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CENTRO INTERDISCIPLINARIO DE CIENCIAS MARINAS

MATERNAL TRANSFER OF ELEMENTS IN TWO PLACENTAL VIVIPAROUS SHARKS AND A YOLK-SAC VIVIPAROUS RAY FROM THE COAST OF BAJA CALIFORNIA SUR

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PRESENTA

ISIS BARÓ CAMARASA

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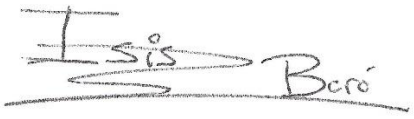

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ABSTRACT

The maternal transfer is the process by which offspring receive essential and non-essential elements during gestation. Transference of essential elements is fundamental for embryo development before parturition. On the other hand, maternal transfer of non-essential trace elements works as an elimination process for the pregnant female that can potentially cause adverse health effects during critical development stages. A significant factor to be considered is the reproductive strategy used. Two of many elasmobranch reproductive strategies are placental viviparity and yolk sac viviparity. This dissertation investigates maternal transfer of major, minor and non-essential elements in two placental viviparous sharks (*Rhizoprionodon longurio* and *Mustelus henlei*) and a yolk sac viviparous ray (*Pseudobatos glaucostigmus*) along the Baja California Sur coast. In addition, nitrogen stable isotope values indicated enrichment of all embryo tissues for *R. longurio* (2.17 ‰ in muscle, 4.39 ‰ in liver, and 0.80 ‰ in blood) compared to the pregnant female. For *P. glaucostigmus*, tissue enrichment was only found in embryo liver (1.22 ‰) and embryo muscle was depleted (-1.22 ‰) compared to the pregnant female. Maternal transfer of mercury (Hg) in the three mentioned species was analyzed. Factors such as embryo total length, total Hg concentration ([THg]) in pregnant female muscle, and nutrition type during gestation influence the [THg] in offspring. Moreover, thirteen essential elements (K, S, P, Na, Ca, Mg, Fe, Zn, Se, Cu, Mn, Cr and Co) and eleven non-essential (As, Sr, Cd, V, Li, U, Tl, Ag, Sn, Sb and Pb) in the muscle and liver of the *R. longurio* pregnant female and embryos were measured. All elements were transferred at detectable concentrations to offspring except for tin (Sn), antimony (Sb), and lead (Pb). Embryo liver concentrations were significantly higher than the pregnant female for all essential elements analyzed, except for magnesium (Mg), Chromium (Cr) and Manganese (Mn). The concentrations of the non-essential elements of strontium (Sr), lithium (Li), thallium (Tl) and silver (Ag) were higher in embryo than pregnant female tissues.

RESUMEN

La transferencia materna es el proceso a partir del cual la descendencia recibe elementos esenciales y no esenciales durante la gestación. La transferencia de elementos esenciales es imprescindible para un buen desarrollo del embrión antes del parto. En cambio, la transferencia materna de elementos no esenciales funciona como detoxificación de la madre y puede causar problemas de salud en el embrión al encontrarse en una etapa importante de desarrollo. Un factor primordial que considerar es la estrategia reproductiva de la especie. Entre los diferentes tipos de reproducción que existen en los elasmobranquios se encuentran la viviparidad placentada y la viviparidad con saco vitelino. El objetivo de este trabajo es investigar la transferencia materna de elementos mayores, menores y no esenciales en dos tiburones vivíparos placentados (*Rhizoprionodon longurio* y *Mustelus henlei*) y en una raya vivípara con saco vitelino (*Pseudobatos glaucostigmus*) en la costa de Baja California Sur, México. Por un lado, se estudió el fraccionamiento de la razón isotópica de nitrógeno durante la transferencia materna y se observó un enriquecimiento en los tejidos de los embriones de *R. longurio* (2.17 ‰ en músculo, 4.39 ‰ en hígado y 0.80 ‰ en sangre) comparados con los de la madre. Para *P. glaucostigmus*, solo se observó enriquecimiento en el hígado de los embriones (1.22 ‰) y el músculo de estos se encontró que estaba empobrecido (-1.22 ‰) en comparación con el de la madre. También se analizó la transferencia materna de mercurio en las tres especies mencionadas, observando que factores como la longitud total del embrión, la concentración de mercurio total ([THg]) en el músculo de la madre y el tipo de nutrición durante la gestación influencian en la [THg] de la descendencia. Además, se estudiaron trece elementos esenciales (K, S, P, Na, Ca, Mg, Fe, Zn, Se, Cu, Mn, Cr y Co) y once no esenciales (As, Sr, Cd, V, Li, U, Tl, Ag, Sn, Sb y Pb) en el músculo e hígado de una hembra preñada de *R. longurio* y sus embriones. Todos los elementos fueron transferidos a la descendencia en concentraciones detectables a excepción del Pb, el Sb y el Sn. La concentración de todos los elementos esenciales en el hígado de los embriones fue significantemente mayor que en el de la madre, a excepción del Mg, Cr y Mn. Los elementos no esenciales Sr, Li, Tl y Ag presentaron concentraciones más elevadas en los tejidos de los embriones que en los de su madre.

CHAPTER 1. GENERAL INTRODUCTION

1.1 Elasmobranchs

Elasmobranchs have played important roles in marine ecosystems (Ferretti *et al.*, 2010). Unfortunately, factors like overfishing, habitat destruction and pollution have negative impacts to their populations, causing the reduction of many of these species worldwide (Bizzarro *et al.*, 2007; Moore *et al.*, 2015).

Due to elasmobranch characteristics such as low reproductive potential, a reduced number of offspring, long gestation periods, slow growth, and a long period to reach sexual maturity, they are susceptible to overfishing and are a highly vulnerable fishing resource (Frisk *et al.*, 2001; Ferretti *et al.*, 2010). Moreover, species of this group have a long life span, which combined which their diets (some are apex predators), make them particularly susceptible to magnifying and accumulating high concentrations of some contaminants in their tissues (van Hees, 2014).

Elasmobranchs are an important source of income in coastal countries or states that depend on fishing, especially where a large amount of the population diet is seafood (Fleming *et al.*, 2006). One of the most important elasmobranch fishing countries in the world is Mexico (Ramírez-Amaro *et al.*, 2013). In 2018, Baja California Sur was the fourth state with the largest total catch of elasmobranchs in Mexico (Conapesca, 2018). As diet can be the main pathway for some contaminants in humans, consumption of elasmobranch meat is a known exposure to some pollutants that are linked to neurotoxicological outcomes, cancer, cardiovascular diseases, reproductive defects, among others (Mozaffarian & Rimm, 2006; Kim *et al.*, 2013).

The main pathway of elasmobranch bioaccumulation and biomagnification of contaminants is their diet (Martins *et al.*, 2021). Another important route of exposure is through maternal transfer of contaminants during gestation (Lyons & Lowe, 2013a; Frías-Espericueta *et al.*, 2014; Dutton & Venuti, 2019). Understanding maternal transfer processes is important as embryos are sensitive to exposure in their early development stage (Mohammed, 2013). An important factor which can influence the maternal

contaminants transfer in elasmobranch is the reproductive strategy of the species (Lyons & Lowe, 2013b).

Elasmobranchs, although having a relative small number of species compared to other vertebrates taxa, had developed a high diversity of reproductive strategies throughout its evolution (Carrier *et al.*, 2004; Hamlett, 2005). Oviparous and yolk sac viviparous are among known reproductive strategies, where nutrients come from yolk (Blackburn, 2014), and placental viviparous, histotrophy and oophagy, where at the beginning of gestation embryos feed on yolk and when depleted, they start a continuous input of nutrients directly from the pregnant females (Hamlett, 2005).

We selected representatives of these two reproductive strategies to determine how elements transfer to the embryo. We describe these species in detail in the next section and in the following chapters we present our detailed studies.

1.2 Ecology and biology of the Pacific sharpnose shark, the brown smooth-hound and the speckled guitarfish

1.2.1 *Rhizoprionodon longurio*

The Pacific sharpnose shark, *Rhizoprionodon longurio* (Fig. 1.1), is distributed from southern California, USA, to Peru (Fig. 1.2; Compagno, 1984). This shark inhabits sandy and muddy bottoms from the intertidal zone to at least 27m depth (Smith *et al.*, 2009). The movements patterns of the species are associated with changes in sea temperature (Márquez-Farias *et al.*, 2005). *R. longurio* feeds on fish, both epipelagic and benthic, cephalopods and crustaceans (Márquez-Farias *et al.*, 2005; Conde Moreno, 2009; Osuna-Peralta *et al.*, 2014; Trejo Ramírez, 2017). The maxim length recorded is 170 cm total length (T_L) (Alatorre-Ramirez *et al.*, 2013). Sexual maturity is at a mean of 101 cm T_L for males and 93 cm T_L for females (Corro Espinoza, 2011), reproduction is placental viviparity with a gestation period of 10 to 12 months (Mejía-Salazar, 2007; Smith *et al.*, 2009). The birthing season is from April to July with litter sizes of 1 to 12 pups (Márquez-Farias *et al.*, 2005).

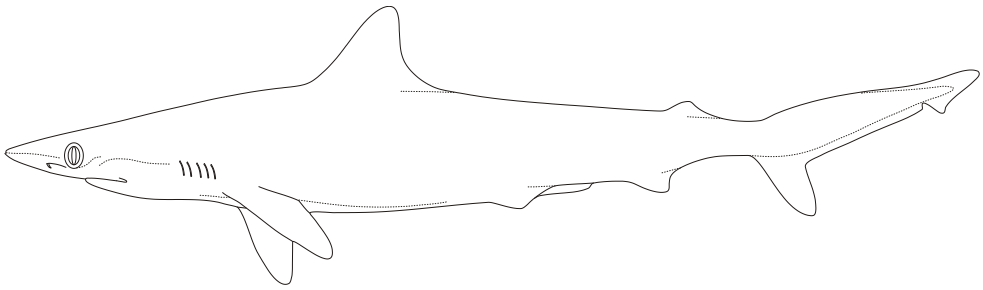

Figure 1.1. Pacific sharpnose shark *Rhizoprionodon longurio*. Image by MSc. Abel Trejo Ramírez.

Figure 1.2. Distribution map of the Pacific sharpnose shark *Rhizoprionodon longurio*. Source: Smith *et al.* (2009).

1.2.2 *Mustelus henlei*

The brown smooth-hound, *Mustelus henlei* (Fig 1.3), is a shark distributed from the Eastern Pacific from Washington, USA, to Peru (Fig 1.4; Pérez-Jiménez & Sosa-Nishizaki, 2008). This species inhabits muddy and sandy bottoms of closed and shallow bays (Rodríguez-Romero *et al.*, 2013). *M. henlei* feeds mainly on fishes, crustaceans and cephalopods (Rodríguez-Romero *et al.*, 2013; Amariles *et al.*, 2017). This species has a maximum length of 153 cm T_L (Soto-López *et al.*, 2018). Females mature at 65.8 cm of T_L and males at 63.5 cm T_L (Soto-López *et al.*, 2018). *M. henlei* is a placental viviparous shark which reproduce annually and has a gestation period of 10 months (Pérez-Jiménez & Sosa-Nishizaki, 2008). The birth season in the west coast of Baja

California Sur is from May to June with litter sizes of 1-20, and embryos measuring around 35 cm T_L at the time of parturition (Soto-López *et al.*, 2018).

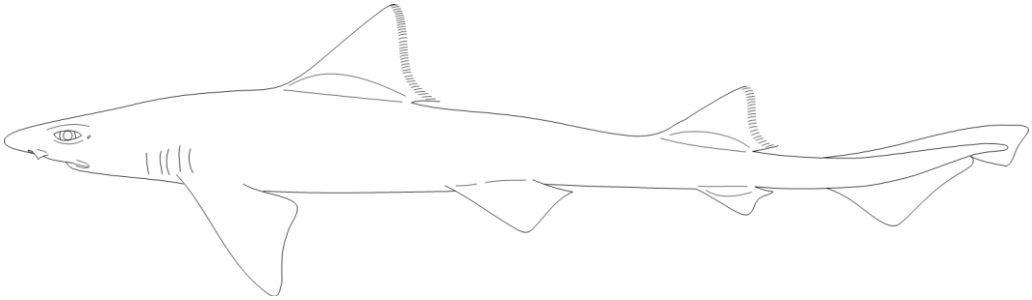

Figure 1.3. Brown smooth-hound shark *Mustelus henlei*. Image by MSc. Abel Trejo Ramírez.

Figure 1.4. Distribution map of the brown smooth-hound *Mustelus henlei*. Source: Pérez-Jiménez *et al.* (2016).

1.2.3 *Pseudobatos glaucostigmus*

The speckled guitarfish (Fig 1.5) is a poorly known ray distributed from Magdalena Bay, Baja California Sur, Mexico, to Ecuador, including the Gulf of California (Fig 1.6; Bizzarro, 2016). This ray inhabits shallow waters on soft bottoms from shallow, nearshore regions to 112 m depth (Rosa-Meza *et al.*, 2013; Bizzarro, 2016). Its diet is dominated by crustaceans, such as stomatopods, decapods and

shrimps and fishes being a complementary component (Valadez-González, 2000; Navarro-González *et al.*, 2012; Rosa-Meza *et al.*, 2013; Lara-Mendoza *et al.*, 2015). Maximum length for this ray is 87 cm (Lara-Mendoza & Márquez-Farías, 2014). Its reproduction is yolk-sac viviparity (Lara-Mendoza & Márquez-Farías, 2014).

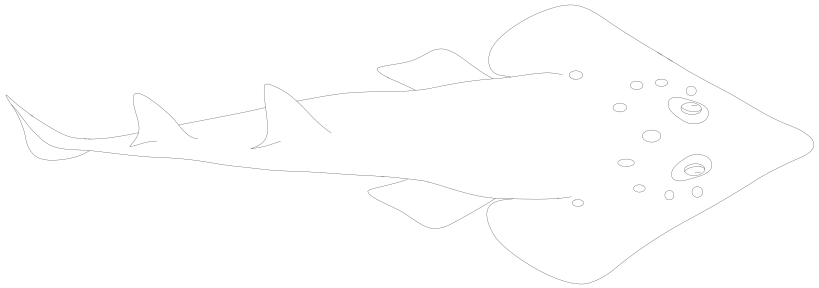

Figure 1.5. Speckled guitarfish ray *Pseudobatos glaucostigus*. Image by MSc. Abel Trejo Ramírez

Figure 1.6. Distribution map of the speckled guitarfish *Pseudobatos glaucostigmus*. Source: Bizzarro (2016)

1.3 Maternal transfer

Maternal transfer is the process through which the offspring receive non-genetic factors from its mother. Over the gestation period, embryos receive different types of nutrients, including major, minor and trace elements (Hasselquist & Nilsson, 2009;

Bakker *et al.*, 2016) and can receive different types of contaminants as microplastics and non-essential elements (Lopes *et al.*, 2019; Fournier *et al.*, 2020). In utero development is a vulnerable phase for many organisms, and the transference of contaminants as non – essential trace elements can cause damage to the developing embryo (Dutton & Venuti, 2019). Embryo nutrition during development can be divided into matrotrophic, when nutrients are provided by the pregnant female over the gestation, or lecithotrophic, when nutrients derive solely from their own yolk sac (Hamlett, 2005). During gestation some species, for example placental sharks, can nourish the embryo through both strategies (Blackburn, 2014).

1.3.1 Nutrients

Stable isotopes of C and N

Stable isotopes analysis (SIA) of carbon (C) and nitrogen (N) are a useful tool to study the ecology of marine organisms (Frankel *et al.*, 2012). It provides a better understanding of the diet and habitat use of an animal, such as trophic webs and energy flow (Jenkins *et al.*, 2001; Polischuk *et al.*, 2001). This technique is based on knowing isotopic carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) proportions of the analyzed sample. Isotopic proportions of the different prey items consumed are reflected in the tissues of the predator, in a proportional manner regarding the amount assimilated for each food source (Habran *et al.*, 2010).

Isotopic fractionation of N and C occurs when predator is enriched with the heavy isotope compared to its prey (Sare *et al.*, 2005). In elasmobranch, mean isotopic fractionation between prey and predator is 3.4 ± 1‰ for $\delta^{15}N$ values and 0.4 ± 1.3‰ for $\delta^{13}C$ values (Post, 2002). Values of $\delta^{15}N$ are relative to the trophic position of an animal and trophic web structure in an ecosystem (Minagawa & Wada, 1984) and in some well-studied systems can be used to calculate an estimated trophic level. Therefore, higher $\delta^{15}N$ values may represent organisms at high trophic levels, while lower $\delta^{15}N$ values are measured in organisms found in lower trophic levels (Sare *et al.*, 2005). On the other hand, $\delta^{13}C$ values can help delineate feeding habitats; benthic or pelagic,

inshore or offshore (Habran *et al.*, 2010). Variations in isotopic fractionation values need to consider diet quality, size, physiologic state, age or lipid extraction (high fat tissues) during analysis (Caut *et al.*, 2009).

In order to improve embryo nutrition knowledge, the technique of SIA is used. During embryo development, nutrients for offspring growth come from the pregnant female (Hay Jr., 1994), weather they are transported through the placenta or stored in a yolk sac. Offspring could be considered as “feeding” on the pregnant female, as nutrients received during gestations are maternally derived. As prey – predator interaction, where metabolic processes occurs, embryos are expected to have higher $\delta^{15}N$ values than the pregnant female, as they are ‘consuming’ her, presenting a higher trophic level than the mother (Jenkins *et al.*, 2001; Habran *et al.*, 2010; Frankel *et al.*, 2012). If protein is simply transferred from the pregnant female to the embryo, $\delta^{15}N$ values would not reflect a change.

Another hypothesis to consider, is that pregnant females can be divided depending on the tactic used to meet the energy requirement associated with reproduction: “income breeding”, where nutrients used had been recently ingested for the pregnant female; and “capital breeding”, where the pregnant female use nutrients previously stored in tissues (Dalerum *et al.*, 2007). When the technique used is “income breeding”, the offspring should not be more enriched than the pregnant female at parturition since ingested nutrients for the female would not been stored in its tissues. Instead, they would be used directly as embryo nutriment. For “capital breeders”, the offspring should have $\delta^{15}N$ values around one trophic level higher than the pregnant female at parturition, and two trophic levels higher during lactation, as milk nutrients will come from female tissue (one level) and newborn will feed on this milk (two levels) (Dalerum *et al.*, 2007). However, more research should be done about the metabolical process involved during embryo and newborn nutrition and its tissue assimilation in both techniques, income breading and capital breeding.

On the other hand, $\delta^{13}C$ embryo values are expected to be depleted in relation to the pregnant female. In general, nutrients used for embryo growth come from maternal lipid stores (liver, milk) (Frankel *et al.*, 2012), and it is known that fatty tissues

often have depleted $\delta^{13}C$ values regarding its diet and other tissues of the predator (Pilgrim, 2007). The determination of the C:N ratio can give an estimation of the percentage of lipids in the sample, as evidenced for aquatic animals, where C:N > 3.5 would indicate a percentage of lipid content higher than 5% (Post *et al.*, 2007).

Essential elements

Essential elements are those compounds, in certain concentrations in the organism, required to perform vital metabolic activities in organisms, maintain normal physiological functions, and ensuring optimal development and health (Goldhaber, 2003; Aliasgharpour & Farzami, 2013). They can be released in the environment through natural sources, as mineral weathering process or hydrothermal inputs, or anthropological sources as mining, combustion processes or fertilizers. Although they are necessary for life, essential trace element can be toxic for the organism when its intake is too high or cause nutritional problems when its intake is too low (Goldhaber, 2003). During pregnancy, the embryo is totally dependent on the pregnant female for nutrient supply, including essential elements, for its development. A deficiency on its transference can cause health problems in the offspring, such as abnormalities in the central nervous system or impaired fetal growth (Hostetler *et al.*, 2003). Complementary information of essential elements can be found in ANNEX I.

1.3.2 Contaminants

Throughout human history, oceans provided different kinds of resources for food and livelihoods. However, an increasing global human population has resulted in a rising pressure on seas and oceans due to an increasing human activity. Human erroneous belief that seas and oceans recycle and absorb all contaminants has resulted in an uncontrolled waste disposed (Borja *et al.*, 2020). Some of the contaminants disposed into the ocean include plastics, persistent organic pollutants (POPs) or non-essential trace elements.

Marine animals can bioaccumulate contaminants during its life through different pathways, such as water or diet, acquiring higher concentrations as they get older (Kalantzi *et al.*, 2014). Once in the organisms, some contaminants can biomagnify through the trophic web, meaning that they are transferred from food to an organism resulting in higher concentrations compared with the source (Gray, 2002; Borgå *et al.*, 2012). Females can bioaccumulate and biomagnify contaminants in their body, being able to transfer them to the offspring during pregnancy (Lyons & Lowe, 2013a; van Hees & Ebert, 2017).

Plastic

Plastic is a relatively new material in the oceans that has become a major environmental pollution problem. The degradation to microplastics (> 100 nm to < 5 mm) and nanoplastics (< 100 nm) makes them prone to uptake in marine species, which are transmitted to humans through the food chain (Borja *et al.*, 2020; Sana *et al.*, 2020). Maternal transfer of nanoplastics has been proved on rats and fishes, demonstrating the ability of crossing the placental transfer (Pitt *et al.*, 2018; Fournier *et al.*, 2020).

