid ester, animals with intensities above 17,000 were 100% male, and those with values below 14,000 were 100% female (Fig. 6c). Finally, by calculating the ratio—$C_{22}H_{32}O_6$/Carboxylic acid— all animals above 0.02 being female and all those below this value being male (Fig. 6d).

In terms of validation, the average R^2 and Q^2 values obtained were 0.99 and 0.54, respectively. Regarding the validation of the metabolomics analysis, R^2 assesses how well the model fits the observed data, while Q^2 assesses its predictive power. As described in the SIMCA users' guide, a Q^2 value greater than 0.5 is acceptable for good predictability[44]. Based on our average results obtained for R^2 and Q^2 (0.99 and 0.54, respectively), we can conclude that the models used fit well and can be considered good predictors.

Discussion

This study successfully decoded sex-specific plasma metabolites using untargeted metabolomic profile analysis to define phenotypic patterns of small molecules from two immature life-stage classes of loggerhead sea turtles. Despite the absence of differences in plasma chemistry analytes by sex, untargeted metabolomic analyses identified several metabolites that consistently differentiated sex in both life-stage classes, with minimal overlap between sexes. Consequently, the utilization of these molecules individually, in combination or as ratios could potentially serve as reliable, minimally invasive biomarkers for sex classification in both post-hatchling and juvenile loggerhead sea turtles.

Traditional plasma chemistry analytes were unable to identify sex differences in either life-stage class in this study. This was not unexpected, since such differences have been reported as relatively subtle in other studies of more mature life-stage classes; Such differences included lower plasma cholesterol and higher uric acid in foraging green turtles in the Bahamas[33], lower LDH and iron as well as higher AST and calcium, in male compared to female green turtles in the Arabian Sea[45], and minimal differences have been documented in studies of foraging adult leatherback sea turtles[32,46]. This illustrates the need for the development of sensitive blood biomarkers for sex classification in both immature and more mature life-stages classes and independent of other factors (e.g., nutrition, habitat).

One of the main objectives stated can be considered fulfilled, as sexual specificity in the metabolome of the loggerhead sea turtle was consistently observed between sexes and across two life-stage classes. Furthermore, aiming to identify potential biomarkers, the metabolites 415.20901RT9.084 ($C_{22}H_{32}O_6$) and 218.1019RT2.792 (pantothenic acid) were significantly higher in females than in males. The former was selected because it was significantly higher in all comparisons made, and the latter, because it shows good identification and higher concentrations in females compared to males, although not significantly higher in juveniles. We also identified the following metabolites of interest: 217.1049RT4.974 (Carboxylic acid ester), 239.08701RT4.972 ($C_7H_{14}N_2O_7$), and 170.0905RT0.669 (1-methylhistidine), which were significantly higher in males than in females (except for the trend observed in juveniles for 1-methylhistidine).

The fold change in $C_{22}H_{32}O_6$ was 2, 2, and 23 times higher in post-hatchlings and juvenile female loggerhead sea turtles, respectively, compared to males in corresponding life-stage classes. Although further efforts to identify this metabolite are needed, there is evidence that this compound belongs to the prostaglandin families[47–49]. Prostaglandins represent a vital group of bioactive lipid compounds in living organisms, particularly significant as they play a crucial role in female reproduction[47,50]. Indeed, other studies corroborate these findings, demonstrating higher baseline prostaglandin concentrations in female humans[51] and fish[52]. In female turtles, oestradiol is detectable during phases of vitellogenesis, whereas prostaglandins are not[53]. However, prostaglandin concentrations significantly increase 15 min post-emergence. This shows significant roles of these mediators for nesting. Thus, this class of lipid mediators within the group of eicosanoids may suggest a correlation with female development in sea turtles, as $C_{22}H_{32}O_6$ exhibited a notable increase from post-hatchling to juvenile female loggerhead sea turtles. The fold change in pantothenic acid was two times higher in females than in males in all comparisons. Pantothenic acid, also known as vitamin B5, is an essential trace nutrient precursor of coenzyme A (CoA) and it has several key roles in some biological processes[54], such as fatty acid oxidation[55]. This connection with fatty acid oxidation suggests a relationship between both metabolites. Other studies relate different vitamin concentrations between males and females[56,57].

