Title: *Accumulation and transfer of environmental pollutants from mothers to newborn sea turtles in Japan*

Members` names:

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1. Aim:

To support conservation of the two dominant species of sea turtles (loggerhead and green turtle) nesting in Japan, the project aimed to increase understanding of the accumulation patterns of organic pollutants during oceanic migrations of these sea turtles, and the rates at which these pollutants are subsequently transferred to offspring via maternal and embryonic transfer. Specifically, we intended to answer the following questions:

1. Do pollution profiles differ between loggerhead and green turtle nesting populations, which are known to have a different migration history?
2. Which pollutants are maternally transferred from mother towards egg albumen and yolk, and does this differ between loggerhead and green turtles?
3. Which pollutants are subsequently embryonically transferred from albumen and yolk towards different embryonic development stages?
4. Is there a relation between the chemical structure of pollutants and their maternal and embryonic transfer rates?

2. Procedure:

Due to the COVID-19 Pandemic and travel restrictions between Japan and The Netherlands, I couldn't come to Ehime University to conduct laboratory work. Consequently, the originally submitted plan for Joint Usage/Research Center, LaMer in Fiscal Year 2020 was adapted in consultation with Prof. Kunisue. We agreed to analyze a subset of samples (by a staff member of the laboratory) as a pilot study, and use the results to inform research to be conducted in a proposed LaMer 2021 project. Additionally, I complemented the laboratory research with a literature meta-analysis, to improve understanding of the mechanisms of maternal transfer. The meta-analysis provides the scientific background to interpret the data analyzed in the current project and informs our future work.

2.1 A meta-analysis of literature data

A systematic search regarding data on bioaccumulation of organic pollutions in coupled internal tissues was conducted for sea turtles. Data were standardized and transformed to allow comparison, a global database was then developed including details on pollution concentrations, biological characteristics of the sea turtles, chemical properties of the pollutants, and characteristics of the studies. This database formed the basis for a meta-analysis of patterns in bioaccumulation among internal tissues and maternal transfer.

2.2 Sample collection

Field sampling of sea turtle tissues (blood, eggs, and carapace) was conducted during 2018-2019. Samples of loggerhead turtles were collected in Kochi Prefecture in collaboration with Prof. Saito and Prof. Ikejima (Usa Marine Biological Institute, Kochi University) and director Wakatsuki (Muroto Schoolhouse Aquarium). Samples for green turtles were collected in Okinawa prefecture in collaboration with Dr. Kameda (Sea Turtle Association of Japan at the Kuroshima Research Station). Field sampling included incidental bycatch by local fishermen cooperatives, targeted sampling using shore nets, and night nesting patrols. Blood samples were analyzed for biochemistry health indicators within 2 hr after sampling in collaboration with Prof. Matsukawa (Kochi University). Carapace samples were dried and stored for stable isotope analysis to trace migratory movements in collaboration with the Kochi Medical School (Prof. Sakamoto) and the JAMSTEC Institute for Core Sample Research (Prof. Ikehara). Samples for pollution analyses were stored at -20°C until transfer into the es-Bank. Together with pollution samples, samples of blood and carapace tissue were taken.

2.3 Analysis of chemical pollution

Chemical analysis targeted PCBs and OCPs in the blood plasma of 1 female loggerhead turtle, and PCBs, OCPs, and PBDEs in 4 of her eggs (yolk and albumen). The analysis of eggs followed the protocol reported by Coelho et al. (2016). Yolk (approximately 20 g) and albumen (6–10 g) samples were freeze-dried and then extracted with hexane/acetone (1:1 v/v) on a SE-100 High-Speed Solvent Extractor (Mitsubishi Chemical Analytech, Japan). The extract was cleaned in gel permeation chromatography for lipid removal (GPC) and activated silica-gel, and then concentrated. PCBs and PBDEs were analysed using a gas chromatograph (GC: Agilent 7980 A) coupled with a quadrupole mass spectrometer (qMS: Agilent 5975C), and OCPs were analysed using a GC (Agilent 7890 A) coupled with a triple quadrupole mass spectrometer (MS/MS: Agilent 7000). The blood plasma sample (2.8 g) was liquid-liquid extracted using hexane/methyl tert-butyl ether (1:1 v/v) according to the protocol by Eguchi et al. (2015), subjected to the same clean-up method described above, and then PCBs and OCPs were determined using a GC (Agilent 6890A) coupled with a magnetic sector high-resolution mass spectrometer (HRMS: JEOL JMS-800D) using instrumental settings reported by Goto et al. (2020). Lipid contents of all tissues were determined gravimetrically using 10% of the extract.