POPs

POPs are pollutants that persist in our environment a long time and transport broadly. They can be pesticides or industrial chemicals, such as polychlorinated biphenyls (PCBs) (Alharbi *et al.*, 2018). Some POPs are transferred to the offspring of elasmobranchs (Weijs *et al.*, 2015) or sea turtles eggs (García-Besné *et al.*, 2015).

Non-essential elements

Non-essential elements are those that even at minimum concentrations can cause adverse effect on the health of organisms. Although main sources of trace

elements in aquatic environments are natural (e.g. volcanic activity), anthropogenic sources can be a major threat (e.g. combustion processes, mining) (Konieczka *et al.*, 2018). Due to its toxicity, maternal transference o non-essential trace elements during pregnancy can cause development retardation, morphological and functional anomalies or even death (Jezierska *et al.*, 2009). Complementary information of non-essential elements can be found in ANNEX I.

One of the non-essential trace elements more studied is mercury (Hg). Its toxicity, even at low concentrations, generates a great concern due to its neurotoxic effects in the organism (Dietz *et al.*, 2013). Mercury in the aquatic environment can come from natural and anthropogenic sources. Natural sources of Hg are geothermal releases, volcanic eruptions and weathering of soils and rocks. On the other hand, anthropogenic sources of Hg can be fuel combustion, mining, intentional or accidental releases of effluents from chlor-alkali plants and mines; also used as pesticide for pest control (Wood *et al.*, 2012; Noël *et al.*, 2015; Ross *et al.*, 2016). The organic form of Hg is methylmercury ($MeHg^{+}$), which is known to be the most toxic and bioavailable of all the forms of this element. It can cause neurotoxic, genotoxic and immunotoxic effects in the organism.

During gestation, Hg can be transferred to the offspring, which work as a total mercury (THg) sink. Maternal transfer of Hg occurs in different reproductive strategies of elasmobranch as placental viviparous, yolk-sac viviparous and oophagy (Le Bourg *et al.*, 2014; van Hees & Ebert, 2017; Dutton & Venuti, 2019). Taking into account that embryos are in a continuous developing stage, effects of Hg in the organism health create a great concern (Brookens *et al.*, 2008; Guirlet *et al.*, 2008).

1.4 Study Area

Samples were taken from three different areas: Santa Rosalía, Las Barrancas and El Saladito. Santa Rosalía, situated in the central part of the western coast of the Gulf of California. It is a mining area where Cu, Co, Zn and possibly Mn are mined (Corporación Ambiental de México, 2006). Old mining activity was characterized as

extractive with the subsequent opening of material banks and the creation of piles of exposed waste material (Carabias Lillo *et al.*, 2000). The waste material, still situated along parts of the coast, is transported to the sea from the drainage area due to rains, by wind or due to direct discharge into the marine environment, which influence the marine sediment composition by rising the specific trace elements concentration (Carabias Lillo *et al.*, 2000; Shumilin *et al.*, 2013). During summer, due to the south winds, seasonal upwellings occur (Arce Acosta, 2015). Samples coming from Santa Rosalía were taken from two different fishing camps: Coloradito (27.03°N; 112.00°W; Fig. 1.7) and San Bruno (27.16°N; 112.16°W; Fig. 1.7).

Las Barrancas (25.99°N; 112.19°W; Fig. 1.7) is situated inside the Ulloa Gulf, which is ubicated in the occidental coast of Baja California Sur. Ulloa Gulf is a Biological Activity Center (BAC) due to its high productivity. The existence of a BAC is related to certain oceanographic events as upwellings or oceanic fronts (González Rodríguez, 2008). In the Ulloa Gulf, the California Current System goes from north to south through its west carrying Pacific subarctic water to the equator. Additionally, a countercurrent penetrates carrying water from the equatorial Pacific to the north. Upwellings are mainly found in the south of the Ulloa Gulf in spring associated with an increase in productivity (del Monte Luna, 2004). Nowadays, no anthropogenic pollution sources exist in the area due to the lack of industries along the coast (López Vera & Maz Courrau, 2006). However, this region is considered for marine mining to extract trisodium phosphate, as the area is known to have natural phosphate deposit along the coast (D'Anglejan, 1967), which is used as a fertilizer (Rodríguez & Violeta, 2017).

El Saladito (24.44°N; 110.69°W; Fig. 1.7) is situated in La Paz Bay, a semi-protected body of water located on the western coast of the Gulf of California. La Paz Bay is an important region for fisheries and tourism (Reyes-Salinas *et al.*, 2003). During summer and fall La Paz Bay presents a strong stratification (Zaytsev *et al.*, 2010). This bay is influenced by wastewater discharges coming from La Paz, the fast-growing capital city of Baja California Sur, and from activities of the phosphorite mining called "Rofomex" (Rodríguez Castañeda *et al.*, 2006; Pérez Tribouillier *et al.*, 2015).

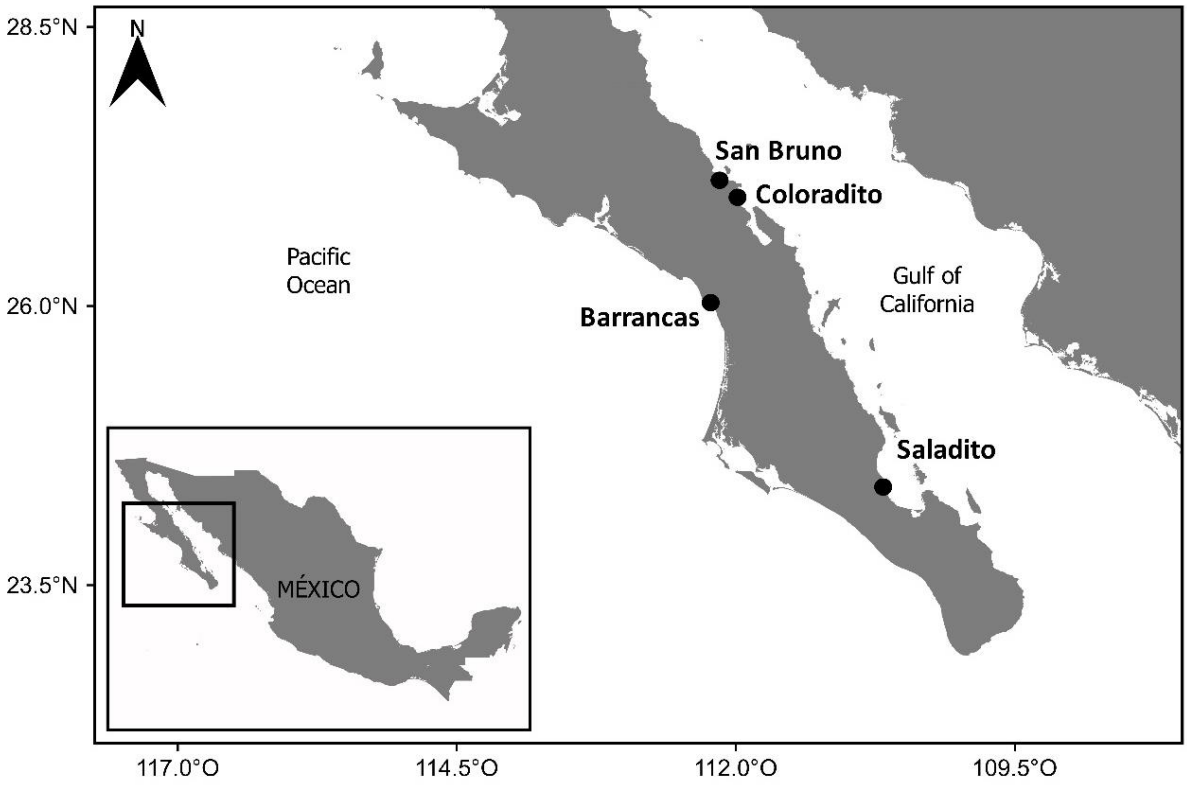

Figure 1.7. Study area. Baja California Sur. México.

1.5 Justification for this study

Until recently, little attention has been paid to maternal transference of elements in elasmobranchs. The high diversity of reproductive strategies in this group and differences on maternal investment of energy during offspring gestation increase the variability of maternal transfer mechanisms of elements between species. Moreover, the presence of non-essential trace elements in embryos, which is a critical stage of development, can cause adverse effects on health and impact survivability and fitness of juveniles, affecting elasmobranch populations (Lyons, 2018).

Understanding maternal transference of essential elements for an optimal offspring development and maternal transference of non-essential elements to evaluate possible health effects of embryos is a challenging task, as multiple parameters can influence this process. Therefore, it is necessary to expand our

knowledge on maternal transfer of elements on different elasmobranch species for a better understanding of this process and which factors have an influence on it.

1.6 Research objective

To investigate the maternal transference of major, minor, and non-essential elements in two placental viviparous sharks (*Rhizoprionodon longurio* and *Mustelus henlei*) and a yolk-sac viviparous ray (*Pseudobatos glaucostigmus*) in the Baja California Sur coast, Mexico.

1.7 Specific objectives

1. Investigate the relationships between $\delta^{15}N$ values of pregnant females and embryos tissues of *Rhizoprionodon longurio* and *Pseudobatos glaucostigmus* and observed differences between reproductive methods.
2. Determine maternal transfer of Hg in *Rhizoprionodon longurio*, *Mustelus henlei* and *Pseudobatos glaucostigmus* to assess different drivers that may explain [THg] in embryo tissues.
3. To know, for both reproductive strategies, the tendencies of $\delta^{15}N$ and [THg] values, in relation with its pregnant female, as embryos increase their size.
4. Determine the concentrations of thirteen essential elements (K, S, P, Na, Ca, Mg, Fe, Zn, Se, Cu, Mn, Cr and Co) and eleven non-essential elements (As, Sr, Cd, V, Li, U, Tl, Ag, Sn, Sb and Pb) in the muscle and liver of the pregnant female ant their respective embryos of *Rhizoprionodon longurio.*

CHAPTER 2. ISOTOPIC ($\delta^{15}N$) RELATIONSHIP OF PREGNANT FEMALES AND THEIR EMBRYOS: COMPARING PLACENTAL AND YOLK-SAC VIVIPAROUS ELASMOBRANCHS

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2.1. ABSTRACT

Nitrogen stable isotopes ratios ($\delta^{15}N$) were determined for selected tissues (muscle, liver, blood and yolk) of pregnant females and their embryos of a placental viviparous species, the Pacific sharpnose shark (*Rhizoprionodon longurio*) and a yolk-sac viviparous species, the speckled guitarfish (*Pseudobatos glaucostigmus).* The *R. longurio* embryo tissues were ^{15}N-enriched compared to the same tissues in the pregnant female, using the difference in $\delta^{15}N$ ($\Delta\delta^{15}N$) between embryo – adult. Mean $\Delta\delta^{15}N$ was 2.17 ‰ in muscle, 4.39 ‰ in liver and 0.80 ‰ in blood. For *P. glaucostigmus*, embryo liver tissue was significantly ^{15}N-enriched in comparison with liver of the pregnant female ($\Delta\delta^{15}N$ mean = 1.22 ‰); whereas embryo muscle was ^{15}N-depleted relative to the muscle of the pregnant female ($\Delta\delta^{15}N$ mean = -1.22 ‰). Both species presented a significant positive linear relationship between $\Delta\delta^{15}N$ and embryo total length (L_t). The results indicated that embryos have different $\Delta\delta^{15}N$ depending on their reproductive strategy, tissue type analysed, and the embryo L_t.

Key words: Isotopic variation, nitrogen stable isotope, *Pseudobatos glaucostigmus, Rhizoprionodon longurio.*

2.2. INTRODUCTION

Nitrogen stable isotope ratios (reported as $\delta^{15}N$) infer different aspects about trophic ecology (e.g. trophic position and habitat use) in aquatic organisms (Bond *et al.*, 2018; Habran *et al.*, 2010; Walker & Macko, 1999). It is useful to understand the nitrogen flow through the different trophic pathways to have a better understanding of food web structures and estimate the trophic level for an organism (Chikaraishi *et al.*,

2010). Enrichment of ^{15}N occurs through amino acid deamination and transamination (synthesis or degradation of protein) (Kim *et al.*, 2011; Schmidt *et al.*, 2004). Between the consumer and its prey (one trophic level) values change between 3 – 4 ‰, due to preferential excretion of the light isotope (^{14}N) over heavy (^{15}N) (Chikaraishi *et al.*, 2010; Post, 2002; Schmidt *et al.*, 2004) .

Maternal transfer of nutrients is a process seen across many species in which offspring receive protein (Bakker *et al.*, 2016; Hasselquist & Nilsson, 2009). The expected $\delta^{15}N$ values for certain tissues for the embryos of the pregnant females are approximately one trophic level higher (Frankel *et al.*, 2012; Jenkins *et al.*, 2001), as proteins are metabolised from one tissue to another (Schmidt *et al.*, 2004). When individuals are born, most start independent feeding changing their $\delta^{15}N$ values that reflect new feeding habits and associated trophic level (Dalerum *et al.*, 2007; Olin *et al.*, 2011).

Elasmobranchs have numerous reproductive strategies, including placental and yolk-sac viviparity (Carrier *et al.*, 2004; Hamlett, 2005). Embryos of placental viviparous species are initially nourished by yolk; once depleted, the yolk stalk elongates to form the umbilical cord that transports maternal nutrients until birth. In contrast, embryos from species with yolk-sac viviparity lack the additional maternal nutritional input beyond yolk (Awruch, 2015; Hamlett, 2005). Depending on reproductive strategy, embryos undergo different nutritional transfer and processing during development, which is reflected by differences in $\delta^{15}N$ values (Borrell *et al.*, 2015; Le Bourg *et al.*, 2014), similar to how stable isotopes are used as dietary indicators (Pilgrim, 2007). In this regard, differences in reproductive strategies can show variations in $\Delta\delta^{15}N$ values ($\Delta\delta^{15}N = \delta^{15}N_{embryo} - \delta^{15}N_{pregnant\ female}$; McMeans *et al.*, 2009) between different maternal tissues and their embryos (Le Bourg *et al.*, 2014).

Few studies have investigated $\Delta\delta^{15}N$ between pregnant females and embryos in elasmobranchs (Broadhurst *et al.*, 2019; Le Bourg *et al.*, 2014; McMeans *et al.*, 2009; Olin *et al.*, 2018; Osgood *et al.*, 2020; Souza-Araujo *et al.*, 2020; Vaudo *et al.*, 2010). Most $\Delta\delta^{15}N$ pregnant female with matched embryos studies in placental viviparous elasmobranchs show embryos have ^{15}N-enrichment in muscle and liver relative to adult

females (McMeans *et al.*, 2009; Vaudo *et al.*, 2010). Exceptions include the bonnethead shark *Sphyrna tiburo* (L. 1758), where embryo muscle is ^{15}N-depleted in relation to muscle of the pregnant female (Olin *et al.*, 2018); and the smalleye smooth-hound *Mustelus higmani* (Springer & Lowe, 1936), where embryo muscle is not different from muscle of pregnant females (Souza-Araujo *et al.*, 2020). Pregnant female to embryo isotopic variation has been investigated in three yolk-sac viviparous species; shortspine spurdog *Squalus megalops* (MacLeay 1881), small-fin gulper shark *Centrophorus moluccensis* Bleeker 1860 and the sixgill shark *Hexanchus griseus* (Bonaterre 1788) (Le Bourg *et al.*, 2014; Osgood *et al.*, 2020). Although interspecific variation exists between them, all species show a depletion of the heavy isotope in embryos tissues relative to maternal tissues.

The elasmobranchs analysed in the present study are the Pacific sharpnose shark *Rhizoprionodon longurio* (Jordan & Gilbert 1882) and the speckled guitarfish *Pseudobatos glaucostigmus* (Jordan & Gilbert 1883). *R. longurio* is a placental viviparous shark with a gestation period of 10 to 12 months, parturition occurs between April and June and litters are up to 12 pups (Márquez-Farias *et al.*, 2005). *P. glaucostigmus* is a yolk-sac viviparous ray (Bizzarro, 2016) and information is lacking regarding its reproduction. This study investigates the relationship between δ^{15}N values of pregnant females and embryos tissues of both species. We hypothesise embryo tissues will be ^{15}N-enriched relative to maternal tissues as proteins are processed for embryo development, and $\Delta\delta^{15}$N values will be different for both species as they have different reproductive strategies. The objectives of this study are: i) investigate and compare δ^{15}N values between pregnant females and embryo tissues during embryonic development, ii) describe relationships between embryo and pregnant females isotopic differences ($\Delta\delta^{15}$N) and embryos total length (L_t), and iii) determine if there are nitrogen isotopic differences between both species studied. The assessment of isotope variation in different tissues between pregnant females and their offspring of these species would give a better understanding of trophic patterns between pregnant females and embryos, and isotopic scenarios by age classes, such as juveniles, regarding their remaining maternal signature as independent consumers (Broadhurst *et al.*, 2019; Olin *et al.*, 2018).

2.3. MATERIALS AND METHODS

2.3.1. Sample collection

Pregnant females of *R. longurio* (n=15), *P. glaucostigmus* (n=8), and three embryos from each specimen (n = 69) were collected in collaboration with the artisanal fisheries fleet. No ethical statement was required, as the specimens were legally harvested for commercial use. For both species, samples were collected in the Gulf of California at two fishing camps situated on the eastern side of the Baja California Sur Peninsula, Mexico. The *R. longurio* samples were collected at the Coloradito fishing camp (27.03°N; 112.00°W) in April, October and November of 2018; whereas the *P. glaucostigmus* were sampled at San Bruno fishing camp (27.16°N; 112.16°W) in October 2017. L_t of each adult and embryo was recorded.

Muscle and liver tissues were collected from each specimen. In addition, blood samples of *R. longurio* were extracted from the caudal vein of six adult females and from 18 embryos. These blood samples were stored in EDTA vacutainer tubes. If the umbilical cord of *R. longurio* embryos was not formed yet, then the yolk was sampled. For the *P. glaucostigmus* specimens, yolk from each embryo was collected. All samples were kept on ice and transported to the laboratory at the Centro Interdisciplinario de Ciencias Marinas from Instituto Politécnico Nacional (CICIMAR – IPN, La Paz, BCS, Mexico) and stored frozen (- 20^{o}C) until analysis.

2.3.2. Sample analysis

The liver, muscle and yolk were sub-sampled and kept in Eppendorf tubes. All samples, including blood tissue, were freeze-dried (Telstar, LyoQuest - 85) for 48 hours. Urea was removed from muscle, liver and yolk tissues using the method proposed by Kim and Koch (2012); these were sonicated three times for 15 minutes with deionized water. However, since liver and yolk samples were being diluted, those tissues were only sonicated twice with deionized water instead of three. Three samples of liver and two samples of yolk were lost during this process. After extraction, samples

were freeze-dried again for 48h and pulverized manually using an Agate mortar. A range of 600 µg to 1400 µg for each sample was placed in tin capsules for isotopic analysis.

Values of $\delta^{15}N$ were determined using a mass spectrometer (Delta V Plus Thermo Scientific) with continuous flow coupled to an elemental analyzer (Elemental Combustion System Costech Instruments) held in the Mass spectrometry Isotopic Laboratory (LEsMA) at CICIMAR-IPN. The conventional standards of reference materials used for N_2 were NIST glutamic acid and IAEA caffeine. The standard deviations of the two standards (n = 32) were 0.13‰ and 0.16‰, respectively. Stable isotope values where represented as δ based on the following equation: $\delta^{15}N = [(R\ sample/\ R\ standard) - 1] * 1000$ where R is the ratio $^{15}N : {}^{14}N$ (Murillo-Cisneros *et al.*, 2019; Park & Epstein, 1961).

2.3.3. Statistical analysis

Statistical analyses were carried out using the R software (Version 3.6.1, R Core Team, 2019). Shapiro-Wilk normality test and Fligner-Killeen Test of Homogeneity of Variances were used as preliminary tests. $\delta^{15}N$ and $\Delta\delta^{15}N$ were not normally distributed ($W_{233} = 0.91$, $p < 0.05$; $W_{153} = 0.97$, $p < 0.05$) and not homoscedastic ($X^2_{3,233} = 35.2$, $p < 0.05$; $X^2_{2,153} = 7.6$, $p < 0.05$), including with data transformation (natural logarithm or squared root, results not shown). Diagnostic plots of residuals by tissue suggest no pattern in variance, therefore we consider that any assumption violations for non-parametric testing for $\delta^{15}N$ or $\Delta\delta^{15}N$ data likely have little effects on outcomes. Therefore, non-parametric tests were used to compare groups (embryo/pregnant female, tissue, species). Mean, standard deviation (SD) and median were calculated for muscle, liver, blood and yolk of adults and embryos of both species. $\Delta\delta^{15}N$ for paired (maternal and embryo) tissues were calculated subtracting the $\delta^{15}N$ value of the pregnant female from the embryo. For each species Wilcoxon tests were used to determine if there were significant differences between the paired tissues of pregnant females and embryos. $\delta^{15}N$ values for all maternal and embryonic tissues for each species were compared using a Kruskal-Wallis test to detect significant differences. A

post-hoc Dunn's test was run to determine the differences between tissues. Multiple linear regression models (MLR) were used to test relationships between $\Delta\delta^{15}N$ embryo L_t and tissues and for each species. Diagnostic plots of MLR suggest residuals are normally distributed and homoscedastic (results not shown). Kruskal-Wallis tests were used to determine significant differences between $\Delta\delta^{15}N$ of matched tissues of *R. longurio* and *P. glaucostigmus* and to determine if $\delta^{15}N$ median values were different between species. To determine which tissues were different a post-hoc Dunn's test was run. Detailed results for Dunn's tests and MLR can be found in Suppl. Table 2.1 – 2.3 in Annex II.