Three metabolites were consistently higher in males, specifically 217.1049RT4.974 (carboxylic acid) on av. 1.1, 239.08701RT10.295 ($C_7H_{14}N_2O_7$) on av. 1.3 and 170.0905RT0.669 (1-methylhistidine) on av. 1.4 higher in males than in females. Carboxylic acid esters play important roles in the biochemistry of living systems[58]. Despite the limited research conducted to date, higher concentrations of two carboxylic acids were also observed in males of the Iberian pond turtle (*Mauremys leprosa*)[59], and these data would be consistent with those observed in this study. Although further research is needed, $C_7H_{14}N_2O_7$ has a potential relationship to phthalates. Phthalates are plasticizers and organic compounds used in many industrial and personal care products; they are known to be endocrine disruptors in humans[60]. $C_7H_{14}N_2O_7$ and other closely related compounds have been proposed as biomarkers for exposure to di-n-butyl phthalate[61]. Since this metabolite is not an endogenous compound but is consistently higher in males of both life-stage classes, it likely has no biological relevance (i.e., as a plasma biomarker for sex differentiation). However, this may indicate that male turtles in this study were exposed to phthalates. Another consideration includes that there is evidence for an association between exposure to phthalate esters and endocrine function, particularly steroid hormone dysregulation[60,62] and its relationship to testosterone[63–66]. Similarly, our findings also showed higher phthalates in males at both life-stage classes. Previous studies have reported the effect of phthalates on sex hormone regulation in turtles. For example, in vitro studies have shown that exposure to phthalates decreases the expression of estrogen receptors in erythrocytes[67]. Furthermore, it is noteworthy that both carboxylic acid ester acid and $C_7H_{14}N_2O_7$ were higher in juvenile males (+36% and +42%, respectively) compared to post-hatchling males, which may be related to the increase in reproductive hormones as turtles grow. Few reports relate this metabolite to reproductive aspects in other species, suggesting that further investigation of its role in the reproductive physiology of these reptiles is warranted. The metabolite 1-histidine is particularly interesting due to its accurate and clear identification, as well as providing higher fold change in males than in females across both life-stage classes. Furthermore, as depicted in Fig. 2 showing the analysis of implicated metabolic pathways, histidine metabolism, along with citrate, holds significant relevance in metabolomic phenotypes. Although no literature has been found directly linking this metabolite to sexual differentiation in turtles, it is intriguing and is proposed as a candidate for future studies. Despite the differences this study has revealed regarding the plasma metabolome, it is particularly interesting to continue investigating other, less invasive matrices such as urine or even tears from these animals[68].

The main conclusions of this study were: (i) the metabolomic profiles of loggerhead sea turtles are influenced by sex, (ii) untargeted metabolomics is a potential tool for sex differentiation, and (iii) specific metabolites, such as $C_{22}H_{32}O_6$, pantothenic acid, carboxylic acid ester acid, and 1-methylhistidine are proposed as potential biomarkers for sex classification in loggerhead sea turtles. These biomarkers have the potential to significantly enhance future sex classification efforts by providing more sensitive and less invasive diagnostic tools compared to traditional techniques (e.g., endoscopy). In addition, they can potentially contribute to health assessments and other studies of immature sea turtle life-stage classes, aiding in the interpretation of population-level data by assessing sex ratios and providing valuable insights into sea turtle health. Furthermore, understanding the variations of these metabolites may be particularly interesting in unraveling the mechanisms of sexual differentiation in different reptile species other than sea turtles.

Methods

Animal ethics statement

Samples were collected from turtles that were either maintained under managed care in a captive rearing program, the post-hatchling group, or from wild turtles that were accidentally caught in local fisheries or rehabilitated for various reasons in Spain, in the case of juveniles. All actions were carried out in full compliance with Spanish regulations. All sea turtles included in this project were under the authority of the "Conselleria d'Agricultura, Desenvolupament Rural, Emergència Climàtica i Transició Ecològica" of the Regional Government of the Valencian Community in collaboration with the Fundación Oceanogràfic for actions of marine fauna conservation. Blood sampling and endoscopies for sex classification were approved by the institution's Animal Care and Use Committee under the number OCE-5-22. All handling and sampling procedures of sea turtles were performed in accordance with general ethical principles of veterinary medicine and animal welfare. We have complied with all relevant ethical regulations for animal use.