3. Results and discussion

3.1 A meta-analysis of literature data

The systematic search identified 26 studies investigating the distribution of organic pollutants among different sea turtle tissues. Only two of these investigated maternal transfer and none considered an embryonic transfer. Hence, there is a critical gap in our understanding of maternal and embryonic transfer processes in sea turtles. Our meta-analysis identified that the concentrations of several tissues can be predicted by the partitioning capacities determined by the lipid content of the tissues. However, egg tissues deviate from this expected equilibrium. This has implications for the toxicity of pollutants in eggs. To improve exposure assessments, we constructed a statistical maternal transfer model to predict egg yolk concentrations from whole blood concentrations for the leatherback turtle ($MT_{leatherback}$ model). To explain deviations from equilibrium partitioning, accurate measurements of lipid contents of different tissues are needed. Nonetheless, our literature review, identified that lipid contents for many sea turtle tissues are poorly known.

3.2 Analysis of chemical pollution

PCBs showed congener-specific accumulation patterns in the three tissues analyzed (Fig. 1). Wet weight concentrations in egg yolk were on average an order of magnitude larger than those in blood plasma and egg albumen, matching the higher lipid content of the yolk. Most of the target PCBs congeners were detected in the yolk, with a decrease in concentration for high chlorinated PCBs (> PCB 188). Meanwhile, in albumen only the lower chlorinated PCBs (< PCB 153) were observed, and in plasma about half (21 of 56) of the analyzed PCBs were observed in quantifiable concentrations. In contrast to PCBs, profiles of OCPs were largely comparable across yolk and albumen (Fig. 1). The highest concentrations were recorded for the DDT metabolite p,p`-DDE. Additionally, trans-Nonachlor (a compound of Chlordane), and the metabolite oxychlordane were found in high concentrations in both egg compartments. Few OCPs were detected in blood plasma (p,p`-DDE, trans-Nonachlor, trans-Chlordane, and HCB). PBDEs were only quantifiable in the yolk (PBDE 28, 100, 153, 154, and 155), with concentrations in albumen below quantification limits.