2.4. RESULTS

For *R. longurio,* the L_t ranged from 104 to 118 cm for pregnant females and 11.5 to 36.5 cm for embryos. For *P. glaucostigmus*, L_t ranged from 61 to 69.5 cm for pregnant females and for embryos ranged from 8.7 to 14 cm.

For *R. longurio,* higher $\delta^{15}N$ mean values were found in embryo tissues relative to pregnant females matched tissues. For *P. glaucostigmus*, only the liver embryo tissue had higher mean values than the pregnant females, while the embryo muscle tissue presented lower values (Table 2.1).

2.4.1 $\delta^{15}N$ variation between mother and embryos

For *R. longurio*, statistically significant differences in $\delta^{15}N$ median values were found between pregnant females and embryos in muscle ($W_{60} = 671.5$, $p < 0.05$), liver ($W_{59} = 660$, $p < 0.05$) and blood ($W_{24} = 101.5$, $p < 0.05$). All embryo tissues had higher mean values for each tissue type, relative to their pregnant females, showing ^{15}N-enrichment of 2.2, 4.4 and 0.8 ‰ in muscle, liver and blood, respectively (Table 2.1). Median values of $\delta^{15}N$ in *R. longurio* showed a rank order (lesser to greater): liver of pregnant female ≤ blood of pregnant female ≤ blood of embryo ≤ muscle of pregnant female ≤ yolk of embryo < liver of embryo ≤ muscle of embryo. No statistically significant differences were observed among medians of all tissues, except for the muscle of

pregnant females and the yolk of embryos and the liver and muscle of embryo tissues ($H = 111.95$, $df = 6$ $p < 0.05$; Dunn's test, $p < 0.05$; Fig 2.1a).

Table 2.1**.** Number of samples (n), $\delta^{15}N$ values of pregnant females and embryos tissues of the Pacific sharpnose shark *Rhizoprionodon longurio longurio* and the speckled guitarfish *Pseudobatos glaucostigmus*, reported as the mean ± standard deviation (SD) and the median. $\Delta\delta^{15}N$, reported as the mean ± SD, are the differences in $\delta^{15}N$ values between embryos and its pregnant female.

	Pregnant females $\delta^{15}N$ (‰)			**Embryos $\delta^{15}N$ (‰)**			**Embryo – Pregnant female $\delta^{15}N$ (‰) ($\Delta\delta^{15}N$)**
	n	**Mean ± SD**	**Median**	**n**	**Mean ± SD**	**Median**	**Mean ± SD**
R. longurio							
Muscle	15	20.85 ± 0.48	20.78*	45	23.03 ± 0.56	23.12*	2.17 ± 0.51†
Liver	15	18.41 ± 0.83	18.67*	44	22.77 ± 0.86	23.05*	4.39 ± 0.81†
Blood	6	19.28 ± 0.28	19.32*	18	20.07 ± 0.46	20.07*	0.80 ± 0.62
Yolk				6	21.42 ± 0.43	21.43	
P. glaucostigmus							
Muscle	8	18.38 ± 0.38	18.43*	24	17.15 ± 0.34	17.12*	-1.22 ± 0.48†
Liver	8	16.38 ± 0.68	16.64*	22	17.57 ± 0.46	17.69*	1.22 ± 0.42†
Yolk				22	16.68 ± 0.42	16.83	

* Denotes significant differences between matched tissues of the same specie (Wilcoxon rank sum test, $p < 0.05$). † Denotes significant differences in $\Delta\delta^{15}N$ of matched tissues between both species (Dunn's test, $p < 0.05$).

For *P. glaucostigmus*, statistically significant differences in $\delta^{15}N$ median values were found between pregnant females and embryos in muscle ($W_{32} = 190$, $p < 0.05$) and liver ($W_{30} = 169$, $p < 0.05$). $\delta^{15}N$ median values of embryo muscle were depleted by -1.2 ‰ relative to the muscle of the pregnant female (Table 2.1). In liver, embryo median values are significantly higher than pregnant females showing ^{15}N-enrichment of 1.2 ‰ (Table 2.1). $\delta^{15}N$ median values of $\delta^{15}N$ in *P. glaucostigmus* showed a rank order: liver of pregnant female ≤ yolk of embryo < muscle of embryo < liver of embryo < muscle of pregnant female. Tissue medians were statistically different, except for liver of pregnant females and embryo yolk ($H = 52.15$, $df = 4$, $p < 0.05$; Dunn's test, $p < 0.05$; Fig 2.1b).

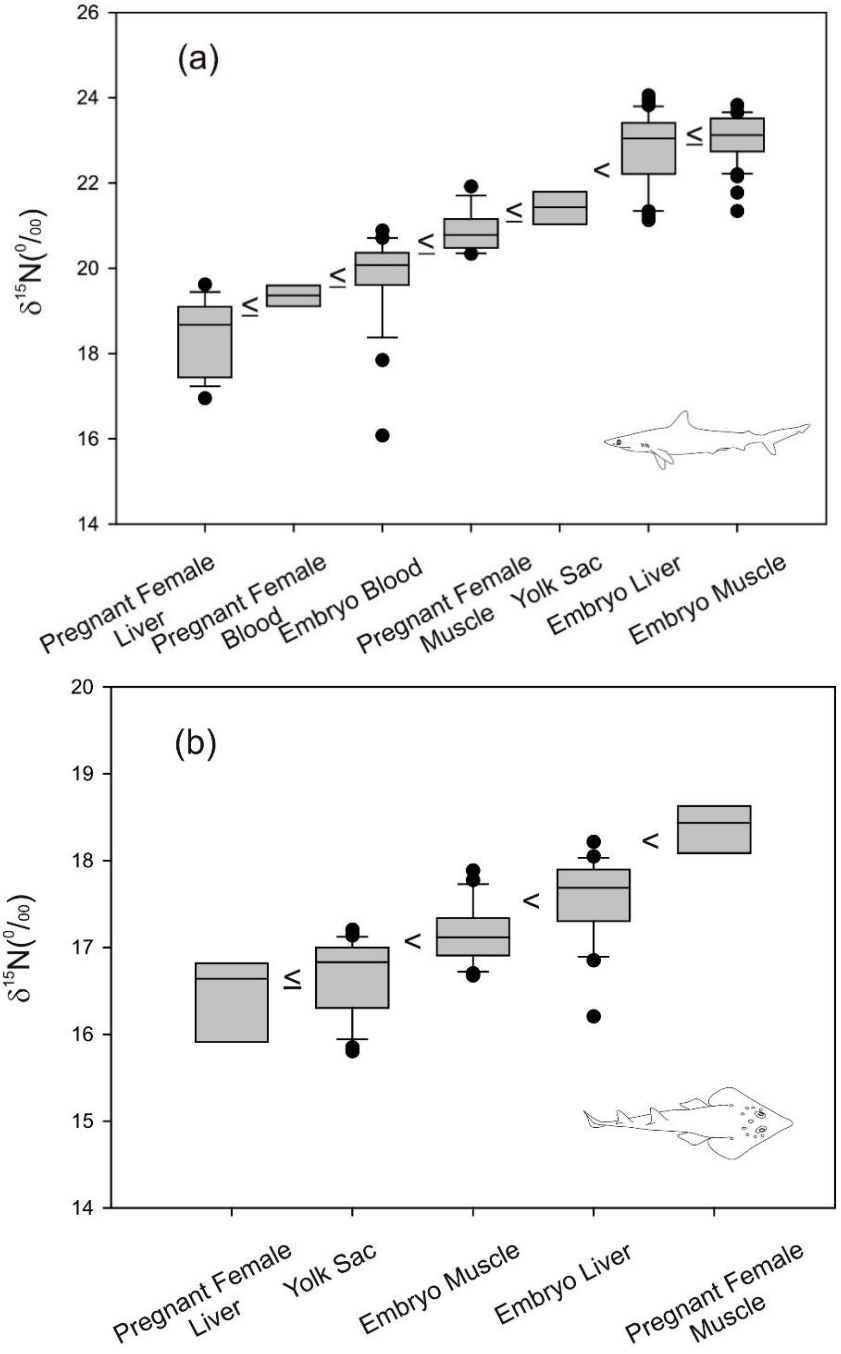


Figure 2.1. Boxplot for pregnant female and embryo tissues $\delta^{15}N$ values ordered from lower to higher $\delta^{15}N$ values of (a) the Pacific sharpnose shark *Rhizoprionodon longurio* and (b) the speckled guitarfish *Pseudobatos glaucostigmus*. Horizontal bar = median; box = 25th to 75th percentile, whiskers = 10th and 90th percentiles and points = outliers.

2.4.2 Relationship between $\Delta\delta^{15}N$ and embryo size

For *R. longurio*, MLR showed that embryo L_t and tissue explained large variability of the $\Delta\delta^{15}N$ ($R^2 = 0.88$; Fig 2.2a). Month was not included in MLR since its contribution was not significant (results not shown). For longer embryos, a higher $\Delta\delta^{15}N$ was observed between embryo and pregnant females. Liver had the highest $\Delta\delta^{15}N$, while blood showed the lowest $\Delta\delta^{15}N$ (Fig 2.2c).

For *P. glaucostigmus*, MLR determined that both variables analysed for this species, embryo L_t and tissue, were significantly related to $\Delta\delta^{15}N$ ($R^2 = 0.90$). Larger embryos had higher values than pregnant females, since $\Delta\delta^{15}N$ became more positive with length (Fig 2.2b). Negative $\Delta\delta^{15}N$ values for muscle tissue denoted higher $\delta^{15}N$ values in pregnant females than in embryos, while a positive $\Delta\delta^{15}N$ in liver indicated higher $\delta^{15}N$ values for embryos (Fig 2.2d).

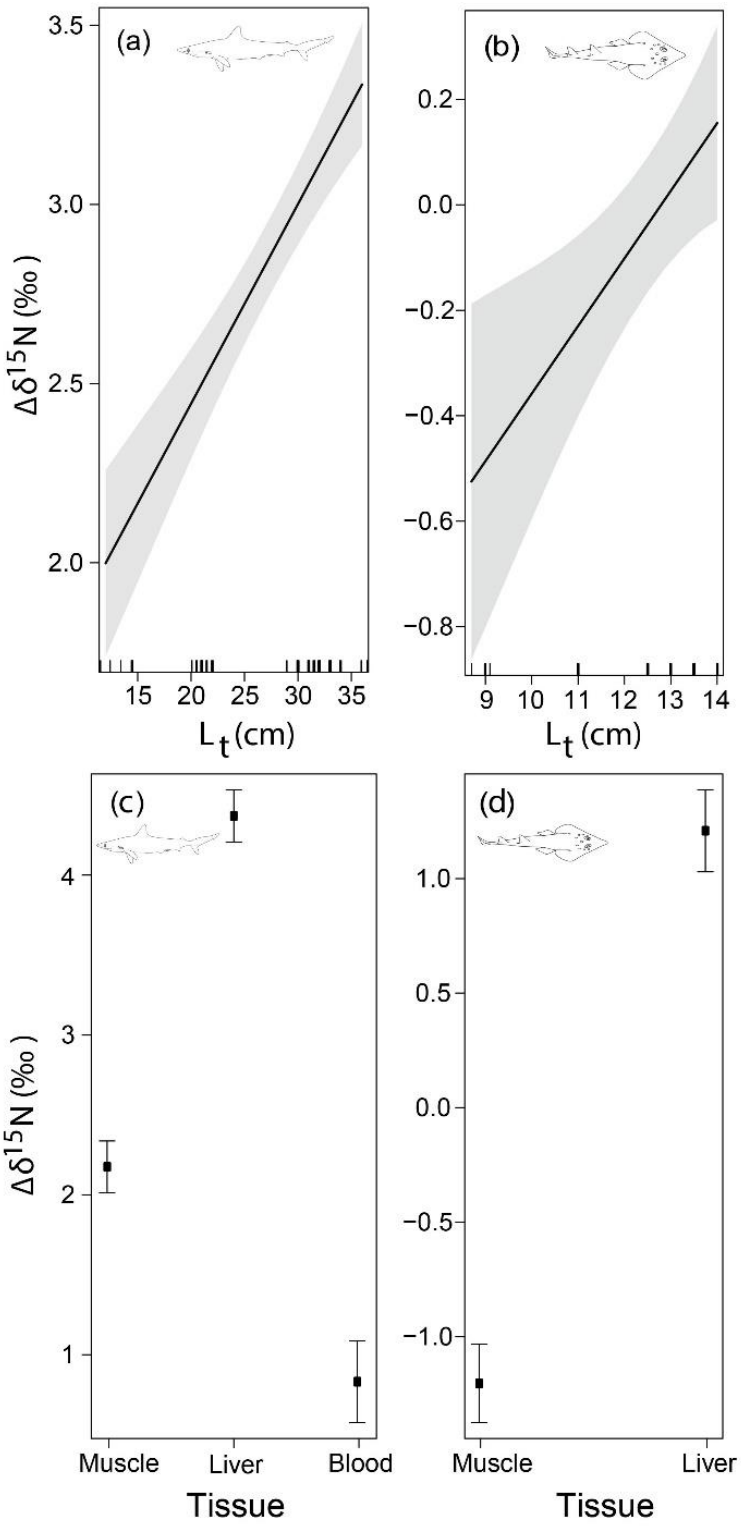


Figure 2.2. Results of the multiple linear regressions models show the effects of (a, b) total length (L_t) and (c, d) tissue on $\Delta\delta^{15}N$ for (a, c) the Pacific sharpnose shark *Rhizoprionodon longurio* and (b, d) the speckled guitarfish *Pseudobatos glaucostigmus*.

2.4.3 Differences between both species

Values of $\Delta\delta^{15}N$ between the matched tissue types of *R. longurio* and *P. glaucostigmus* differed statistically ($H = 117.72$, $df = 3$, $p < 0.05$; Dunn's test, $p < 0.05$; Table 2.1). Moreover, *R. longurio* $\delta^{15}N$ median values of matched tissues were statistically higher than *P. glaucostigmus* ($H = 182.96$, $df = 9$, $p < 0.05$; Dunn's test, $p < 0.05$).

2.5. DISCUSSION

2.5.1 $\delta^{15}N$ variation between mother and embryos

Significant differences in median $\delta^{15}N$ values between embryo and pregnant female pair matched tissue types, for both species, were observed. The *R. longurio* embryos had statistically higher values than pregnant female matched tissues, up to approximately one trophic level (3-4‰) (Kim *et al.*, 2011). The ^{15}N-enrichment of embryo muscles have been reported for other elasmobranch species that are placental, such as the Atlantic sharpnose shark *Rhizoprionodon terraenovae* (Richardson 1836), the scalloped hammerhead *Sphyrna lewini* (Griffith & Smith 1834) and the blacktip shark *Carcharhinus limbatus* (Valenciennes 1839) (McMeans *et al.*, 2009; Vaudo *et al.*, 2010). On the other hand, *S. tiburo* embryos presented lower $\delta^{15}N$ values in muscle relative to pregnant females (Olin *et al.*, 2018). There is evidence of ^{15}N-enrichment in *R. terraenovae* embryo liver ($\Delta\delta^{15}N$ mean = 1.7 ‰) (McMeans *et al.*, 2009), as shown in our results; however the liver $\Delta\delta^{15}N$ in *R. longurio* (our study) was higher ($\Delta\delta^{15}N$ mean = 4.4 ‰). Enriched embryo tissue values of $\delta^{15}N$, around one trophic level higher relative to their pregnant females, are expected as they are continuously "feeding on maternal tissue", emulating a predator-prey relationship (Habran *et al.*, 2010; Jenkins *et al.*, 2001).

As in *H. griseus, P. glaucostigmus* yolk $\delta^{15}N$ values were significantly lower than muscle of the pregnant female and not statistically significant relative to the liver of the pregnant female (Osgood *et al.*, 2020). Muscle $\delta^{15}N$ values in embryos were lower,

compared with those of their pregnant females. This tendency was shown in the yolk-sac viviparous species *S. megalops* and *H. griseus,* but not in *C. moluccensis,* in which embryo muscle values were not statistically different from their pregnant females (Le Bourg *et al.*, 2014; Osgood *et al.*, 2020). Values of $\delta^{15}N$ in embryo liver were ^{15}N-enriched compared with their pregnant female, as in *C. moluccensis* but not in *S. megalops,* where values did not differ from the pregnant female, and *H. griseus*, where values were ^{15}N-depleted (Le Bourg *et al.*, 2014; Osgood *et al.*, 2020). Variability in results for muscle and liver could be due to a direct protein yolk source for yolk-sac viviparous embryos, typically formed before start of gestation. Since yolk proteins come from maternal liver (Reading *et al.*, 2017), which has a high metabolic rate, $\delta^{15}N$ values in pregnant females represent a short time frame (MacNeil *et al.*, 2005) and not the period of yolk formation. Although the turnover rate of muscle is slower, the female liver transfers nutrients to yolk for embryo development (van Hees & Ebert, 2017). Thus, $\delta^{15}N$ values in maternal muscle might not be reflected by embryo tissues. Results for the smallest *R. longurio* embryos and for *P. glaucostigmus* are consistent with what is known; the main constituent of yolk is vitellogenin, which is formed in the liver (Carrier *et al.*, 2004; Hamlett, 2005). ^{15}N-enrichment in the muscle of pregnant females, compared to the embryo tissues with the lowest $\delta^{15}N$ value, in the placental and the yolk-sac viviparous elasmobranchs under analysis, may indicate maternal muscle might not be as relevant as maternal liver for protein transfer during embryo development.

2.5.2 Relationship between $\Delta\delta^{15}N$ values and embryo size

For *R. longurio*, $\Delta\delta^{15}N$ increased with embryo L_t (Fig 2.2a). These findings were also evident in liver and muscle of *R. terraenovae* (McMeans *et al.*, 2009) and *S. tiburo* (Olin *et al.*, 2018), but not in *M. higmani* (Souza-Araujo *et al.*, 2020). An increase in differences between embryo and pregnant female $\delta^{15}N$ values, as the embryo grows, has been attributed to a shift in nutrient source (McMeans *et al.*, 2009). The smallest embryos feed on yolk; however as they grow, a placental connection with the pregnant female is established (Hamlett, 2005). A similar result was found for the pygmy devilray

Mobula kuhlii cf. *eregoodootenkee* (Valenciennes 1841), where $\delta^{15}N$ values in embryo muscle changed over offspring development, compared with the muscle of the pregnant female. When embryos were small, $\delta^{15}N$ values were lower than maternal muscle and as they grew values increased to exceed those of the pregnant female (Broadhurst *et al.*, 2019). *M. kuhlii* cf. *eregoodootenkee* is an elasmobranch whose reproductive strategy is histotrophic, which consists of yolk-sac nutrition at the beginning and after a lipid-rich histotroph is provided by specialized secretory cells (trophonemata), which are found in the maternal uterus (Hamlett, 2005). This transition in embryo $\delta^{15}N$ values is probably due to this nutritional change during gestation, although it could also be due to a dietary shift by gravid females (Broadhurst *et al.*, 2019; McMeans *et al.* 2009).

For the yolk-sac viviparous elasmobranch *S. megalops*, the negative relationship between $\delta^{15}N$ and $\delta^{13}C$ values in embryo muscle and their L_t was attributed to a progressive dilution of heavy isotopes in the embryo body as they grow due to the absence of supplementary maternal transfer of nutrients (Le Bourg *et al.*, 2014). A positive relationship exists in *P. glaucostigmus* between embryo L_t and $\Delta\delta^{15}N$ (Fig 2.2b). Smaller embryos presented $\delta^{15}N$ values lower than their respective pregnant females; however, the largest embryos sampled had higher $\delta^{15}N$ signatures than pregnant females. Unlike *S. megalops*, *P. glaucostigmus,* $\delta^{15}N$ values increased as they grew, not showing a progressive ^{15}N-dilution. $\delta^{15}N$ embryo tissue values were higher than yolk at less than 1 ‰. This small difference between protein source and embryo tissues suggests that proteins could be transferred mostly unchanged to form the new tissues.

2.5.3 Differences between both species

R. longurio had a higher median $\Delta\delta^{15}N$ in muscle and liver matched tissue compared to *P. glaucostigmus*. For the first species, differences were approximately one trophic level and embryos had consistently higher $\delta^{15}N$ values than their mothers. However, *P. glaucostigmus* showed $\Delta\delta^{15}N$ similar to those found in previous studies of placental elasmobranchs, although it is a yolk-sac viviparous which are between 0.82

and 1.7 ‰ (McMeans *et al*., 2009; Olin *et al*., 2018; Vaudo *et al*., 2010). Moreover, muscle of pregnant females of *P. glaucostigmus* had higher values than both embryo tissues analysed.

Embryos, for which yolk is the protein source for both species (shortest *R. longurio* embryos and all *P. glaucostigmus* embryos) had differences lower than 2 ‰ between yolk and muscle and liver tissue embryos (*R. longurio* embryos which blood was drawn are different from those which yolk was sampled). Those small differences suggest that proteins are mostly transferred unchanged from yolk to embryo tissues.

The position of the muscle of the pregnant female in the rank order of tissues differs between species; however, higher values were observed in both, relative to the embryo tissue. A similar rank order of tissue values may indicate that nutrients follow similar paths as observed in our results, with liver the possible main nutrient source.