Animals and sampling

After hatching, turtles were included in a rearing program at the Oceanogràfic Aquarium in Valencia, Spain. Juveniles were sampled during the rehabilitation process when admitted to the rescue center of the same institution. The animals (from different locations of the Mediterranean) were classified according to their life-stage class as post-hatchlings (1.3 kg, 16.3 cm, 15.7 cm, 17.2 cm, and 13.9 cm for weight, long straight carapace, wide straight carapace, long curved carapace and wide curved carapace, respectively) and juveniles (10.6 kg, 38.0 cm, 31.8 cm, 42.0 cm, and 38.5 cm for weight, long straight carapace, wide straight carapace, long curved carapace and wide curved carapace, respectively), more information is available in Supplementary Table 2. This classification was done according to the known age of individuals from the head-start program and according to the straight carapace length (SCL), based on the criteria published by Casale et al.[69,70]. Sex classification was performed by laparoscopy following Wyneken et al.[14] no mortality was observed in the sampled animals.

All blood samples were collected during routine veterinary examinations from May 2021 to May 2023. Blood samples were collected during the morning hours. Blood was collected from the dorsal cervical sinus using a 1 to 5 mL syringe and a 25G to 21G hypodermic needle (Henry Schein®). Blood samples were transferred to 1 mL lithium heparin tubes (Aquisel®) for immediate processing. Plasma was obtained through centrifugation at 2000 rpm for 5 min and stored at −80 °C until analysis. Plasma samples were analyzed by routine chemistry analysis and untargeted metabolomics (Fig. 7) as outlined below.

Plasma chemistry analysis

Plasma chemistry analysis included non-esterified fatty acids (NEFA), albumin, total protein, glucose, urea, triglycerides, uric acid, cholesterol, phosphorus, and calcium. NEFA were determined using the Wako, NEFA C ACS-ACOD assay method. Plasma chemistry analyses were performed using an ADVIA 1800 ®Chemistry System autoanalyzer (Siemens Medical Solutions, Tarrytown, NY 10591, USA). For NEFA, triglycerides, and uric acid, intra-and inter CV% variation was below 2.5 and 3.1%, respectively. Correspondingly, for albumin, creatinine, total protein, glucose, urea, cholesterol, phosphorus, and calcium, CV% variation was below 2.2 and 2.8% in all instances.

Untargeted liquid chromatography–mass spectrometry (LC-MS) metabolomics analysis of plasma

A total of 48 plasma samples (12 from female hatchlings, 12 from male hatchlings, 12 from female juveniles, and 12 from male juveniles) were randomly selected. Plasma was prepared by deproteinizing 150 µL of sample with 450 µL ice-cold acetonitrile (100% ACN) containing an internal standard mixture of glycocholic acid (glycine-1-13C) and p-chlorophenylalanine to a final concentration of 0.01 mg/mL. Samples were prepared in 96-well plates with 1 mL wells. Plates were mixed for 1 min, incubated at 4 °C for 10 min and centrifuged at 2250 g and 4 °C for 25 min. Approximately 400 µL of supernatant was transferred to Phenomenex 96-square well filter plates under vacuum and the filtrate was collected in a collection plate. The filtered supernatant was transferred to two 200 µL 96-well plates (65 µL per well) and the plates were vacuum centrifuged to dryness (~2.5 h, 805 g, and 30 °C). Samples were resuspended in a mixture of H_2O:ACN:FA (95:5:0.1) using the same volume as before evaporation. A protective foil was sealed to the plate using a heat sealer and