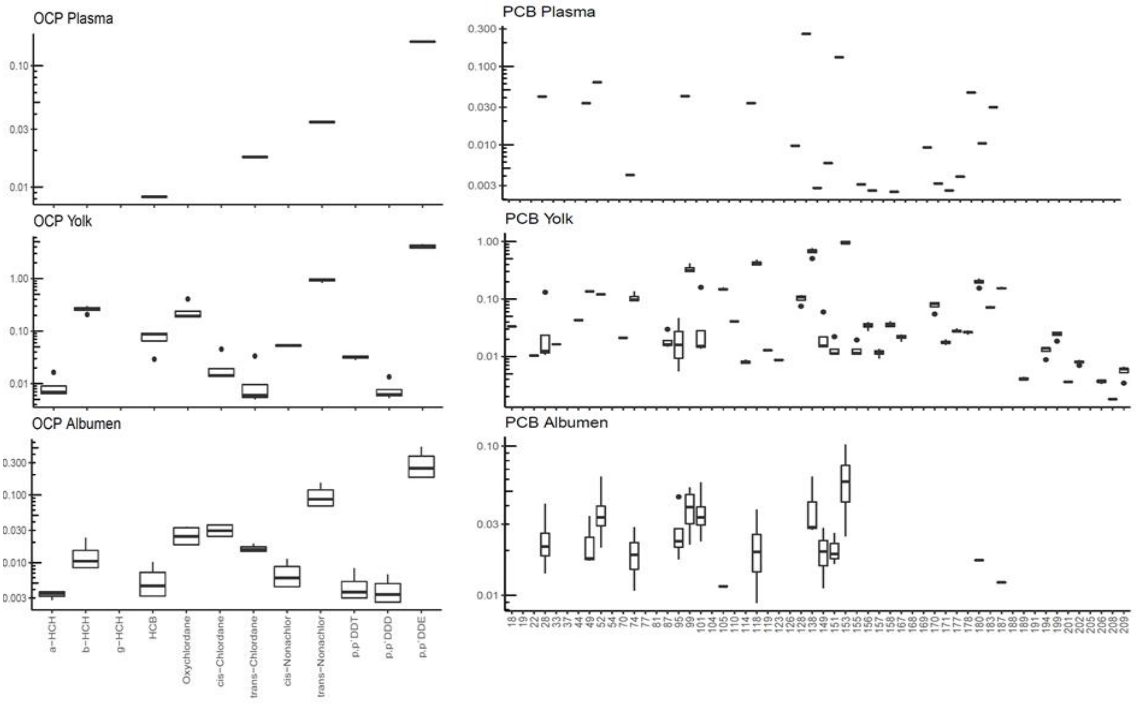

Fig. 1 Compound-specific concentrations of OCPs (left panel) and PCBs (right panel) in ng/g wet mass.

To compare tissue distributions based on their partitioning capacity for lipophilic compounds, we lipid normalized the observed concentrations. Measured lipid contents in albumen varied more than an order of magnitude among observations, ranging from 0.56 % to 0.014 %. This wide range affected the comparability of data after lipid normalization (Fig. 2). Very few data on albumen lipid contents are available in the scientific literature for reptiles. The only known data to compare with are lipid contents in aquatic snakes (Sato et al. 1973) of 0.02 % in albumen.

This paucity of data makes the measurement of lipid contents in the current project valuable to the overall scientific literature. Additionally, accurate measurement of lipid contents is also needed to compare tissue partitioning capacities. In line with the measurements of Sato et al. (1973), we took the average lipid content among eggs T2 and T3, leading to a lipid percentage of 0.016 %, which we used to lipid normalize our albumen data.

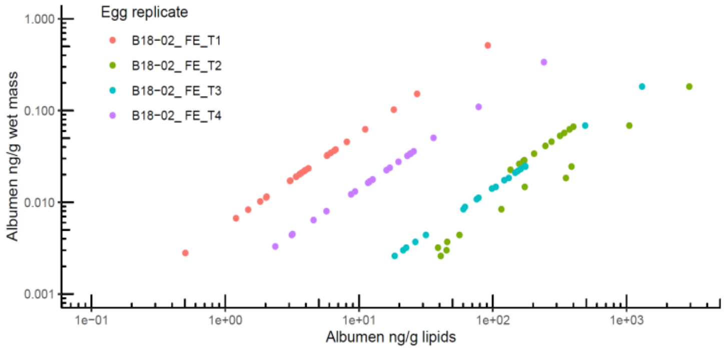

Fig 2 Comparison of concentrations in albumen on a lipid vs. wet mass basis, for the 4 eggs analyzed.

The partitioning of lipophilic compounds, such as OCPs, PCBs, and PBDEs, among tissues under steady-state, is expected to reach an equilibrium where lipid normalized concentrations are equal in both tissues (Russell et al. 1999, MacKay 2001). However, lipid normalized concentrations of PCBs and some OCPs were higher than expected based on lipid determined partitioning in blood plasma than in egg yolk (Fig 3A). Potentially, an affinity for blood proteins, especially known for some PCBs, could underlie this pattern. In our literature meta-analysis, we identified a similar pattern of affinity among lipid normalized PCB and OCP compounds for whole blood compared to yolk within leatherback turtles. In comparison, the pattern observed here for plasma in loggerheads is less strong than for whole blood in leatherback turtles.
The opposite pattern was identified for albumen, where the affinity of lipid normalized PCB and OCP compounds is higher than for blood plasma. Consequently, an affinity for albumen is also greater than for yolk.