Our findings showed variation between both reproductive strategies under analysis and are consistent with several studies investigating pregnant female to embryo isotopic variation in placental sharks; embryos have higher $\delta^{15}N$ values than pregnant females (McMeans *et al.*, 2009; Vaudo *et al.*, 2010). However, embryo ^{15}N-enrichment in *P. glaucostigmus,* the yolk-sac viviparous elasmobranch, was only present in liver but not muscle. A positive relationship between $\Delta\delta^{15}N$ and embryos L_t in the viviparous placental shark supported the hypothesis by McMeans *et al*. (2009); embryo values reflect nutrient source shift from yolk to a placental connection. However, it is important to perform more studies on pregnant female to offspring isotopic variation in elasmobranchs, as well as considering different species.

CHAPTER 3: MERCURY MATERNAL TRANSFER IN TWO PLACENTAL SHARK AND A YOLK-SAC RAY FROM THE BAJA CALIFORNIA SUR COAST

3.1 ABSTRACT

We measured total mercury concentration ([THg]) in muscle and liver of two placental viviparous sharks, Pacific sharpnose shark (*Rhizoprionodon longurio*) and brown smooth-hound shark (*Mustelus henlei*); and muscle, liver, and yolk of the yolk-sac viviparous speckled guitarfish ray (*Pseudobatos glaucostigmus*) across the coast of the Baja California Sur, to determine which factors could be involved in maternal transfer and resultant [THg]. Higher [THg] were found in pregnant female than embryos in paired tissues. [THg] of embryo tissues decreased with embryo total length (T_L) except for Pacific sharpnose shark muscle. Embryo muscle [THg] was positively related to pregnant female muscle [THg]. Higher concentrations of THg relative to the pregnant female were found in placental viviparous embryos than yolk-sac viviparous embryos. The embryo T_L, muscle [THg] of the pregnant female, percentage of THg in embryos (embryo [THg]/pregnant female [THg]*100), and reproductive strategy are factors necessary to better understand [THg] in embryo tissues.

Key Words: Maternal transfer, mercury, placental viviparous, yolk-sac viviparous, elasmobranchs, Mexican Pacific.

3.2 INTRODUCTION

Mercury (Hg) is a non-essential trace element with known toxicosis concerns for marine ecosystems, especially through the bioaccumulation and biomagnification of the monomethyl mercury ($MeHg^+$) form (Hauser-Davis *et al.*, 2020). Dietary $MeHg^+$ is rapidly absorbed by marine animals as the main exposure pathway in piscivorous humans and wildlife (Gworek *et al.*, 2016; van Hees & Ebert, 2017), and comprises approximately 80% or more of the total mercury concentrations ([THg]) in fish muscle (Storelli *et al.*, 2002; Horvat *et al.*, 2014; Harley *et al.*, 2015).

Maternal transfer of Hg (e.g., *in utero*, lactation) is another key exposure pathway during development. During gestation females transfer various nutrients to offspring vital for embryo growth and development (Hamlett *et al.*, 1985; Manta-Vogli *et al.*, 2018), concurrent with $MeHg^{+}$ (Lahaye *et al.*, 2007; Guirlet *et al.*, 2008), which crosses the transplacental barrier (Caserta *et al.*, 2013; Frías-Espericueta *et al.*, 2015). Females transport a proportion of their THg tissular pool and more recent circulating diet sources (diet – dam circulation – transplacental) to the embryos during gestation (Dutton & Venuti, 2019). These mechanisms represent an *in utero* THg "sink" or form of maternal elimination (Brookens *et al.*, 2007). THg exposure during the embryonic stage (rapid development) can cause adverse effects to the offspring including delayed growth and psychomotor retardation (Devlin, 2006; Rice *et al.*, 2014). Some elasmobranchs are prone to bioaccumulate and biomagnify high tissue [THg] due to life traits including long lifespan, slow growth, high trophic status, inherent tissue tropisms, and low fecundity (Kim *et al.*, 2016; Murillo-Cisneros *et al.*, 2018).

Elasmobranchs have numerous species-dependent reproductive strategies (Carrier *et al.*, 2004; Hamlett, 2005). Two of the known reproductive strategies are placental and yolk-sac viviparity. Embryos of placental viviparous species are initially nourished by yolk; once depleted, the yolk stalk elongates to form the umbilical cord that transports maternal nutrients until birth. In contrast, embryos from species with yolk-sac viviparity lack the additional maternal nutritional input beyond yolk (Awruch, 2015; Hamlett, 2005). Maternal transfer of Hg occurs in both reproductive strategies; placental viviparous (Adams & McMichael Jr, 1999; Frías-Espericueta *et al.*, 2015) and yolk-sac viviparous (Pethybridge et al., 2010; Le Bourg et al., 2014; van Hees & Ebert, 2017; Hauser-Davis et al., 2020). Profound differences are noted between both reproductive strategies, but also between species using the same reproductive strategy. There are various factors influencing maternal transfer of Hg, such as the amount of nutrition invested in embryos (van Hees & Ebert, 2017).

Mercury transfer to embryos varies between species driven by different biological characteristics. Three species assessed in this study include the Pacific sharpnose shark *Rhizoprionodon longurio*, the brown smooth-hound shark *Mustelus*

henlei, and the speckled guitarfish ray *Pseudobatos glaucostigmus.* The Pacific sharpnose shark is a viviparous placental shark with a gestation period between 10 – 12 months producing 1 – 12 pups (Márquez-Farias *et al.*, 2005; Smith *et al.*, 2009). The brown smooth-hound is a viviparous placental shark with gestation of 10 months having 1 – 21 pups (Pérez-Jiménez & Sosa-Nishizaki, 2008; Soto-López *et al.*, 2018). We are lacking information regarding gestation and pup production for the speckled guitarfish, a yolk-sac viviparous ray (Bizzarro, 2016).

We evaluated maternal transfer of Hg in three different species of elasmobranchs with the aim to assess key drivers that may explain varying [THg] in embryo tissues. These included [THg] in muscle, liver and/or yolk of the pregnant female relative to their embryo(s); embryo total length (T_L); percentage [THg] between maternal and embryo tissues; and reproductive strategy (placental or yolk sac viviparous) of analyzed individuals.

3.3 MATERIAL AND METHODS

3.3.1 Sample collection

Pregnant females and respective embryos of Pacific sharpnose shark (female n = 9, embryo n = 67), brown smooth-hound shark (female n = 4, embryo n = 40) and speckled guitarfish ray (female n = 6, embryo n = 34) were donated and sampled from an artisanal fishery using gill nets. As noted in Figure 3.1, Pacific sharpnose shark specimens were collected in the San Bruno fishing camp (27° 10’ 13” N; 112° 10’ 02” W) from October 2017 to October 2018; brown smooth-hound specimens were sampled in the Barrancas fishing camp (26° 03’ 48’’ N; 112° 16’ 28’’ W) in April 2018; speckled guitarfish rays were sampled in the San Bruno fishing camp on October 2017 except for one pregnant female which was sampled on December 2018 in the Saladito fishing camp (24° 26’ 33’’N; 110° 41’ 16’’ W). Total length (T_L), sex and uterus (#1, #2, or unknown) for each embryo were determined. Samples of muscle, liver and embryos of each pregnant female were collected and placed in plastic bags, kept on ice in coolers and transported to the laboratory at Centro Interdisciplinario de Ciencias

Marinas del Instituto Politécnico Nacional (CICIMAR-IPN, La Paz, BCS, Mexico) where they were stored frozen (-20°C).

In the laboratory, muscle and liver of the pregnant females were subsampled (range 0.2 – 20 g) on an acid-washed (5% HNO_3) surface using a clean stainless-steel scalpel then stored at -20°C in acid-washed plastic containers. For each embryo, we sampled muscle and liver, and yolk for the speckled guitarfish. Samples were freeze dried (Telstar, LyoQuest - 85) for 48 h and homogenized using an agata mortar and pestle as well as a stainless-steel ball grinder (Retsch, CryoMill). Weight of each sample before and after freeze drying was determined to calculate the percent water.

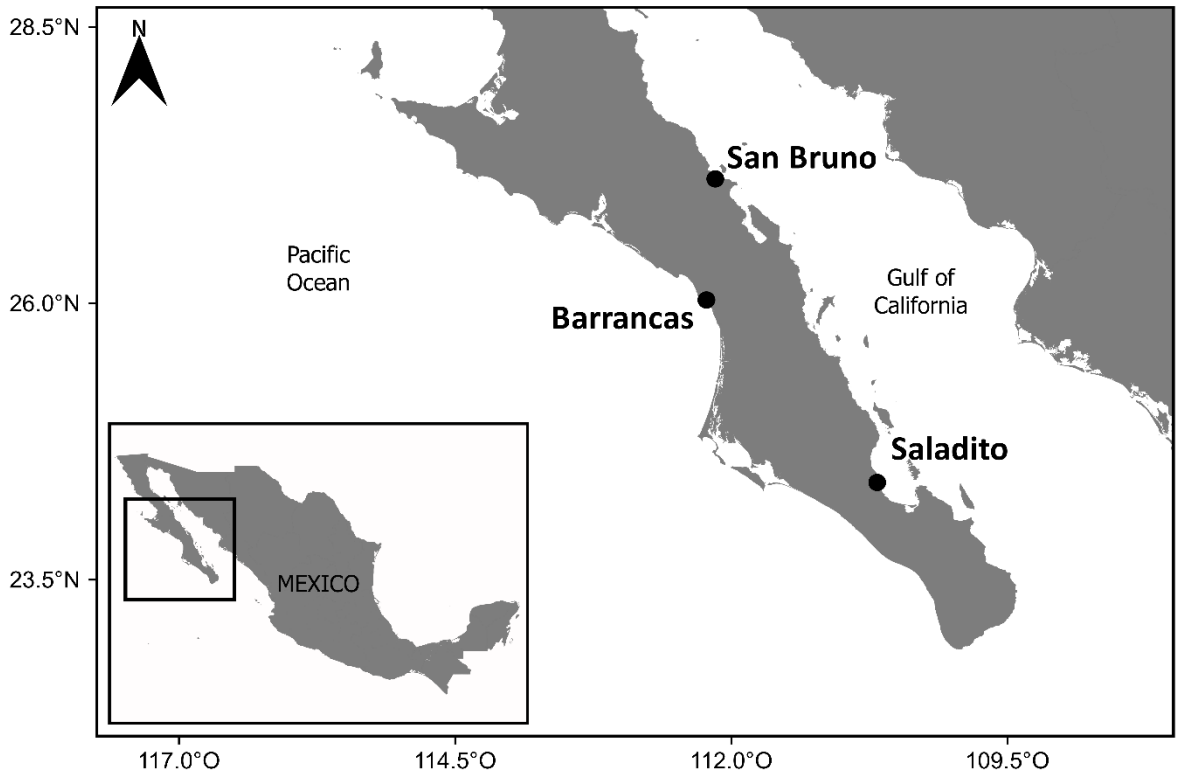


Figure 3.1. Map indicating study locations in Baja California Sur, México.

3.3.2. Total mercury concentration ([THg]) determination

Tissue samples were analyzed for [THg] at the Wildlife Toxicology Laboratory (WTL) at the University of Alaska Fairbanks (UAF), USA, using a direct mercury analyzer (DMA – 80, Milestone Scientific, Inc.) according to USEPA method 7473, as in Murillo-Cisneros et al*.* (2018). The dual cell DMA-80 had a detection limit of 0.5 ng THg and the tri-cell DMA-80, used for low concentration samples, had a 0.2 ng THg detection limit. Samples were freeze dried again, on site at the WTL, before each run to remove any potential residual moisture obtained from storage or transport. Samples below 0.2 ng of THg detected were considered below detection limit (BDL) and not considered for statistical analysis (n = 18 for embryo liver of the brown smooth-hound shark, n=14 for muscle and n=27 for embryo liver of the speckled guitarfish ray). Blanks, aqueous standards (1 ng at 0.01 mg Kg^{-1}, 10 ng at 0.1 mg kg^{-1}, Perkin-Elmer), and standard reference materials (DORM-4: National Research Council Canada, Ottawa ON, Canada; Lake Superior Fish: LSF, National Institute of Standards and Technology) were analyzed for each analytical run for quality assurance. Percent recoveries of standard reference materials and aqueous standards ranged from 89–114%. Pregnant female samples were analyzed in triplicate using the dual cell DMA-80 with the 0.5 ng THg detection limit (12-15 mg mass each). Depending on maternal [THg], embryo tissues were analyzed on either the dual (12-15 mg mass each) or tri-cell (16-20 mg mass each) DMA-80. The coefficient of variation for triplicate and duplicate samples was less than 15%. When samples BDL were less than 50% of a specie-tissue group, a proxy value was calculated using ½ the detection limit of the DMA 80 (ng) divided by the mass of the sample (g). When more than 50% of the samples were BDL, values were not used for statistical analyses, just reported as BDL.

3.3.3 Statistical analyses

Statistical analyses were carried out using R software (Version 4.0.3, R Core Team, 2020). Data were not normally distributed ($W_{259} = 0.35$, $p < 0.05$) and not homoscedastic ($X^2_{259} = 25.0$, $p < 0.05$), therefore non-parametric tests were used.

Mean ± standard deviation (SD) and median [THg] were calculated for muscle and liver of pregnant females, embryos of each species, and embryo yolk of speckled guitarfish ray. Wilcoxon tests were used to determine differences in [THg] between pregnant females and embryos matched tissues for each species, muscle and liver of embryos of the same species and between sexes and uterus (undetermined uteri omitted) for each tissue and embryo of each species. The percentage of THg (% [THg]) for each species and tissue was calculated using the median [([THg] of embryo tissue/[THg] of matched pregnant female tissue) * 100] of each offspring. Differences of % [THg] between tissues of each species was determined using Wilcoxon tests. Two Kruskal-Wallis tests were done, one using embryos values and other using pregnant females' values, to test for differences in [THg] between species and tissues. Post-hoc Dunn's test analyses were used to determine the differences. Multiple linear models were used to describe the effect of predictor variables (embryo T_L, [THg] of pregnant female tissues, and embryo tissues) on the response variable ([THg] of the embryo) for the three species analyzed. Tissue was also used as interaction term with embryo T_L and [THg] of pregnant female to assess more complex relationships of predictor variables on the response variable. Models can be expressed as follows:

$$\hat{y}_i = \alpha + \beta_1(embryo\mathrm{TL}_i) + \beta_2\left([THg]_{pregnant\ female_i}\right) + \beta_3(EmbryoTissue_i) + \beta_4(embryo\mathrm{TL}_i : EmbryoTissue_i) + \beta_5\left([THg]_{pregnant\ female_i} : EmbryoTissue_i\right)$$

Where $\hat{y}_i$ is the expected value of response variable (embryo [THg] of the species analyzed); α is the intercept; β_n are the coefficients; and ":" denotes interaction term. Diagnostic plots (not shown) suggest normality and homoscedasticity of residuals; therefore, assumptions of regression models were not violated.

3.4 RESULTS

3.4.1 Pregnant females and embryos [THg]

A total of 19 pregnant females and 141 embryos from three different species of elasmobranchs were analyzed for [THg]. Biometric data (mean ± SD, median [THg] for each tissue, and percentage of [THg] for female-embryo matched tissues of each species are noted in Table 3.1. Values of liver [THg] for embryos of the speckled guitarfish ray were not reported as 79.4% of the samples were BDL The [THg] in muscle and liver of pregnant females of the three species studied were significantly higher than embryo [THg] matched tissues ($p < 0.05$). Embryo muscle [THg] was significantly higher than embryo liver [THg] for the Pacific sharpnose shark and for the brown smooth-hound shark ($p < 0.05$). No significant differences between sexes or uteri were noted for any embryo tissues in any species analyzed ($p > 0.05$).

3.4.2 Percentage of total mercury: embryos/pregnant females

For the Pacific sharpnose shark, embryos median % [THg] of muscle relative to pregnant females was significantly lower (median = 6.3 %) than liver (median = 20.0 %; $p < 0.05$; Table 3.1). The brown smooth-hound shark exhibited higher % [THg] in muscle tissues (median = 16.1 %) than in liver (median = 6.4 %; $p < 0.05$; Table 3.1). The speckled guitarfish ray had the lowest % [THg] in muscle (median = 1.9 %; Table 3.1), and although liver [THg] in pregnant females was similar between the brown smooth-hound shark and the speckled guitarfish ray, most livers of the speckled guitarfish ray embryos had [THg] and thus, % [THg], BDL. These results indicate proportions of [THg] between pregnant females and embryos varies between tissues, reproductive strategy, and species.

3.4.3 Total mercury concentration comparison between species

As noted in Table 3.1, Pacific sharpnose shark muscle of pregnant females had significantly higher median [THg] than the other two species ($p < 0.05$), whereas no

significant differences were detected between the brown smooth-hound shark and the speckled guitarfish ray ($p > 0.05$). The hepatic [THg] of pregnant females was significantly higher in the Pacific sharpnose shark than the speckled guitarfish ray ($p < 0.05$). No significant differences existed between [THg] in liver of the brown smooth-hound shark and the other two species ($p > 0.05$). For embryos, muscle [THg] ranked Pacific sharpnose shark > brown smooth-hound shark > speckled guitarfish ray and differed statistically between all species ($p < 0.05$). For embryos, liver of Pacific sharpnose shark had significantly higher [THg] than brown smooth-hound shark ($p < 0.05$).

Table 3.1: Mean ± standard deviation (SD), and range (minimum-maximum) for total length (T_L) of females and embryos, and total mercury concentration ([THg]; mg kg^{-1} wet weight);and median [THg] (mg kg^{-1} wet weight) for muscle and liver of pregnant females and embryos and percent of median [THg] (% of [THg]) of embryo relative to pregnant female for muscle and liver of the Pacific sharpnose shark *R. longurio*, the brown smooth-hound *M. henlei* and muscle of the speckled guitarfish *P. glaucostigmus*.

				Muscle			Liver		
		n	T_L (cm)	Mean [THg] ± SD (range)	Median [THg]	% of [THg] (median)	Mean [THg] ± SD (range)	Median [THg]	% of [THg] (median)
R. longurio	Pregnant females	9	110.2 ± 6.1 (100 – 120)	0.730 ± 0.358 (0.287 – 1.277)	0.704 [a d]		0.144 ± 0.150 (0.030 – 0.462)	0.070 [a e]	
	Embryos	67	21.7 ± 5.5 (14.5 – 30)	0.060 ± 0.042 (0.006 – 0.126)	0.060 [a b †]	6.3 [c]	0.021 ± 0.018 (0.005 – 0.095)	0.015 [a b ‡]	20.0 [c]
M. henlei	Pregnant females	4	84.8 ± 1.2 (83 – 86)	0.138 ± 0.022 (0.124 – 0.172)	0.128 [a d]		0.045 ± 0.025 (0.025 – 0.081)	0.037 [a]	
	Embryos	40	22.5 ± 1.2 (19.5 – 25)	0.023 ± 0.008 (0.011 – 0.036)	0.025 [a b †]	16.1 [c]	0.003 ± 0.001***** (0.001 – 0.006)	0.004 [a b ‡]	6.4 [c]
P. glaucostigmus	Pregnant females	6	68.7 ± 6.2 (58 – 78)	0.095 ± 0.08 (0.035 – 0.262)	0.069 [a d]		0.068 ± 0.103 (0.021 – 0.279)	0.026 [a e]	
	Embryos	34	14.8 ± 2.1 (12 – 18.5)	0.002 ± 0.001***** (0.001 – 0.005)	0.002 [a †]	1.9 [c]	- ****** (0.001 – 0.008)	-	-

n: number of samples, T_L: total length, SD: standard deviation.
% of [THg] = ([THg] of embryo tissue/[THg] of matched pregnant female tissue) * 100
* < 50% BDL: below detection level (n= 18 for liver of *M. henlei* embryos and n=14 for muscle of *P. glaucostigmus* embryos).
** > 50% BDL: below detection level (n=27 for liver of *P. glaucostigmus* embryos).
[a] [THg] in muscle and liver of all pregnant females of the three species were significantly higher than embryo [THg] matched tissues ($p < 0.05$).
[b] Embryo muscle [THg] was significantly higher than embryo liver [THg] for *R. longurio* and for *M. henlei* ($p < 0.05$).
[c] % of [THg] differed statistically between tissues of each species with reported values ($p < 0.05$).
[d] *R. longurio* muscle of pregnant females had significantly higher median [THg] than the other two species analyzed ($p < 0.05$), with no significant differences between *M. henlei* and *P. glaucostigmus* ($p > 0.05$).
[e] Hepatic [THg] of pregnant females was significantly higher in *R. longurio* than *P.glaucostigmus* ($p < 0.05$).
† For embryos, muscle [THg] ranked *R. longurio* > *M.* henlei > *P. glaucostigmus* and differed statistically between all species ($p < 0.05$).
‡ For embryos liver, *R. longurio* showed significant higher [THg] than *M. henlei* ($p < 0.05$).

3.4.4 Embryo total length & maternal [THg] related to embryo [THg]

The multiple regression model used to describe the effect of predictor variables on the [THg] of embryo of the Pacific sharpnose shark is graphically summarized in Fig. 3.2. This model explains a large proportion of the variance (R^2 = 0.83, $F_{5,127}$=127, $p<0.05$) of [THg]. According to this model, there is a strong and linear relationship between embryo T_L and [THg], with a positive relationship for muscle and a negative relationship observed for liver (Fig. 3.2, Suppl. Table 3.1). A strong and positive relationship is noted between [THg] of pregnant females and its embryos for both tissues, for this variable this model indicates that liver has higher values than muscle (Fig. 3.2, Suppl. Table 3.1).