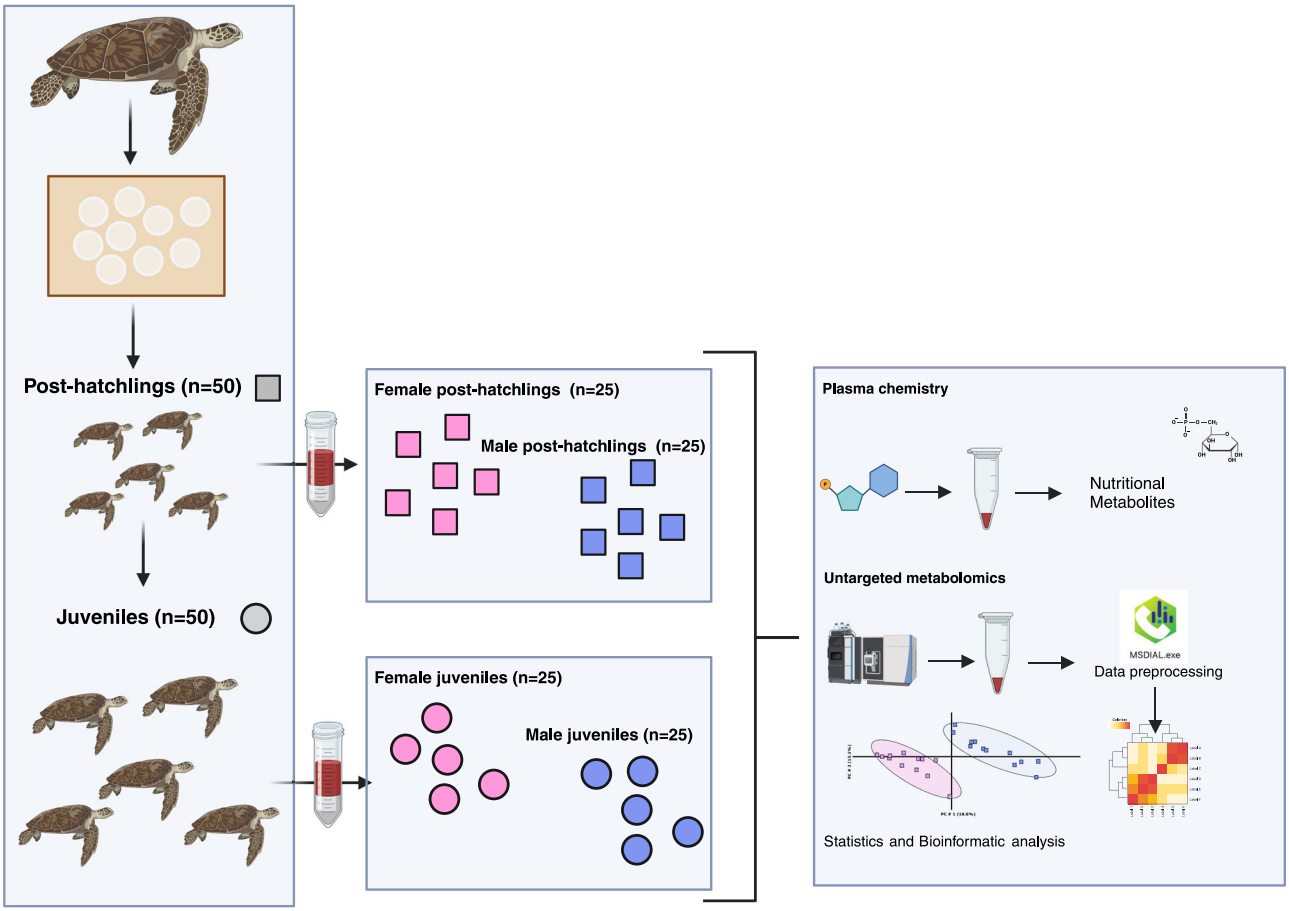


Fig. 7 | Experimental design. Samples from a total of 100 loggerhead sea turtles (*Caretta caretta*) belonging to both life-stage classes were used: post-hatchlings and juveniles, with the shapes of square and circle, respectively. From each of the groups, 25 females and 25 males were used to form the four experimental groups: : female post-hatchlings, : male post-hatchlings, : female juveniles, and : male juveniles. Blood samples were analyzed by both plasma chemistry analysis and untargeted metabolomics. *Plasma chemistry analysis:* All animals ($n = 100$) were used. Non-esterified fatty acid, albumin, total protein, glucose, urea, triglyceride, uric acid, cholesterol, phosphorus, and calcium were analyzed. *Untargeted metabolomics:* Blood samples from randomly selected animals ($n = 48$; 12 per group) were analyzed by ultra-high-performance liquid chromatography and mass spectrometry. Created with BioRender.com.

the plates were centrifuged at 3700 rpm, 4 °C for 25 min before the LC-MS analysis.

Samples were analyzed by UHPLC using a Nexera X2 LC coupled to an LCMS-9030 Q-TOF MS system (Shimadzu Corporation, Kyoto, Japan) using both positive and negative electrospray ionization (ESI). Chromatographic separations were performed on an Acquity HSS T3 column (1.7 µm 100 × 2.1 mm, Waters Ltd., Elstree, UK). The column temperature was set at 40 °C, the samples were held at 10 °C and 3 µL aliquots were injected onto the column. The chromatographic system used a binary gradient of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid) at a flow rate of 0.4 mL/min. A linear gradient was used from 5% B to 100% B over 12 min, with a hold at 100% for 1 min before returning to the initial conditions of 5% B for 3 min to re-equilibrate the column. This resulted in a total analysis time of 16 min per sample. MS detection was performed using a data-independent acquisition (DIA) method for both MS and MS/MS analyses. The method acquired a single time-of-flight (TOF) MS scan (m/z 50–900) followed by 33 MS/MS mass scans over a mass range of m/z 40–900; each MS/MS mass scan had a precursor isolation width of 25.2 Da and a collision energy range of 10–30 V, resulting in a cycle time of 0.9 s. This allowed collection of fragmentation data from all masses in the spectra over the entire LC gradient. The following MS parameters were used: ion source temperature, 300 °C; heated capillary temperature, 250 °C; heating block temperature, 400 °C; electrospray voltage, 4.5 kV (ESI+) or −3.5 kV (ESI−); electrospray nebulizer gas flow, 3 L/min; drying gas flow, 10 L/min; detector voltage, 2.02 kV. Mass calibration was performed externally using a sodium iodide solution (400 ppm in methanol) from m/z 50–1000. Data acquisition was performed using LabSolutions software version 5.114 (Shimadzu Corporation, Kyoto, Japan).