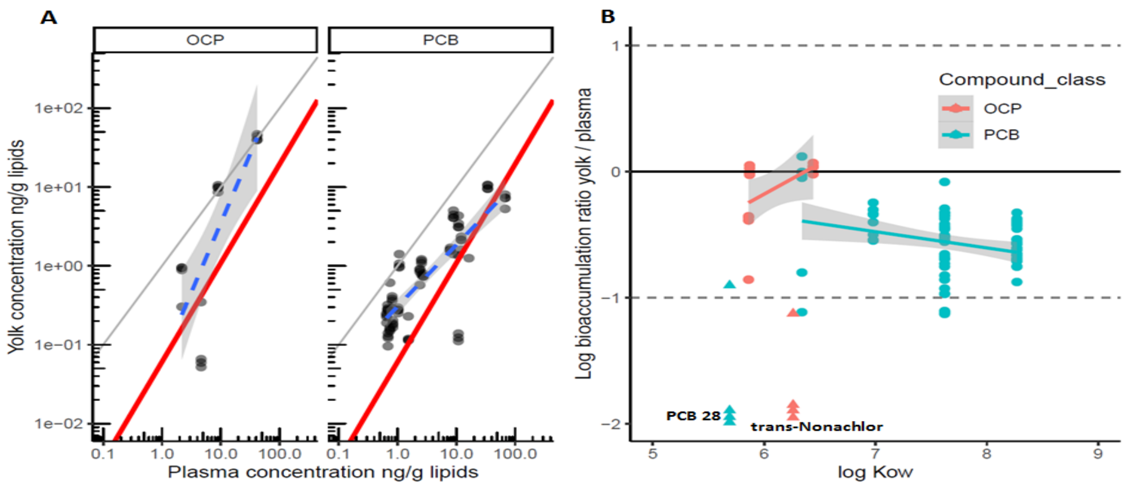

Fig. 3 A) Lipid normalized concentrations of OCPs and PCBs among plasma and yolk. Grey line: partitioning equilibrium model, red line: $MT_{leatherback}$ model, blue dashed line: linear model fitted to our data with a 95% credibility interval. **B)** Relation between log K_{ow} and the bioaccumulation ratio of egg yolk/blood plasma. Linear regressions with 95% credibility intervals are plotted. PCB 28 and

trans-Nonachlor, indicated as outliers in three eggs, were excluded from the regression.

Tissue distributions are influenced by chemical properties (Fig. 3B). Bioaccumulation ratios of PCBs decreased as the octanol-water partition coefficient (K_{ow}) increased, matching observations from our literature review. Maternal transfer of PCB 28 was lower than expected, requiring further replication in other eggs to determine underlying causes.

Analysis of stable isotopes and blood chemistry

Our stable isotope results indicate that the currently sampled turtle has been in the adult stage for at least 5 years and undergoes sporadic seasonal migration patterns between nesting and foraging locations. Our blood chemistry results indicated that the female is comparable in health to other loggerheads within the population of Kochi prefecture, which is composed of mixed sub-adult and adult stages.

4. Publication/conference presentation

Publication in review: **Muñoz C.** Saito T. and Vermeiren P**.** Population age structure and individual resource specialization in a long-lived marine species with ontogenetic migrations. *Marine Ecology Progress Series.*

The research was presented to the Faculty of Science, Radboud University, December 2019, and will be presented at SETAC Europe, 31st Annual Meeting (abstract submitted)

5. Perspective in future

Aim 1 and 3: Laboratory analysis for pollution is planned for the 2021 fiscal year LAMER project as a response to the Covid-19. (Migratory history for aim 1 is already analyzed using stable isotopes)

Aim 2: We established differences in maternal transfer among leatherback (literature data) and loggerhead turtles (our samples).

- We plan further replication in loggerheads to establish the statistical validity of this pattern of PCBs, OCP, and PBDE. For comparison, we will also analyze samples from green turtles. These analyses are planned for the 2021 fiscal year LAMER project.
- We will put extra attention to the analysis of lipid contents during these analyses.

Aim 4: Our results indicated that transfer rates depend on chemical properties (log K_{ow}), we will include additional chemical properties (derived from literature and databases) into our statistical analyses.

Additionally, in a parallel project, mathematical modeling is conducted to increase the mechanistic understanding of maternal and embryo transfer. The chemical analysis conducted with the LaMer program will be valuable as validation data for such models.

A publication on the meta-analysis of literature data in the ET&C journal is planned for 2021, and a presentation at the British Ecological Society: “Ecology Across Borders 2021” conference.