For the brown smooth-hound shark, the model (Fig. 3.2, Suppl. Table 3.1) explains a large proportion of the variance (R^2 = 0.93, $F_{5,56}$ = 151, $p<0.05$) of [THg] and suggests a weak linear relationship of embryo T_L and [THg] of pregnant females in liver, but a strong linear relationship in muscle (Fig. 3.2, Suppl. Table 3.1). In the latter, a negative relationship is observed between [THg] of the embryo and its body size; meanwhile a positive relationship is observed between [THg] of pregnant females and their embryos (Fig. 3.2, Suppl. Table 3.1).

For the speckled guitarfish ray, the model (Fig. 3.2) explains a low proportion of the variance (R^2 = 0.24, $F_{2,17}$ = 2.7, $p>0.05$) of [THg], however suggests linear relationships between [THg] of embryo muscle with embryo T_L and [THg] of pregnant female muscle. According to this model [THg] of embryo muscle decreases with increasing size (Fig. 3.2, Suppl. Table 3.1). On the other hand, [THg] in muscle of embryos is higher if [THg] of pregnant female muscle is also high (Fig. 3.2, Suppl. Table 3.1). While we were unable to statistically assess embryo liver tissue of the speckled guitarfish ray, a high proportion (>50%) of embryo liver samples BDL was noted.

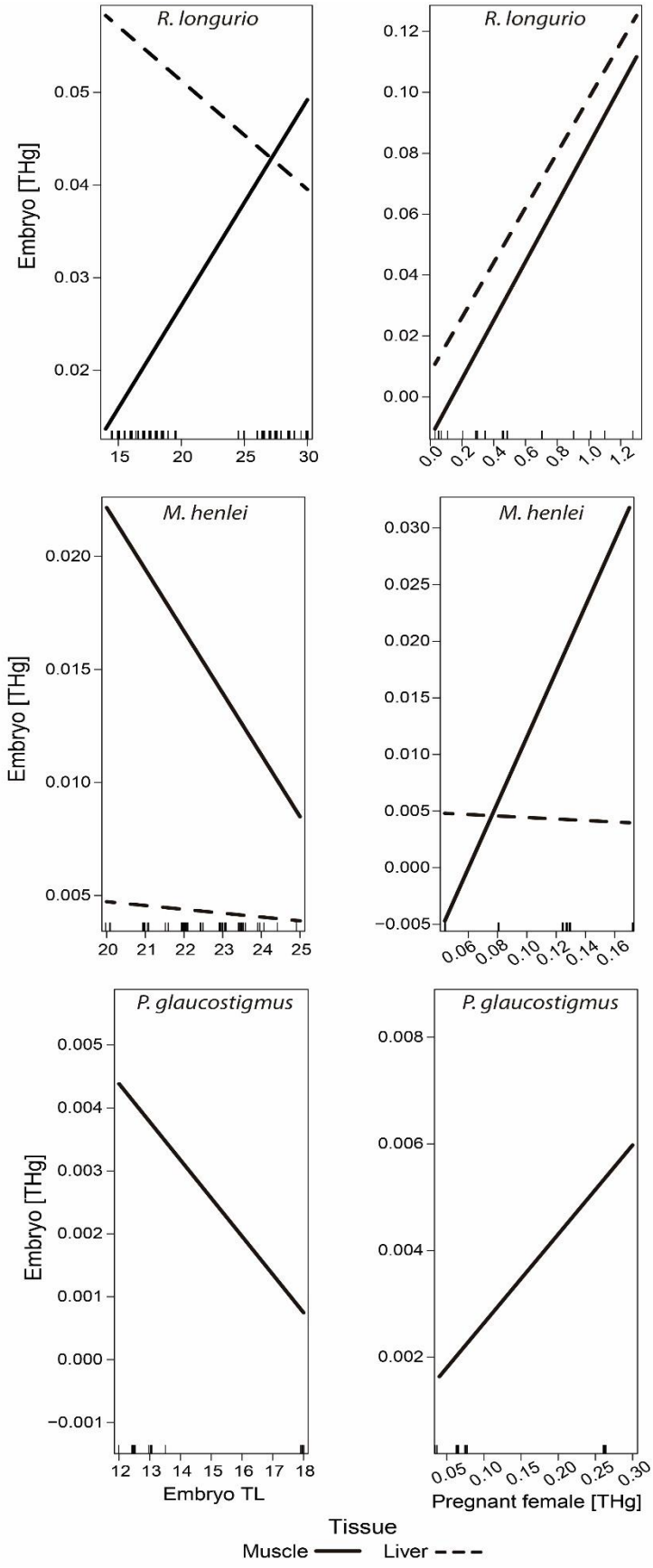


Figure 3.2. Results of multiple linear regressions models show effects of embryo total length (T_L) and pregnant female total mercury concentration ([THg]) on embryo [THg] for the sharonose shark *Rhizoprionodon longurio*, the brown smooth-hound *Mustelus henlei* and the speckled guitarfish *Pseudobatos glaucostigmus*. Details in Suppl. Table 3.1 in Annex II.

3.5 DISCUSSION

3.5.1 Pregnant females and embryos [THg]

The determination of [THg] in embryo tissues provides empirical evidence of maternal transfer during gestation for both reproductive strategies under analysis; placental and yolk-sac viviparity. This process facilitates contaminant elimination for pregnant female vertebrates via embryos and fetuses (Wagemann *et al.*, 1988; Brookens *et al.*, 2007); yet higher [THg] were noted in pregnant female tissues relative to embryo tissues in this study. We found variation of [THg] among the species, tissues and between females and embryos that includes size (T_L) of the embryos as a significant factor.

Despite transfer (maternal elimination) of Hg to the embryos, three pregnant females of Pacific sharpnose shark exceeded the human consumption threshold of 1 mg $kg^{-1}$of THg in muscle established for top predators in Mexico (NOM-242-SSA1-2009, 2011). Furthermore, considering the possible harmful effects of Hg to other early life stage organisms (Fjeld *et al.*, 1998; Matta *et al.*, 2001), elasmobranch embryos may be at risk of adverse effects from Hg even at lower [THg] relative to the females. Pregnant females and embryos for each species studied had higher [THg] in muscle than in liver. Moreover, embryos of the speckled guitarfish ray showed a higher percentage of BDL in liver relative to muscle (Table 3.1). This tendency is in agreement with previous studies in other elasmobranchs species (Pethybridge *et al.*, 2010; Lyons & Lowe, 2013a; Le Bourg *et al.*, 2014; Frías-Espericueta *et al.*, 2015). These differences between tissues are likely related to their depuration rates; European seabass (*Dicentrarchus labrax*) liver has shown higher depuration rates than muscle (Maulvault *et al.*, 2016), or that THg is deposited and stored in muscle tissue mainly as $MeHg^+$ (Amlund *et al.*, 2007; Pethybridge *et al.*, 2010). Lower [THg] in liver can be related to an effective process of demethylation of $MeHg^+$ and inorganic mercury formation by reaction with selenium (Se) to form mercuric selenide (Storelli & Marcotrigiano, 2002). A key function of liver is metabolism (biotransformation) and elimination of compounds (Hinton *et al.*, 2001). All samples of yolk analyzed for the yolk-sac viviparous species speckled

guitarfish ray were BDL. This result could be influenced by the relatively high lipid content of the yolk (Wrisez *et al.*, 1993). However, yolk contains Hg as some embryos of the speckled guitarfish ray indicated detectable [THg] and yolk is the only nutrient input during gestation. Sex of individuals does not influence the tissue [THg] in embryos before parturition (Taguchi et al*.,* 1979). No differences between uterus in any species confirmed the lack of inter-uterine differences during gestation, which have been previously reported by Frías-Espericueta et al*.* (2015) for the Pacific sharpnose shark.

3.5.2 Percentage of total mercury: embryos/pregnant females

Higher percentages of THg occurred for embryos of placental viviparous species relative to yolk-sac viviparous (Table 3.1). Similar tendencies were found in previous studies, where embryos of placental viviparous elasmobranchs had between 8-60% of THg in relation to the pregnant female (Adams & McMichael Jr, 1999) and yolk sac viviparous embryos had between 4-10% of THg in relation to the pregnant female (Pethybridge *et al.*, 2010; Dutton & Venuti, 2019; Hauser-Davis *et al.*, 2020). As Pethybridge et al*.* (2010) hypothesized, higher percentages of THg in offspring in placental viviparous elasmobranch could be expected as they have a continuous input of nutrients (thus Hg) during gestation.

3.5.3 Total mercury concentration comparison between species

Differences in [THg] in pregnant female by species were found in this study for both tissues. Variations in [THg] between adult organisms among species result from multiple factors. Foraging ecology and trophic position are known to be main factors determining [THg], including elasmobranchs (Murillo-Cisneros *et al.*, 2018). However, other ecological and biological factors may influence tissue concentration, such us differences in metabolism, biotransformation, environmental characteristics of a given ecosystem, source of contamination, among others (Hisamichi *et al.*, 2010; Pethybridge *et al.*, 2010; Le Bourg *et al.*, 2019), which may lead to [THg]

variation among species. Differences between species in [THg] of embryo tissues could be due to different factors mentioned above, plus other factors such as time of gestation, number of embryos per litter, physiology, among others (Lyons & Lowe, 2013b; Lyons *et al.*, 2013, 2019). Higher concentrations in placental viviparous elasmobranch could be related to the well-known ability of $MeHg^+$ to cross the placental barrier (Mansour *et al.*, 1973; Caserta *et al.*, 2013).

3.5.4 Effect of embryo total length & maternal [THg] on embryo [THg]

Pacific sharpnose shark embryos that were analyzed comprised both types of nutrient uptake (yolk and placental) as a reproductive strategy. A significant positive relationship between embryo T_L and [THg] in muscle tissue of Pacific sharpnose shark showed an increase during gestation (Fig. 3.2). The smallest embryos, still nourished on yolk, had the lowest concentrations in muscle during this early stage of gestation, likely as yolk is the only nutrient source. As liver of the pregnant female is where yolk components are synthesized, it is unlikely that a major proportion of muscle derived Hg ends up in yolk relative to hepatic sources (Lyons & Lowe, 2013a; Souza-Araujo *et al.*, 2020). When nutrient uptake took place directly through the umbilical cord of the pregnant female, embryos presented higher [THg] in muscle tissue. $MeHg^+$ can cross the placental fetal – maternal barrier (Caserta *et al.*, 2013). Thus, the embryos of elasmobranchs of placental viviparous reproduction have higher [THg] during mid and late gestation. However, embryo liver tissue of the Pacific sharpnose shark had a negative relationship between embryo T_L and [THg] (Fig. 3.2), possibly as a result of several factors, such as embryonic growth dilution, development of mechanisms of elimination, detoxification or distribution to non-hepatic tissues (Brookens *et al.*, 2007), and high fat content of liver.

Brown smooth-hound embryos sampled represent mid and late gestation periods (Pérez-Jiménez & Sosa-Nishizaki, 2008), potentially explaining different trends in relation to the Pacific sharpnose shark. Negative relationships between embryo T_L and [THg] are seen in both tissues of brown smooth-hound shark, although a stronger linear relationship was found in muscle than liver (Fig. 3.2).

These results showed a potential dilution of [THg] as embryos become larger in late gestation. This tendency was reported for the non-essential trace elements Al, As and Pb in embryo muscle of *M. higmani* (Souza-Araujo *et al.*, 2020) and for Hg in the yolk-sac viviparous embryos muscle of *S. megalops* (Le Bourg *et al.*, 2014). Similarly, a negative relationship between T_L and [THg] was found in this study for the yolk sac speckled guitarfish ray (Fig. 3.2). Le Bourg et al. (2014) attributed this negative relationship to Hg dilution in the body as embryos grew.

Strong correlations between muscle [THg] of pregnant females and muscle [THg] of embryos were found for the three species analyzed (Fig. 3.2). Van Hees & Ebert, (2017) found in a yolk sac viviparous elasmobranch (*Triakis semifaciata*) that [THg] of female muscle was a stronger indicator for embryo Hg concentration than [THg] in female liver, contrary to expectation. Embryo nutrition in yolk-sac viviparous elasmobranchs is basically yolk, which is formed in the liver of the pregnant female during vitellogenesis (Reading *et al.*, 2017). Thus, a stronger correlation with liver [THg] of pregnant female would be expected. In the current study, no correlation between pregnant female and embryo [THg] in liver could be explored, because most of the embryos of the speckled guitarfish ray samples were BDL. Our findings indicate we need a more in-depth assessment of how maternal nutrients and contaminants are mobilized and move to the embryo and developing fetus among the diverse reproductive strategies of elasmobranchs.

3.6 CONCLUSIONS

The presence of Hg in embryos of the three species analyzed confirmed maternal transfer of this element. Sex and location in uterus of the embryo did not influence the [THg] of the individual during gestation. Embryo T_L was an important factor for determining [THg] in both tissues, though the degree may be species dependent. The nutrient delivery to the embryo during gestation was likely another important factor. For example, the smallest embryos, feeding on yolk, had low [THg], and when placental nutrient uptake took place, higher [THg] were observed in both tissues. Muscle [THg] in pregnant females was closely related with muscle [THg] in

embryos. A higher relative percentage of THg (embryo/maternal) was evidenced for the placental offspring relative to yolk-sac viviparous species.

Species-specific differences in maternal transfer of Hg to embryos, as studied here, may be affected by [THg] in pregnant female tissues, relative percentage of [THg] between mom and pups, reproductive strategy of the species, and embryo T_L. In addition, but not evaluated in this study, time of gestation, number of embryos per litter, and/or the physiology and ability of Hg elimination/biotransformation of the species analyzed may be involved in embryos demonstrating different [THg]. More studies in different species and including more reproductive strategies are needed to understand which are the main factors that determine the [THg] in elasmobranch embryo tissues.

CHAPTER 4. TENDENCIES OF $\delta^{15}N$ VALUES AND TOTAL MERCURY CONCENTRATIONS OF *RHIZOPRIONODON LONGURIO* AND *PSEUDOBATOS GLAUCOSTIGMUS* EMBRYOS AS THEY INCREASE THEIR SIZE

4.1 ABSTRACT

Nitrogen isotopic values ($\delta^{15}N$) and total mercury concentrations ([THg]) were analyzed in muscle and liver of pregnant females and their respective embryos of *Rhizoprionodon longurio*, a placental shark, and *Pseudobatos glaucostigmus,* a yolk-sac placental ray. Differences between embryos and pregnant females in terms of $\delta^{15}N$ ($\Delta\delta^{15}N$) and the biomagnification factor (BMF) were calculated to examine trends as embryos become larger. *R. longurio* embryos had higher $\delta^{15}N$ values than the pregnant female, opposite of *P. glaucostigmus*. $\Delta\delta^{15}N$ values were higher for longer the embryo for both species. BMF were lower than 1 for both species. For *R. longurio* muscle, BMF were higher in longer embryos, contrary to *R. longurio* liver and *P. glaucostigmus* muscle.

4.2 INTRODUCTION

Contaminants, such as some trace elements, transferred from food to organisms can result in higher concentrations than the source. This process is known as biomagnification, or in lower concentration, known as biodilution (Gray, 2002; Sun *et al.*, 2020). During development, pregnant females transfer, in addition to nutrients, a proportion of their total Hg pool to the embryos (Dutton & Venuti, 2019).

Nitrogen isotope ($\delta^{15}N$) values are used to track the nutrient flow through the food web (Kim *et al.*, 2011). During prey ingestion, values of $\delta^{15}N$ can vary around 3 - 4 ‰ (one trophic level) between prey and consumer (Post, 2002; Schmidt *et al.*, 2004), which can be used to estimate the trophic position within the food web

(Ikemoto *et al.*, 2008). During maternal transfer of nutrients, $\delta^{15}N$ values for certain embryo tissues are expected to be one trophic level higher (Jenkins *et al.*, 2001) as proteins from embryo development come from the pregnant female, emulating a prey – predator interaction, as long as proteins are metabolized and not just simply transferred. It is noted that ^{15}N enrichment for trophic level might be correlated with trace elements concentration (Pereira *et al.*, 2010). This correlation is assumed to be caused by biomagnification due to food intake (Gray, 2002).

A factor that influences biomagnification factor values (BMF; [contaminant] predator/ [contaminant] prey) of the contaminants and $\delta^{15}N$ is the kind of food that the organism consumes (Ambrose, 1991; Gray, 2002). Elasmobranch embryos receive a different kind of nutrition depending on the reproductive method of the species. Depending on the input that embryos intake, the synthesis varies, as well as the composition of nutrients (Hamlett, 2005). Variations in nutrition can influence the BMF and $\delta^{15}N$ values, resulting in variations between their tendencies.

Two species with different reproductive strategies were studied in this chapter: the Pacific sharpnose shark *Rhizoprionodon longurio*, which is viviparous placental and the speckled guitarfish *Pseudobatos glaucostigmus* ray, which is yolk-sac placental. This chapter aimed to know, for both reproductive strategies, which are the $\delta^{15}N$ tendencies and total mercury concentrations ([THg]) values, in relation to its pregnant female, as embryos increase their size.

4.3 MATERIALS AND METHODS

4.3.1 Sample collection

For the stable isotope analysis, pregnant females of *R. longurio* (n=15), *P. glaucostigmus* (n=8), and three embryos from each specimen (n = 69) were collected in collaboration with the artisanal fisheries fleet. The *R. longurio* samples were collected at the Coloradito fishing camp (27.03°N; 112.00°W) in April, October, and November of 2018; whereas the *P. glaucostigmus* were sampled at San Bruno fishing camp (27.16°N; 112.16°W) in October 2017. For THg analysis, pregnant

females of *R. longurio* (n= 9) and *P. glaucostigmus* (n=6) and the respective embryos of *R. longurio* (n= 67) and *P. glaucostigmus* (n= 34) were sampled from an artisanal fishery using gill nets. *R. longurio* specimens were collected in San Bruno fishing camp (27° 10' 13" N; 112° 10' 02" W) from October 2017 to October 2018 and *P. glaucostigmus* all specimens were sampled in San Bruno fishing camp in October 2017, except for a pregnant female that was sampled in December 2018 in Saladito fishing camp (24° 26' 33''N; 110° 41' 16'' W). Total length (T_L) for each individual was determined. Samples of muscle, liver and embryos of each pregnant female were collected and placed in plastic bags, kept on ice in coolers and transported to the laboratory at Centro Interdisciplinario de Ciencias Marinas from Instituto Politécnico Nacional (CICIMAR-IPN, La Paz, BCS, Mexico) where they were stored frozen (-20°).

4.3.2 Sample analysis $\delta^{15}N$

Liver and muscle were sub-sampled and kept in Eppendorf tubes. All samples were freeze-dried (Telstar, LyoQuest - 85) for 48 h. Urea was removed from muscle and liver tissues using the method proposed by Kim and Koch (2012); these were sonicated three times for 15 min with deionized water. However, since liver samples were being diluted, they were only sonicated twice with deionized water instead of three. Three samples of liver were lost during this process. After extraction, samples were freeze-dried again for 48 h and pulverized manually using an Agate mortar. A range of 600 µg to 1400 µg for each sample was placed in tin capsules for isotopic analysis.

Values of $\delta^{15}N$ were determined using a mass spectrometer (Delta V Plus Thermo Scientific) with continuous flow coupled to an elemental analyzer (Elemental Combustion System Costech Instruments) held in the Mass spectrometry Isotopic Laboratory (LEsMA) at CICIMAR-IPN. The conventional standards of reference materials used for N_2 were NIST glutamic acid and IAEA caffeine. The standard deviations of the two standards (n = 32) were 0.13‰ and 0.16‰, respectively. Stable isotope values were represented as δ based on the following equation: $\delta^{15}N = [(R$

sample/ R standard) – 1] * 1000 where R is the ratio ^{15}N : ^{14}N (Murillo-Cisneros *et al.*, 2019; Park & Epstein, 1961).

4.3.3 Analysis of [THg]

In the laboratory, muscle and liver of the pregnant females were subsampled (range 0.2 – 20 g) on a HNO_3 (5%) acid-washed surface using a stainless steel scalpel then stored at -20 °C in acid-washed plastic containers. For each embryo, we sampled muscle and liver. Samples were freeze dried (Telstar, LyoQuest - 85) for 48 h and homogenized using an agata mortar and pestle as well as a stainless-steel ball grinder (Retsch, CryoMill). The weight of each sample before and after freeze-drying was determined to calculate the percent water.