Sample quality control and metabolomics data preprocessing

The quality of the chromatographic runs, the stability of UPLC system and the accuracy of sample preparation were monitored using quality control samples (QCs). Plasma sample QCs were prepared by pooling an aliquot of all samples and subjecting this pooled sample to the same sample preparation protocol as the samples. The QCs were injected multiple times throughout the analysis as well as at the beginning and end of the analysis and were used in the data preprocessing for signal drift correction. Blanks were injected during the chromatographic analysis to monitor for external contamination from solvents, eluents, and carryover effects. Sample order was randomized for the chromatographic analysis to eliminate bias in the results and to ensure that each sample group was equally affected.

MS-DIAL software[71] was used to perform peak detection, alignment, and gap filling for the data files. The MS-DIAL generated data matrix was exported to Excel and filtered to eliminate peaks present in blanks, and retention time was truncated to include only portions containing chromatographic peaks, while masses greater than 700 m/z were discarded.

An initial principal component analysis was performed using LatentiX 2.12 (LATENTIX Aps., Gilleleje, Denmark) to check the quality of the data set and eliminate potential outliers. PLS-DA models were constructed to determine the metabolites responsible for the differences between

experimental groups (female vs males, post-hatchling females vs post-hatchling males, and juvenile females vs juvenile males) of loggerhead sea turtles. Model validation was performed using repeated random sub-sampling validation. Models were evaluated using the explained variation in Y, plots showing actual and predicted values, and the proportion of variation explained (R^2). Variables for identification were selected using VIP scores.

Chemical solvents and standards for metabolomics analysis

For untargeted metabolomics analysis, high-performance liquid chromatography (HPLC)-grade solvents and eluents were used as follows: HPLC-grade acetonitrile (VWR, West Chester, PA, USA), formic acid (FA, Fluka, Merck KGaA, Darmstadt, Germany), and MilliQ grade water (MilliporeSigma, Burlington, MA, USA). Internal standards included in the sample preparation were glycocholic acid (glycine-1-13C), and 4-chloro-DL-phenylalanine (Sigma, Merck KGaA, Darmstadt, Germany). To validate the PLS-DA models obtained, cross-validation was performed using 5 as the maximum number of components to be searched along with the five-fold CV method. Both R^2 and Q^2 were calculated for each generated PLS model.

Metabolite identification

Metabolites were identified by querying the online HMDB (http://www.hmdb.ca) to obtain possible chemical structures based on accurate mass and mass spectrometric fragmentation patterns. Lab Solutions and Sirius were also used.

Statistics and reproducibility

Three statistical analyses were performed in both chemistry analysis and untargeted metabolomics. The aim was to study sex-specific metabolomic biomarkers in females and males (independently of life-stage class), a study within the group of post-hatchlings (comparing post-hatchling females and post-hatchling males), and to unravel sex-specific metabolomic biomarkers in juveniles (comparing juvenile females and juvenile males).

In plasma chemistry and untargeted metabolomics analyses, metabolites were fitted to a normal distribution and analyzed as dependent variables using a GLM model from SAS, including sex, life-stage class, and their interaction as a main fixed effect. Least-square means comparisons were performed by t-test (Supplementary Tables 1, 2).

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files). Source data for all the figures and plots in the manuscript can be found in Supplementary Data 1.

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Author contributions

Conceptualization and experimental design were performed by P.J.M.G., D.G.P., J.L.C.P., L.L., M.C.L, E.B., J.J.P., and M.S.H. Data were collected by D.G.P. and J.L.C.P. and analyzed by P.J.M.G., T.L., and M.S.H. The manuscript was written by P.J.M.G. and N.I.S. and reviewed by all authors, who approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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