Tissue samples were analyzed for [THg] at the Wildlife Toxicology Laboratory (WTL) at the University of Alaska Fairbanks (UAF), USA, using the Milestone Direct Mercury Analyzer 80 (DMA - 80) according to USEPA method 7473, with thermal decomposition, amalgamation, and atomic absorption spectrophotometry as in Murillo-Cisneros *et al.* (2018). One DMA - 80 had the detection limit of 0.5 ng THg and calibrated using 17-point calibration curve ranging from 0.5 to 500 ng THg. The other instrument had a lower detection limit (0.2 ng THg) and calibrated using a 16-point calibration curve ranging from 0.15 to 300 ng THg. Samples were freeze-dried again, on site at the WTL, before each run to remove any potential residual moisture. Samples below 0.2 ng of THg detected were considered below detection limit (BDL) and not considered for statistical analysis (n=14 for muscle and n=27 for liver of *P. glaucostigmus* embryos). Blanks, aqueous standards (1 ng at 0.01 mg Kg^{-1}, 10ng at 0.1 mg kg^{-1}, Perkin-Elmer), and standard reference materials (DORM-4: National Research Council Canada, Ottawa ON, Canada; Lake Superior Fish: LSF, National Institute of Standards and Technology) were analyzed for each analytical run for quality assurance. An HNO_3 at 3.5% and aqueous standards were included after every 16-18 samples. Percent recoveries of standard reference materials and aqueous standards were within 89–114%. Pregnant female samples were analyzed in triplicate using the DMA-80 with the 0.5 ng THg detection limit (12-15 mg each).

Depending on the [THg], embryo tissue samples were analyzed in duplicate using the DMA-80 with the detection limit of 0.5 ng THg (12-15 mg each); or the DMA-80 with the lower detection limit of 0.2 ng THg (16-20 mg each). The coefficient of variation for triplicate and duplicate samples was less than 15%. Using this approach, we determined [THg] in all samples analyzed.

4.3.4 Statistical analyses

As values of $\delta^{15}N$ and [THg] were not from samples of the same organism, statistical analyses were done separately. For $\delta^{15}N$ analysis, was used the difference between embryo value and the pregnant female value; $\Delta\delta^{15}N$. $\Delta\delta^{15}N$ for paired (maternal and embryo) tissues were calculated subtracting the $\Delta\delta^{15}N$ value of the pregnant female from the embryo. To know if there were differences between $\Delta\delta^{15}N$ values as embryos got larger, data was treated different depending on the specie. *R. longurio* embryos were pooled in 3 different groups sorted by length (12.5 – 14.5 cm, 20 – 22 cm and 29 – 36.5 cm) and Kruskal Wallis rank sum test were done followed by a post hoc Dunn test for each tissue. Division of the groups was done influenced by sampling dates. For *P. glaucostigmus*, liver tissue values were omitted because there was a low number of samples found higher than the BDL for THg analyses. To know the behaviour of $\Delta\delta^{15}N$ values of *P. glaucostigmus* as embryos got larger, a spearman correlation was done.

THg biomagnification was assess based on the following equation;

$$BMF = \frac{[THg]Predator}{[THg]Prey}$$

For this study, the equation was modified, replacing the predator by the embryo values and the prey with the pregnant female values:

$$BMF = \frac{[THg]\ Embryos}{[THg]Pregnant\ females}$$

To know if there were differences between BMF values as embryos increased their size, they were pooled in two different groups sorted by length (14.5 – 17.5 cm and 25 – 30 cm for *R. longurio* embryos and 12 – 13.5 cm and 18 – 18.5 cm for *P. glaucostigmus* embryos), highly influenced by the sampling dates. Wilcoxon tests were performed to compare values of both groups for each tissue and species.

A basic description of what would be the molar ratio of Se:Hg for *R. longurio* was done for both tissues. However, we acknowledge that this is only an approximation of the reality, since samples do not belong to the same organisms. Values of Se were extracted from the next chapter, which embryos ranged between 14-16.5 cm T_L. For a better comparison, only the [THg] of the smallest embryos (14.5 - 17.5 cm T_L) were used. To determine the molar ratio, Se and Hg concentrations were divided by their molecular weight (200.59 for Hg and 78.96 for Se) and then divided Se:Hg (Grajewska *et al.*, 2019).

4.4 RESULTS

$\Delta\delta^{15}N$ and BMF for muscle and liver tissues of *R. longurio* and *P. glaucostigmus* were calculated based on $\delta^{15}N$ values and [THg] (Table 4.1).

Table 4.1. Median and range values of $\Delta\delta^{15}N$ and BMF for muscle and liver tissues of the Pacific sharpnose shark *Rizoprionodon longurio* and the speckled guitarfish *Pseudobatos glaucostigmus* sorted by body size groups.

	$\Delta\delta^{15}N$					**BMF**				
	Length (cm)	**Muscle**		**Liver**		**Length (cm)**	**Muscle**		**Liver**	
		n	**Median (range)**	**n**	**Median (range)**		**n**	**Median (range)**	**n**	**Median (range)**
Rhizoprionodon longurio	12.5 - 14.5	6	1.81 [a] (0.99 – 2.42)	5	2.90[a] (2.56 – 3.46)	14.5 - 19.5	37	0.05 [a] (0.02 – 0.09)	37	0.25 [a] (0.16 – 0.41)
	20 - 22	9	2.07 (1.63 – 2.49)	9	4.41[b] (3.69 – 5.19)	25 - 30	30	0.10 [b] (0.04 – 0.15)	29	0.13 [b] (0.03 – 0.27)
	29 - 36.5	30	2.44[b] (1.31 – 3.13)	30	4.62[b] (3.48 – 6.01)					
Pseudobatos glaucostigmus	8.7 - 14	24	-1.21 (-1.93 – -0.55)	-	-	12 -13.5	14	0.05 [a] (0.04 – 0.08)	-	-
						18 - 18.5	6	0.01 [b] (0.008 – 0.014)	-	-

a-b: Significant differences ($p < 0.05$) between groups of the same tissue and species.

For *R. longurio*, $\Delta\delta^{15}N$ values of muscle and liver of the longest embryos were statistically higher than the smallest embryos ($p < 0.05$; Fig. 4.1a and 4.1b). For liver, medium size embryos were statistically higher than the smallest embryos ($p < 0.05$; Fig 4.1b). For BMF muscle values, the longest *R. longurio* embryos had significantly higher values than the smallest embryos ($p < 0.05$; Fig. 4.1a). For liver of *R. longurio*, BMF values are statistically higher in the smallest embryos than the longest ($p < 0.05$; Fig. 4.1b). Se:Hg molar ratio for *R. longurio* embryos in muscle was 246.45:1 and in liver 679.47:1. For *P. glaucostigmus*, $\Delta\delta^{15}N$ values of muscle showed a significant positive correlation as the embryo is longer ($r = 0.690$, $p < 0.05$). BMF muscle values of *P. glaucostigmus* were significantly higher in the smallest embryos than the longest ($p < 0.05$; Fig. 4.2).

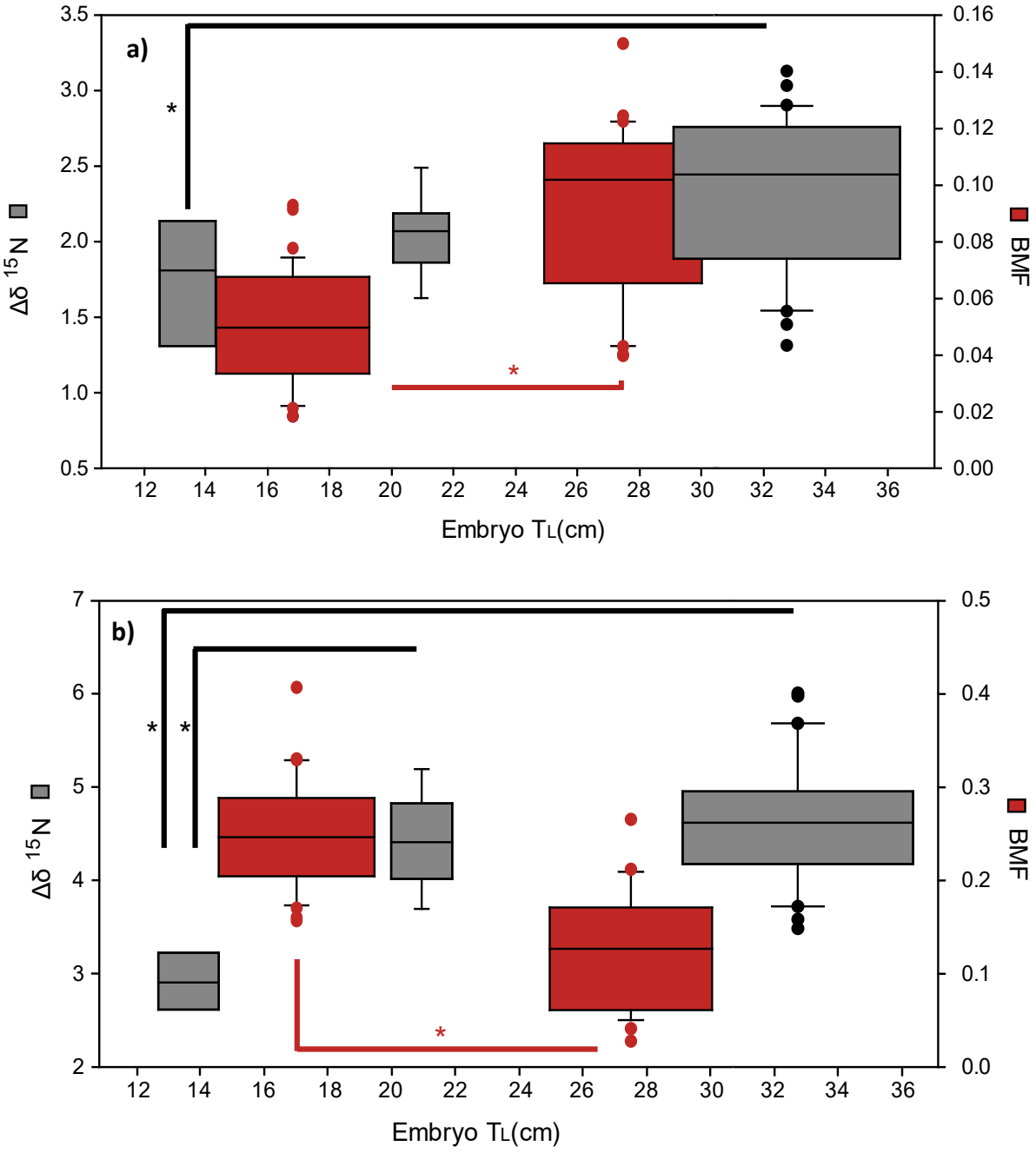


Figure 4.1. Boxplot for $\Delta\delta^{15}N$ (grey) and BMF (red) values ordered by embryo total length (L_T) for (a) muscle and (b) liver of the Pacific sharpnose shark *Rhizorpionodon longurio*. Horizontal bar = median; box = 25th to 75th percentile, whiskers = 10th and 90th percentiles and points = outliers. * $p < 0.05$.

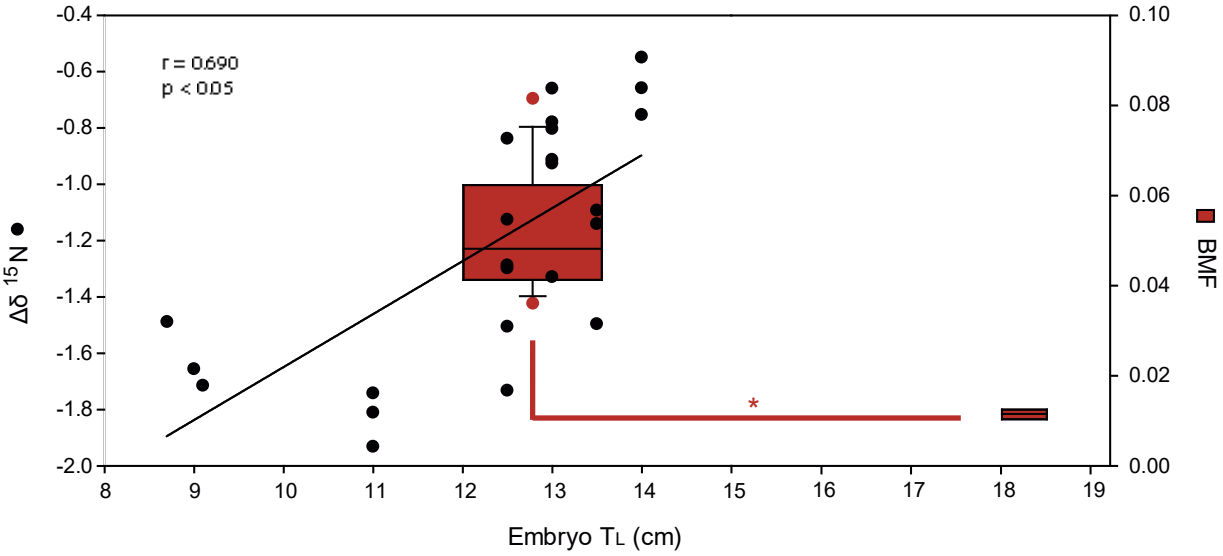


Figure 4.2. Linear regression for $\Delta\delta^{15}N$ (dots) and boxplot for BMF (red) values ordered by embryo total length (L_T) for muscle of the speckled guitarfish *Pseudobatos glaucostigmus* Horizontal bar = median; box = 25th to 75th percentile, whiskers = 10th and 90th percentiles and points = outliers. * $p < 0.05$.

4.5 DISCUSION

For *R. longurio*, while $\Delta\delta^{15}N$ values were higher as embryos increased their size for both tissues, BMF tendency varied on each tissue. For muscle, the BMF was higher in the longest embryos than the shortest ones and, in liver, the BMF was lower in the longest embryos than the shortest ones. For both tissues, the BMF was lower than 1, meaning that Hg is biodiluted (Sun *et al.*, 2020), despite $\Delta\delta^{15}N$ values representing more than a trophic level higher than the pregnant female. Although $\delta^{15}N$ values of embryos, compared with the pregnant female, are similar to what can be seen between predator – prey interactions, Hg did not biomagnify as would be expected, considering nitrogen values (Ikemoto *et al.*, 2008; Murillo-Cisneros *et al.*, 2019b; Sun *et al.*, 2020). Hg is hardly eliminated from the organism, which makes it prone to biomagnify with the increase of trophic levels (Sun *et al.*, 2020). Differences in BMF between tissues could be due to its physiological functions. Main liver functions are to remove, metabolise and excrete compounds. This organ is more efficient with demethylation of $MeHg^+$ processes and transform inorganic Hg to

mercuric selenide (Storelli & Marcotrigiano, 2002). On the other hand, muscle is known to bioaccumulate and biomagnify $MeHg^{+}$. Therefore, those differences between tissues in BMF tendencies could be due to liver being more efficient in removing and excreting Hg as the embryo is developing, and muscle is accumulating Hg as embryos are larger. Our results showed that embryos might have ratios of Se:Hg that far exceed from 1:1. Selenium is known to have the capacity to counteract Hg toxicity, as it forms HgSe and precipitates (Ralston *et al.*, 2008; Gerson *et al.*, 2020). Se:Hg ratios above 1 will protect Hg from adverse effects (Burger & Gochfeld, 2011). Thus, *R. longurio* embryos might be highly protected against Hg toxicity as Se is found in high quantities proportionally to Hg concentrations.

For *P. glaucostigmus*, embryos $\delta^{15}N$ values were lower than the pregnant female, although $\delta^{15}N$ differences reduced as embryos grew, showing a significant positive relationship. However, the BMF showed the opposite tendency; embryos reduced their [THg] compared to the pregnant female. Hg concentration being diluted as embryo got larger was been seen in *Squalus megalops*, which is a yolk-sac elasmobranch (Le Bourg *et al.*, 2014). This dilution is also attributed to the absence of supplementary maternal transfer of nutrients during development, as all come from the yolk sac (Le Bourg *et al.*, 2014). Hg may be assimilated early in gestation, and as the embryos get larger, their mass increase but not the amount of Hg, which is diluted in the body.

4.6 CONCLUSION

The present results show that although the placental shark present $\delta^{15}N$ embryo values as high as one trophic level of difference, [THg] do not show the BMF values expected according to $\delta^{15}N$ values of previous biomagnification studies of Hg. While $\Delta\delta^{15}N$ values increased as embryos were larger, BMF values varied. The tendency of the BMF values in muscle of *R. longurio* was to increase as embryos were longer, instead BMF values of liver tissue of *R. longurio* and muscle of *P. glaucostigmus* decreased. *R. longurio* embryos seem to be highly protected against Hg toxicity, as Se:Hg molar ratios are far above 1. More studies are needed as

embryo tendencies are not the same as adult elasmobranchs, so it is not plausible to extrapolate information from $\delta^{15}N$ values to know BMF values from previous studies of born elasmobranch.

CHAPTER 5. MATERNAL TRANSFER OF MAJOR AND TRACE ELEMENTS IN *RHIZOPRIONODON LONGURIO* FROM THE GULF OF CALIFORNIA IN BAJA CALIFORNIA SUR

5.1 ABSTACT

Major and trace elements (essential and non-essential) are transferred during gestation to embryos. This study aimed to determine and compare the concentrations of thirteen essential elements (K, S, P, Na, Ca, Mg, Fe, Zn, Se, Cu, Mn, Cr and Co) and eleven non-essential elements (As, Sr, Cd, V, Li, U, Tl, Ag, Sn, Sb and Pb) in the muscle and liver of a pregnant female and their respective embryos of the Pacific sharpnose shark *Rhizoprionodon longurio.* Except for tin (Sn), antimony (Sb), and lead (Pb) which were below the detection limit, all other elements were found in the muscle and the liver of embryo tissues in measurable amounts. All major elements analyzed had significantly higher concentrations in embryos liver than the pregnant female, except for magnesium (Mg). Higher concentrations in embryo tissues than the pregnant female tissues were found for the non-essential trace elements of strontium (Sr), lithium (Li), thallium (Tl) and silver (Ag). More studies need to be done focusing on which concentrations of non-essential elements can cause damage to the offspring and how the reproductive strategy and nutrition type during embryo gestation influences the maternal transfer process.

5.2 INTRODUCTION

All aquatic animals require essential elements for normal metabolic and physiological functions (Halver & Hardy, 2002). Also, top predators as elasmobranchs, due to their position at the trophic level, exhibit high tissue concentrations of non-essential elements, which mainly come via dietary uptake (Mathews & Fisher, 2009). During gestation, pregnant females transfer both classes of elements to their embryos. Essential elements are necessary for an optimal development and health of organisms, although a deficiency or excess of them can

cause negative effects in organism fitness (Fraga, 2005). Non – essential elements are not required for embryo growth and the transference of minimum concentrations can cause health problems (Bosch *et al.*, 2015).

Elasmobranchs have a wide variety of reproductive strategies, as placental viviparity, which depends on the species. Placental viviparity strategy is characteristic of around 30% of all shark species, which have several similarities with mammal reproduction (Haines *et al.*, 2006). In placental viviparous elasmobranch, embryos initially are dependent upon yolk. Once the yolk sac is depleted it is implanted into the uterine wall to form the placenta that will facilitate nutrient exchange between the pregnant female and their embryos until parturition (Haines *et al.*, 2006).

Just four studies have investigated the maternal transference of more elements than Hg in elasmobranchs. These performed in *Rhizoprionodon longurio*, *Narcine brasilensis, Mustelus higmani* and *Alopias vulipnus* species (Frías-Espericueta *et al.*, 2014; Dutton & Venuti, 2019; Lopes *et al.*, 2019; Souza-Araujo *et al.*, 2020). Transference of elements to embryos through the pregnant female has been demonstrated for several elements. Higher concentrations in embryo muscle than pregnant female muscle has been found of essential elements as copper (Cu), iron (Fe), manganese (Mn), selenium (Se) and zinc (Zn) in elasmobranchs. Moreover, besides Hg, other non-essential trace elements, such as cadmium (Cd), lead (Pb), silver (Ag), thallium (Tl) and uranium (U), have been observed in maternal transfer (Frías-Espericueta *et al.*, 2014; Dutton & Venuti, 2019; Lopes *et al.*, 2019; Souza-Araujo *et al.*, 2020).

The Pacific sharpnose shark is a small coastal shark that inhabits sandy and muddy bottoms (Smith *et al.*, 2009). It feeds on different kind of prey as cephalopods, fish and crustaceans (Márquez-Farias *et al.*, 2005; Osuna-Peralta *et al.*, 2014). Females reach the maturity size at 93 cm and litters are up to 12 pups (Márquez-Farias *et al.*, 2005; Corro Espinoza, 2011). The Pacific sharpnose shark has a placental viviparous reproductive strategy with an annual reproductive cycle, with

embryos fully developed and a total length (T_L) around 31 – 39 cm at parturition time (Corro Espinoza, 2011).

This study aimed to determine and compare the concentrations of 13 essential elements (potassium (K), sulfur (S), phosphorus (P), sodium (Na), calcium (Ca), magnesium (Mg), Fe, Zn, Se, Cu, Mn, chromium (Cr), cobalt (Co)) and eleven non-essential elements (arsenic (As), strontium (Sr), Cd, vanadium (V), lithium (Li), U, Tl, Ag, tin (Sn), antimony (Sb), Pb) in muscle and liver of a pregnant female and their respective embryos of *R. longurio,* which were in an early stage of development, still feeding on yolk. This investigation analyzes the transference of several elements as K, S, P, Na, Ca (essential) Sr, Li, Sn and Sb (non-essential) that had never been studied before in elasmobranchs. Moreover, inferences were made on possible processes according to different element ratios.

5.3 MATERIALS AND METHODS

5.3.1. Sample collection

A pregnant female of *R. longurio* and their respective embryos (n= 10) were collected from artisanal fishery captured using gill nets. *S*pecimens were collected in San Bruno fishing camp (27° 10’ 13” N; 112° 10’ 02” W) on the 26th of October 2017. T_L for each individual and sex (male and female) for each embryo was defined. Samples of muscle and liver from the pregnant female and whole embryos were collected and placed in plastic bags, kept on ice in coolers and transported to the laboratory at Centro Interdisciplinario de Ciencias Marinas del Instituto Politécnico Nacional (CICIMAR-IPN, La Paz, BCS, Mexico) where were stored frozen (-20º). In the laboratory, muscle and liver of the pregnant female were sub-sampled (range 0.2 – 20 g) on a HNO_3 (5%) acid-washed surface and stainless steel scalpel and stored at -20 °C in acid-washed plastic containers. For each embryo, we sampled muscle and liver. Samples were freeze-dried (Telstar, LyoQuest - 85) for 48 h and homogenized using an agata mortar. The weight of each sample before and after freeze-drying was determined to calculate the percent water. Moisture contents for

the pregnant female sample were 80.77% in muscle and 57.64% in liver. In embryos samples, mean ± standard deviation (s.d) moisture contents were 63.3 ± 0.8% for muscle and 76.51 ± 2.69% in liver.

5.3.2. Sample analysis

Digestion of the samples and ICP-MS analysis were performed in the Instituto de Investigacións Mariñas (Vigo, Spain; IIM-CSIC). For digestion, were weighted subsamples between 0.025 – 0.8 g in a Denver Instrument TP-214 scale with a precision of 0.1 mg. Pregnant female samples of muscle and liver were analyzed in triplicate and each embryo, due to the small amount of sample available, was just analyzed once. When coefficient of variation (CV) of pregnant female triplicates was higher than 20%, the outlier was removed to reach a lower CV. Subsamples were introduced in Teflon bombs, where were add 7 ml of HNO_3 (Hiperpur 69 % Panreac AppliChem, 721037.0012) and 3 ml of H_2O_2 (Hydrogen Peroxide for ultratrace analysis solution 1691-250 ml-F Sigma Aldrich). Sample digestion was done in a microwave oven Ethos Easy (Milestone) using Sk-15 program (from the general methods of the brand; Table 5.1). Accuracy of the analytical procedures was ensured using DORM-4 and DOLT-5 standard reference material (SRM), prepared by the National Research Council of Canada (NRCC). Digestion of the certified reference material was performed using the same procedure.

Table 5.1. Procedures used for digestion samples in a microwave oven Ethos Easy (Milestone) using Sk-15 program.

Step	T1 ºC	T2 ºC	Time min
1	110	60	5
2	160	90	10
3	200	110	10
4	200	110	15
5	0	0	

Once samples were digested, the resulting solutions were transferred to polypropylene centrifuge vials (Labcon MetalFree^{TM}) and were diluted with Mili-Q water until 50 mL. Elements were determined using an ICP-MS (inductively coupled plasma-mass spectrometry) using an Agilent 7900, equipped with an inert sample introduction system (nebulizer and PFA spray chamber, sapphire injector and platinum cones). Before analysis, equipment was optimized to obtain maximum sensibility without compromising the production of oxides and double charged species (formation of interfering species). Monitored isotopes for chemical elements determination were: ^{7}Li, ^{23}Na, ^{24}Mg, ^{31}P, ^{34}S, ^{39}K, ^{44}Ca, ^{51}V, ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{63}Cu, ^{66}Zn, ^{75}As, ^{78}Se, ^{88}Sr, ^{107}Ag, ^{111}Cd, ^{118}Sn, ^{121}Sb, ^{205}Tl, ^{208}Pb, ^{238}U. Moreover, germanium (^{72}Ge), rhodium (^{103}Rh) and iridium (^{193}Ir) were added as internal standard to correct the drift of the instrument sensitivity during the determination process. Each chemical element concentration was carried out by external calibration. Isotopes below an atomic mass of 80, which are more susceptible to interferences generated by the sample matrix, were analyzed using the collision cell with the addition of helium (4.3 mL/ min) which minimize such interferences. Results were calculated as µg/ g dry weight (d.w.). Percent recoveries of SRM were within 85 – 115 %, except for Li, U and Sn (Table 5.2). Wet weight (w.w) results were calculated to be contrasted with international standards stablished for different areas.

5.3.3. Statistical analyses

Statistical analyses were carried out using R software (Version 4.0.3, R Core Team, 2020). Data distribution was verified through the Shapiro-Wilk test, which was not normally distributed and therefore non-parametric tests were used. Concentration values of one embryo muscle sample were removed for all statistics as was an outlier for Ca and Sr. High concentrations of those elements in a sample indicates salt contamination of marine water. Wilcoxon tests for one sample were used for comparison between pregnant females and embryos concentration of each element and tissue, and comparison between tissue embryo concentration of each

element. Differences were considered significant at $p < 0.05$. Ratios for each element and matched tissue were calculated by dividing: embryos tissue concentration / pregnant female concentration. Ratios higher than 1 mean that element concentration was higher in embryo tissue and less than 1 that element concentration was higher in the pregnant female tissue. Moreover, results in wet weight basis were contrasted with international standards.

Table 5.2. Certified, observed values and recoveries for DORM-4 and DOLT-5 determined by an ICP-MS.

Element	DORM - 4			DOLT - 5		
	Certified value (µg/g)	**Observed value (µg/g)**	**Recovery (%)**	**Certified value (µg/g)**	**Observed value (µg/g)**	**Recovery (%)**
Essential						
K	15000	13010 ± 940	86.73	14400	15610 ± 890	108.40
S	-	9405 ± 260	-	-	19380 ± 725	-
P	8000	8160 ± 220	102.00	11500	12030 ± 390	104.61
Na	14000	13400 ± 400	95.71	9900	9760 ± 470	98.59
Mg	910	855 ± 25	93.96	940	870 ± 40	92.55
Ca	2360	2260 ± 34	95.76	550	560 ± 21	101.82
Fe	341.0	327 ± 12	95.89	1070	1056 ± 27	98.69
Zn	51.60	50.5 ± 1.5	97.87	105.3	107 ± 2	101.61
Se	3.56	4.08 ± 0.17	114.61	8.3	8.79 ± 0.06	105.90
Cr	1.87	1.79 ± 0.16	95.72	2.35	2.04 ± 0.02	86.81
Mn	3.1700	2.95 ± 0.13	93.06	8.91	8.72 ± 0.24	97.87
Cu	15.90	14.4 ± 0.6	90.57	35	33 ± 0.8	94.29
Co	0.25	0.242 ± 0.007	96.80	0.267	0.237 ± 0.008	88.76
Non-essential						
As	6.80	6.87 ± 0.3	101.03	34.6	35.1 ± 0.6	101.45
Sr	10.100	9.56 ± 0.39	94.65	3.73	3.89 ± 0.14	104.29
V	1.57	1.62 ± 0.06	103.18	0.51	0.47 ± 0.01	92.16
Cd	0.306	0.316 ± 0.012	103.27	14.5	14.4 ± 0.2	99.31
Li	1.21	0.34 ± 0.06	28.10*	-	0.022 ± 0.005	-
U	0.05	0.061 ± 0.004	122.00*	0.082	0.082 ± 0.001	100
Tl	-	0.011 ± 0.003	-	0.013	0.013 ± 0.002	100
Ag	-	-	-	2.05	2.05 ± 0.06	100
Pb	0.416	0.38 ± 0.01	91.35	0.162	0.15 ± 0.01	92.59
Sb	-	0.007 ± 0.003	-	0.013	0.0125 ± 0.0001	96.15
Sn	0.056	0.084 ± 0.009	150.00*	0.069	0.086 ± 0.02	124.64*

*Recoveries outside the limits of 85-115%

5.4 RESULTS

T_L of the pregnant female sampled of *R. longurio* was 105 cm. First maturity size for females of *R. longurio* is set at 92 cm T_L (Corro Espinoza *et al.*, 2011) which point out it is not its first reproduction event. Embryos T_L sampled (n = 10) ranged between 14.5 - 16 cm and were still feeding on yolk. All elements analyzed were detected in pregnant female tissue and all were transferred to embryos tissues except for non-essential elements of Sn, Sb and Pb, which were BDL (below detection limit) for both embryo tissues (Table 5.3).

Significant different concentrations between muscle of the pregnant female and muscle of the embryo were found for all elements analyzed except for Ca, which concentrations were similar. K, S, P, Fe, Mg, Mn, Cr, Co, As, Cd, V and U elements had significant higher concentrations in muscle of the pregnant female than embryo muscle. Significant higher concentrations were found in the muscle of the embryos for Na, Se, Cu, Zn, Sr, Li Tl and Ag (Table 5.3; Fig 5.1). In the liver, except for Mg, Mn and Cr, all elements had statistically significant differences. K, S, P, Na, Ca, Fe, Co, Zn, Cu, Sr, Li and Tl were higher in embryo liver and Se, As, Cd, V and U in the pregnant female liver (Table 5.3; Fig 5.1). Comparations between muscle and liver concentrations of the embryo showed that all elements had significantly different concentration except for Ca, Se, Mn, Co, Sr and Cd (Table 5.3). Higher concentrations were found in embryo muscle for Na, S, P, K, Mg, Zn, Cr, Sr, Cd, V and U and in embryo liver for Fe, Cu, As, Li, Tl and Ag. Higher rates of transference were found in liver than muscle (Table 5.4). For Ag, Tl, Li, Ca, Na, Cu, Sr and Se element ratios were higher than 1 for both tissues. For P, K, S, Co, Zn, Fe and Mg transference was higher than 1 just in liver tissue. Cr, Mn, As, U, V and Cd were the elements less transferred to the offspring with ratios lower than 1 for both tissues. Finally, for Sn, Sb and Pb elements ratios could not be calculated as embryo tissues concentration were BDL. (Table 5.4).

According to the results of this study and the limit of different international standards, As was the trace element, which concentrations in muscle of the pregnant female, exceed all limits established (Table 5.5). Moreover, As muscle

concentrations of embryos already exceed the New Zealand limit. Concentrations of Se in embryo muscle also exceed the international standards set (Table 5.5). Muscle concentrations for the rest of trace elements with a limit stablished (not all trace elements have fixed limits) were below the international standards.

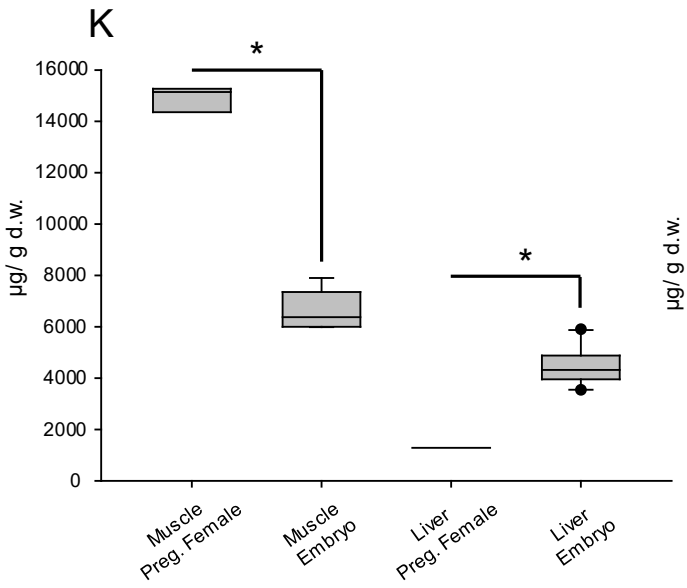
Table 5.3. Mean, median or range concentrations of essential and non-essential elements in muscle and liver tissues for a pregnant female of the Pacific sharpnose shark *Rhizoprionodon longurio* and their embryos. Range was used when triplicates of pregnant female tissue exceed 20% of CV (coefficient of variation) and the outlier was removed.

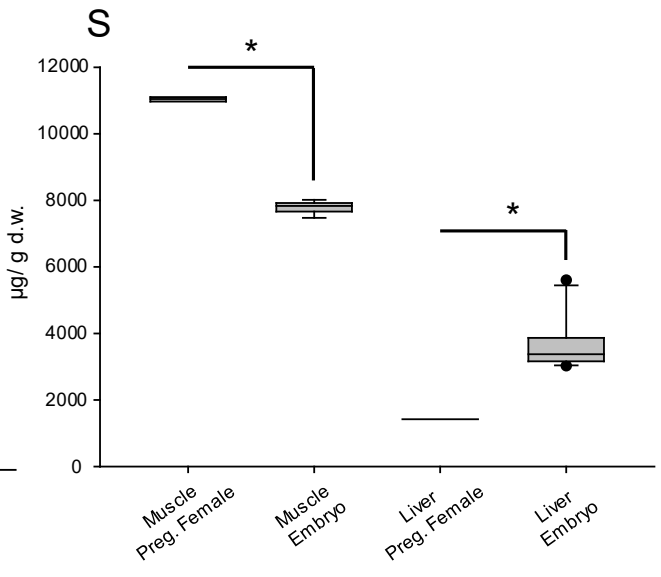
	Muscle				Liver			
	Pregnant female		Embryo		Pregnant female		Embryo	
Essential	**Mean**	**Median or range**	**Mean**	**Median**	**Mean**	**Median or range**	**Mean**	**Median**
K	14924 ± 495	15144	6623 ± 748	6675*	1283 ± 133	1189 - 1377	4466 ± 760	4319*†
S	11040 ± 68	11048	7795 ± 174	7832*	1422 ± 176	1298 - 1547	3629 ± 758	3375*†
P	9304 ± 208	9322	6792 ± 619	6891*	1337 ± 112	1257 - 1416	4683 ± 1008	4309*†
Na	4747 ± 160	4671	21365 ± 1727	21095*	1036 ± 80	979 - 1093	13598 ± 1798	13085*†
Mg	962 ± 105	976	312 ± 144	266*	69.2 ± 2.1	67.7 - 70.7	102 ± 94	85.5†
Ca	602 ± 60	617	633 ± 96.2	657	65.5 ± 5.5	68.3	743 ± 305	704*
Fe	112.9 ± 21.0	98.0 - 127.8	53.9 ± 10.8	53.7*	119.1 ± 11.1	111.2 - 127.0	232 ± 71	219.4*†
Zn	17.78 ± 3.27	15.47 - 20.09	25.79 ± 1.98	26.26*	9.18 ± 0.24	9.01 - 9.35	19.83 ± 4.68	18.82*†
Se	8.77 ± 1.32	7.84 - 9.70	27.79 ± 2.64	27.82*	16.43 ± 1.02	15.71 - 17.15	28.07 ± 6.11	27.93*
Cr	2.42 ± 0.014	2.42 - 2.43	0.21 ± 0.12	0.12*	0.11 ± 0.02	0.10 - 0.13	0.064 ± 0.054	0.04*†
Mn	1.23 ± 0.21	1.08 - 1.38	0.49 ± 0.14	0.46*	0.94 ± 0.16	0.83 - 1.06	0.54 ± 0.32	0.40*
Cu	1.04 ± 0.124	1.13 - 0.95	3.59 ± 0.32	3.46*	1.44 ± 0.11	1.36 - 1.52	17.90 ± 7.09	16.01*†
Co	0.065 ± 0.001	0.064 - 0.065	0.03 ± 0.003	0.03*	0.013 ± 0.001	0.012 - 0.014	0.03 ± 0.021	0.02*
Non-essential								
As	52.07 ± 0.49	51.91	11.27 ± 0.98	10.8*	32.32 ± 3.71	33.67	16.35 ± 1.83	16.41*†
Sr	2.77 ± 0.43	2.63	3.61 ± 0.39	3.63*	0.47 ± 0.07	0.43	3.37 ± 1.91	3.07*
V	0.107 ± 0.021	0.091 - 0.121	0.01 ± 0.003	0.01*	0.05 ± 0.005	0.045 - 0.053	0.004 ± 0.002	0.003*†
Cd	0.095 ± 0.001	0.094 - 0.096	0.03 ± 0.025	0.02*	1.42 ± 0.05	1.39 - 1.46	0.017 ± 0.008	0.01*
Li	0.009 ± 0.001	0.008 - 0.01	0.037 ± 0.005	0.036*	0.003 ± 0.0002	0.003 - 0.003	0.052 ± 0.007	0.05*†
U	0.005 ± 0.0002	0.005	0.001 ± 0.00003	0.001*	0.001 ± 0.00001	0.001 - 0.001	0.001 ± 0.0002	0.0005*†
Tl	0.002 ± 0.0004	0.002	0.005 ± 0.001	0.005*	0.0004 ± 0.00003	0.0004 - 0.004	0.014 ± 0.009	0.01*†
Ag	0.001 ± 0.00003	0.001	0.0014 ± 0.0004	0.001*	0.002 ± 0.0001	0.002 - 0.002	0.102 ± 0.03	0.11*†
Pb	0.177 ± 0.008	0.171 - 0.182	BDL	BDL	0.006 ± 0.001	0.006	BDL	BDL
Sb	0.019 ± 0.002	0.018 - 0.020	BDL	BDL	0.001 ± 0.0001	0.001 - 0.001	BDL	BDL
Sn	0.03 ± 0.002	0.029 - 0.031	BDL	BDL	0.013 ± 0.001	0.012 - 0.013	BDL	BDL

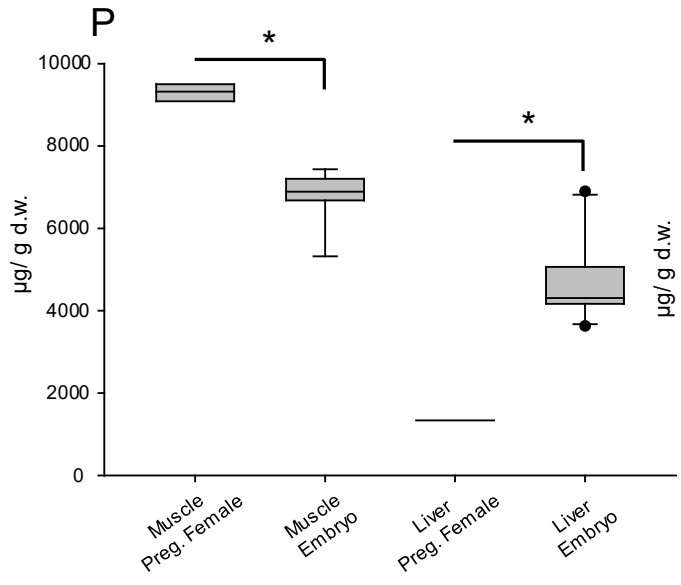
* Significant differences between median concentrations of the pregnant female and their embryos matched tissues.
† Significant differences between median concentrations of embryo paired tissues.

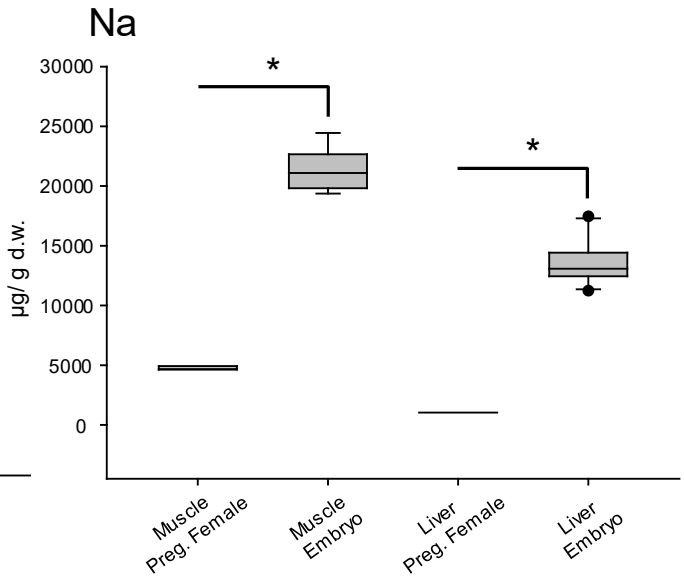
Table 5.4. Element ratio (embryos tissue concentration / pregnant female concentration) in muscle and liver tissues of the Pacific sharpnose shark *Rhizoprionodon longurio*.

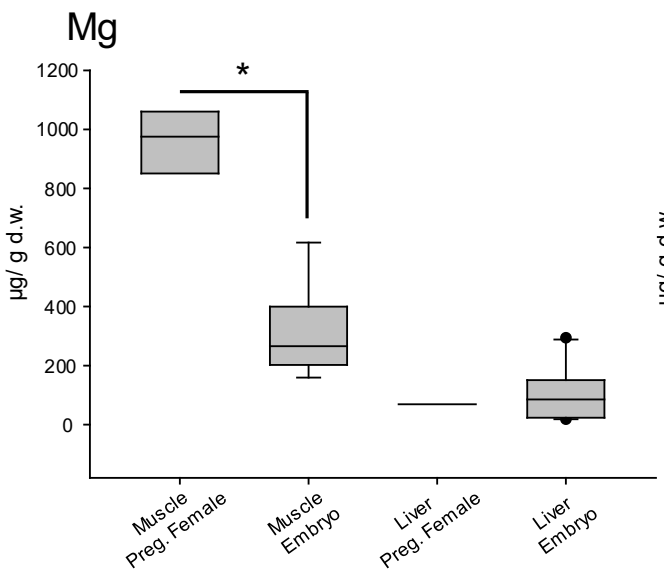
	Muscle	Liver
Ag	2.67	60.15
Tl	2.21	27.88
Li	3.59	13.39
Ca	1.05	11.35
Na	4.50	11.15
Cu	4.04	10.79
Sr	1.30	7.24
P	0.73	3.07
K	0.44	2.94
S	0.71	2.21
Co	0.48	1.96
Zn	0.99	1.89
Fe	0.56	1.73
Mg	0.32	1.65
Se	3.72	1.24
Cr	0.08	0.80
Mn	0.46	0.64
As	0.22	0.51
U	0.17	0.34
V	0.11	0.06
Cd	0.37	0.01
Sn	-	-
Sb	-	-
Pb	-	-

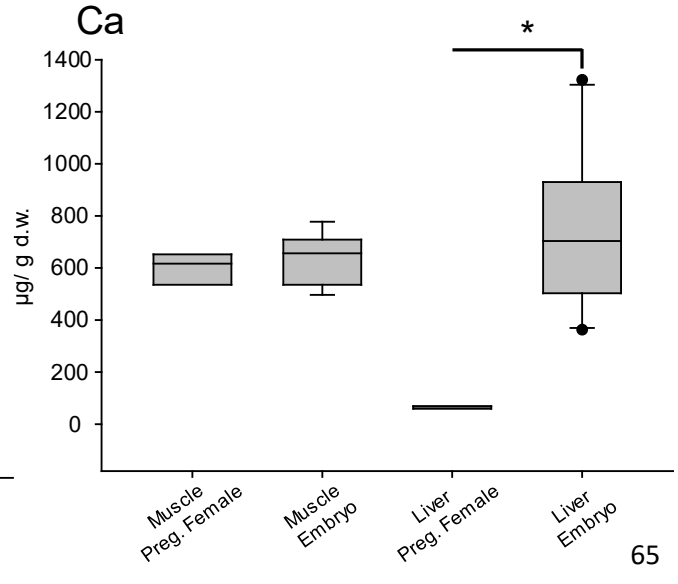

K
µg/ g d.w.
16000
14000
12000
10000
8000
6000
4000
2000
0
*
*
Muscle Preg. Female
Muscle Embryo
Liver Preg. Female
Liver Embryo

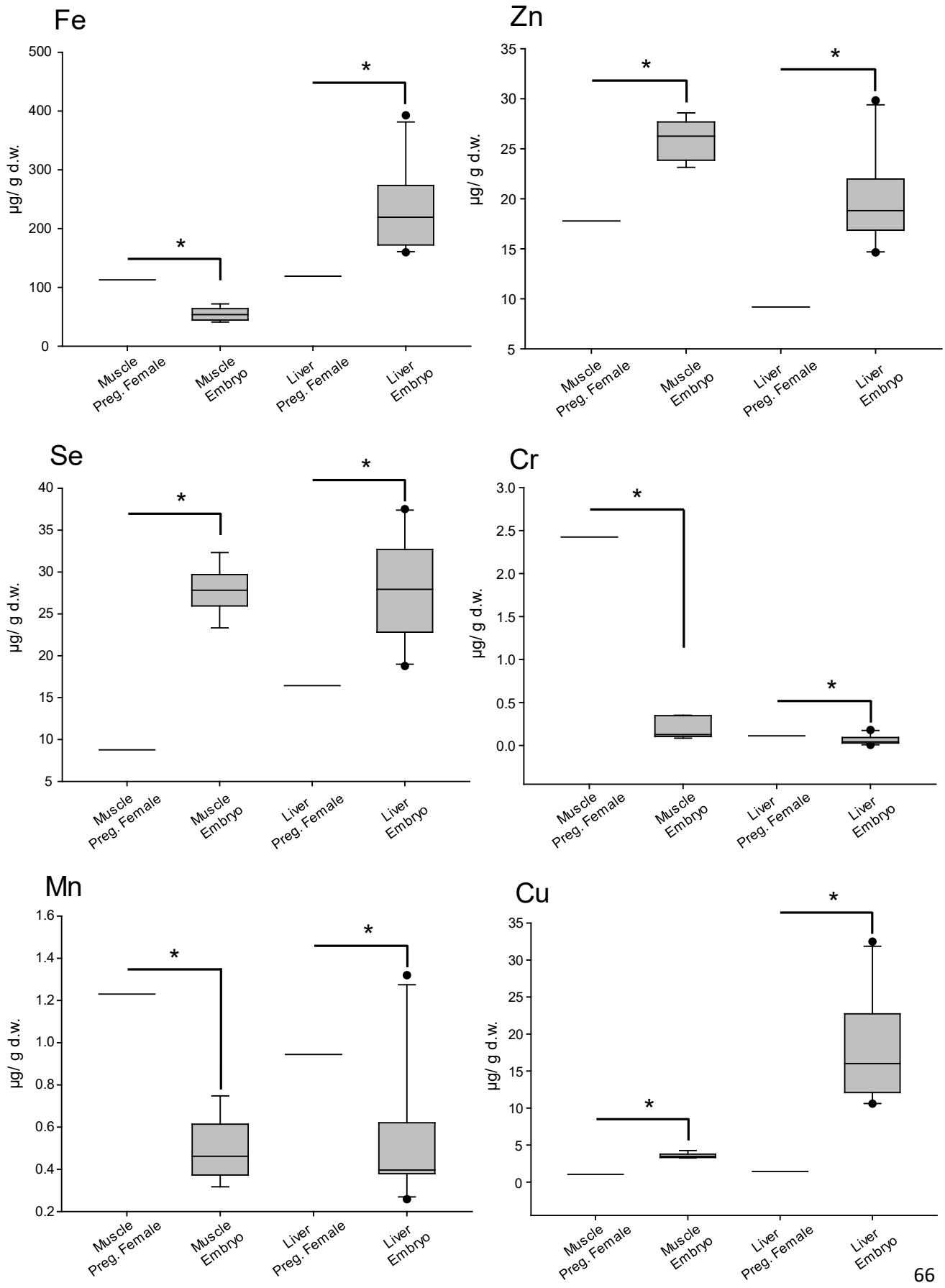

S
µg/ g d.w.
12000
10000
8000
6000
4000
2000
0
*
*
Muscle Preg. Female
Muscle Embryo
Liver Preg. Female
Liver Embryo

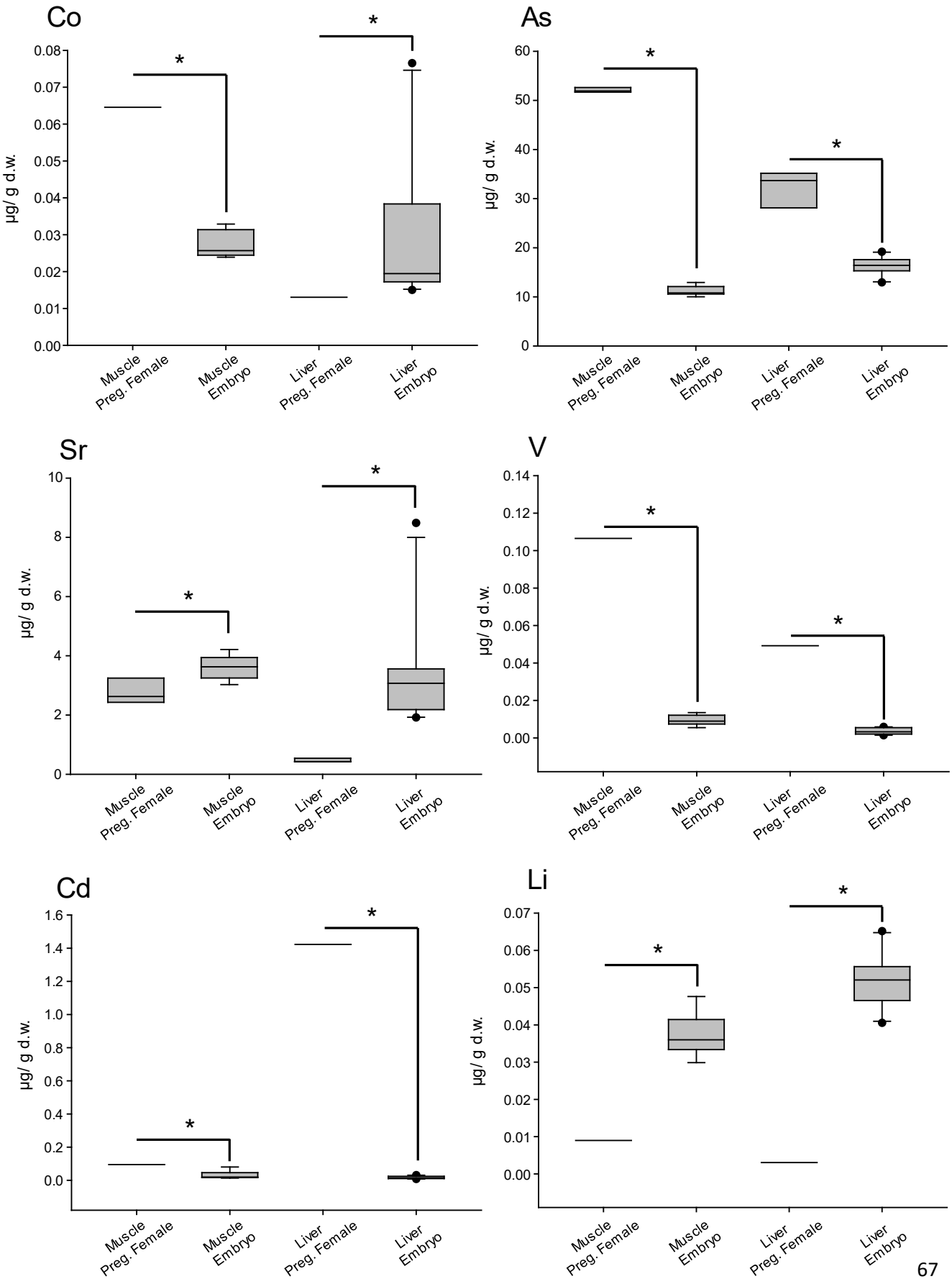

P
µg/ g d.w.
10000
8000
6000
4000
2000
0
*
*
Muscle Preg. Female
Muscle Embryo
Liver Preg. Female
Liver Embryo


Na
µg/ g d.w.
30000
25000
20000
15000
10000
5000
0
*
*
Muscle Preg. Female
Muscle Embryo
Liver Preg. Female
Liver Embryo


Mg
µg/ g d.w.
1200
1000
800
600
400
200
0
*
Muscle Preg. Female
Muscle Embryo
Liver Preg. Female
Liver Embryo


Ca
µg/ g d.w.
1400
1200
1000
800
600
400
200
0
*
Muscle Preg. Female
Muscle Embryo
Liver Preg. Female
Liver Embryo


Fe
µg/ g d.w.
500
400
300
200
100
0
*
*
Muscle Preg. Female
Muscle Embryo
Liver Preg. Female
Liver Embryo
Zn
µg/ g d.w.
35
30
25
20
15
10
5
*
*
Muscle Preg. Female
Muscle Embryo
Liver Preg. Female
Liver Embryo
Se
µg/ g d.w.
40
35
30
25
20
15
10
5
*
*
Muscle Preg. Female
Muscle Embryo
Liver Preg. Female
Liver Embryo
Cr
µg/ g d.w.
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Muscle Preg. Female
Muscle Embryo
Liver Preg. Female
Liver Embryo
Mn
µg/ g d.w.
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
*
*
Muscle Preg. Female
Muscle Embryo
Liver Preg. Female
Liver Embryo
Cu
µg/ g d.w.
35
30
25
20
15
10
5
0
*
*
Muscle Preg. Female
Muscle Embryo
Liver Preg. Female
Liver Embryo


Co
As
Sr
V
Cd
Li
µg/ g d.w.
Muscle Preg. Female
Muscle Embryo
Liver Preg. Female
Liver Embryo
*

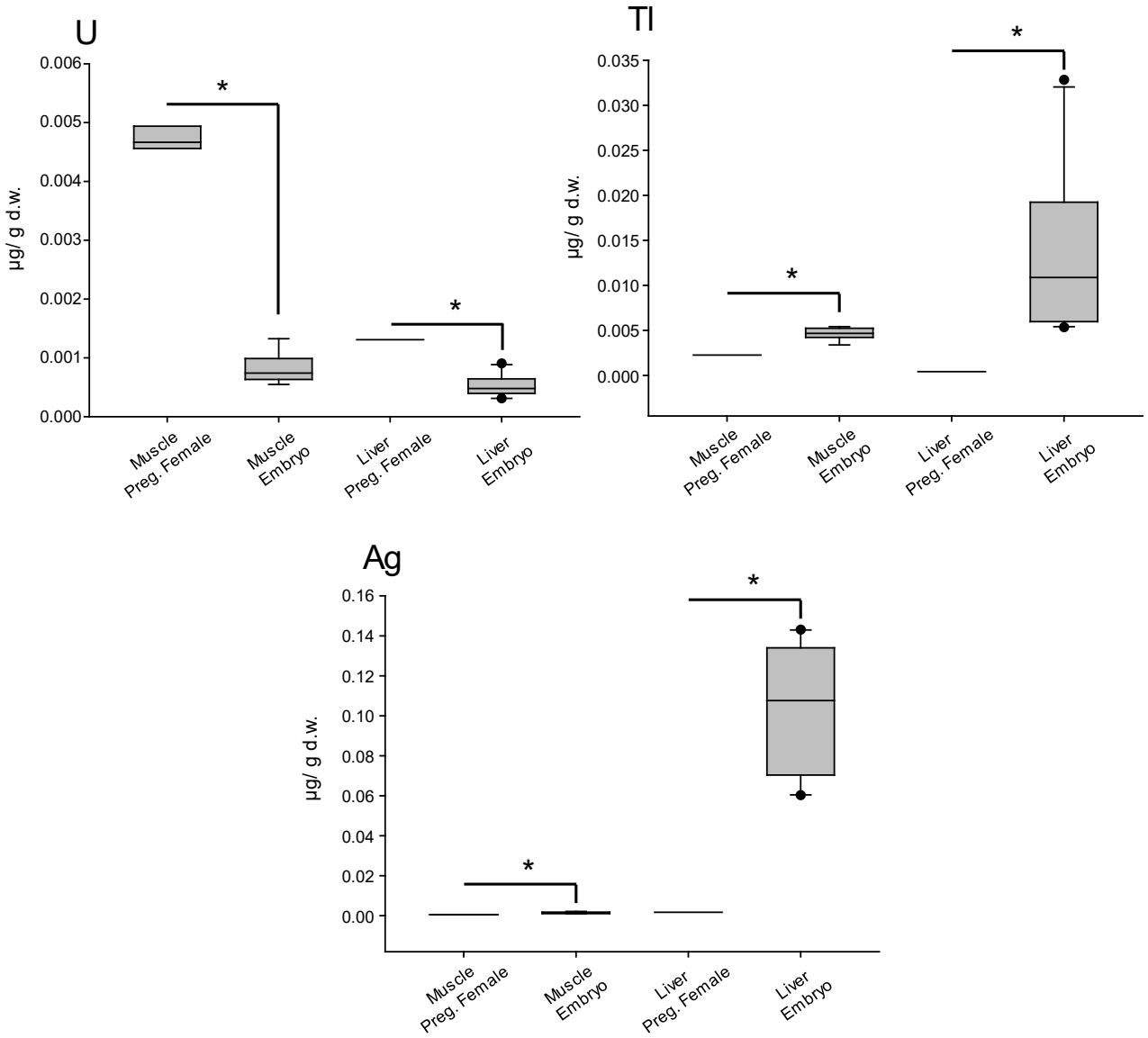


Figure 5.1. Boxplots for the pregnant female and embryo tissues element concentrations (µg/g d.w.). Horizontal bar = median; box = 25th to 75th percentile, whiskers = 10th and 90th percentiles and points = outliers. * Significant differences between pregnant female and embryo matched tissues ($p < 0.05$).

Table 5.5. Median concentration for the pregnant female and their embryos of this study and limit concentrations set for certain elements in different areas of the world.

Trace element	Present study (Median)		Limit	Standard	Area	Reference
	Pregnant female	Embryo				
Zn	3.86	3.5	40	FAO	New Zealand	(Nauen, 1983)
			100	FAO	Zambia	(Nauen, 1983)
			150	FAO	Australia	(Nauen, 1983)
Se	1.50	3.7	1	FAO	Australia	(Nauen, 1983)
			2	FAO	New Zealand	(Nauen, 1983)
Cr	0.47	0.03	1	FAO	Hong Kong	(Nauen, 1983)
Cu	0.18	0.48	10	FAO	Australia	(Nauen, 1983)
			30	FAO	New Zealand	(Nauen, 1983)
			100	FAO	Zambia	(Nauen, 1983)
As	9.98	1.6	1	FAO	New Zealand	(Nauen, 1983)
			3.5	FAO	Canada	(Nauen, 1983)
			3	FAO	Philippines	(Nauen, 1983)
			5	FAO	Zambia	(Nauen, 1983)
			5	FAO	Finland	(Nauen, 1983)
			6	FAO	Hong Kong	(Nauen, 1983)
V*	0.09	0.01	0.5	WHO		(Yazdanabad *et al.*, 2014)
Cd	0.02	0.003	0.05	FAO	Netherlands	(Nauen, 1983)
			0.25	Commission regulation	UE	(Commission of the European Communities, 2006)
			0.2	FAO	Australia	(Nauen, 1983)
			0.5	Norma Oficial Mexicana	Mexico	(NOM-242-SSA1-2009, 2011)
			1	FAO	New Zealand	(Nauen, 1983)
			2	FAO	Hong Kong	(Nauen, 1983)
Pb	0.03	-	0.2	Codex alimentarius	Slovak Republic	(Andreji *et al.*, 2006)
			0.3	Commission regulation	UE	(Commission of the European Communities, 2008)
			0.5	Norma Oficial Mexicana	Mexico	(NOM-242-SSA1-2009, 2011)

			0.5	FAO	Canada	(Nauen, 1983)
			0.5	FAO	Netherlands	(Nauen, 1983)
			0.5	FAO	Philippines	(Nauen, 1983)
			1	FAO	Sweden	(Nauen, 1983)
			1.5	FAO	Australia	(Nauen, 1983)
			2	FAO	United Kingdom	(Nauen, 1983)
			2	FAO	New Zealand	(Nauen, 1983)
			6	FAO	Hong Kong	(Nauen, 1983)
			10	FAO	Zambia	(Nauen, 1983)
Sb	0.003	-	1	FAO	Hong Kong	(Nauen, 1983)
			1	FAO	New Zealand	(Nauen, 1983)
			1.5	FAO	Australia	(Nauen, 1983)
Sn	0.006	-	150	FAO	New Zealand	(Nauen, 1983)
			200	FAO	Philippines	(Nauen, 1983)
			230	FAO	Hong Kong	(Nauen, 1983)
			250	FAO	Sweden	(Nauen, 1983)

*All concentrations and limits are in µg/g w.w. except for V which are in µg/g d.w.

5.5 DISCUSSION

Biological factors (reproductivity strategy, time of gestation, number of pups, tissue, etc.) and element properties (species, solubility, lipophilicity, polarity, etc.) can affect maternal transference of elements in marine animals. Those drivers influence the element concentrations in embryos during gestation among tissues, individuals and the species (Lahaye *et al.*, 2007; Lopes *et al.*, 2019; Guzman *et al.*, 2020; Baró-Camarasa *et al.*, Unpublished data) .

Embryos, as living organisms, are expected to contain all chemical elements in different concentrations. In this type of approach, it is important to consider the composition of chemical elements in animal life, but also the comparison between concentrations found in the pregnant female and its litter. Our study found that all elements were shared between females and its offspring but with wide differences between chemical elements. The highest concentrations in the pregnant female and embryos were in the macroelements K, S, P, Na, Ca and Mg, which are needed in the body in large quantities. All major elements analyzed had significantly higher concentrations in embryos liver than the pregnant female, except for Mg, which embryo liver concentration was higher but not significantly different from the pregnant female. An optimal transfer of these elements is necessary for the best development of the embryo, so each element can fulfill its function. K and Na are important elements for the organism as one of their roles is to maintain the osmotic pressure of the cell (Cîrtîna & Capatîna, 2018). The main constituent of elasmobranchs skeleton is cartilage and not bone as mammals, so high concentrations of S are expected to be transferred to the embryo as an essential component of cartilage is chondroitin sulfate (Sim *et al.*, 2007), which will be needed during embryo development. P is a macroelement that is fundamental to be transferred during offspring development due to its important role in the structure of adenosine mono-, di- and triphosphate acid (AMP, ADP and ATP) and is an universal source of energy in biological successes (Cîrtîna & Capatîna, 2018). Higher Mg concentrations were found in muscle of both stages analyzed than in liver. Transference of Mg during gestation is important, since a deficiency of this trace element increases neonatal mortality and morbidity, as observed in rodents (Almonte

et al., 1999). Higher Ca concentrations were found in embryos tissues relative to pregnant female tissues. This element during gestation might be used for tooth formation in the embryos, as around 30% of its composition is Ca (Enax *et al.*, 2012), as well as in calcifications spots from the cartilage in the vertebral column and jaws (Moss, 1977).

All essential trace elements were transferred to the embryo in the early stage of gestation, when yolk is its only nutritional input. The presence of Fe is important in both stages as it transports oxygen from tissues to cellular respiration (Srai *et al.*, 2002). Similar results to this study were find for Fe concentrations in Souza-Araujo *et al.* (2020), where liver embryo had higher concentrations than muscle embryo. Higher concentrations in liver are expected as is a major site of iron storage and for heme synthesis within the body (Bonkovsky, 1991). Higher concentrations of Zn were found in embryos than in mother tissues despite the presence of Cd. Cadmium induces the synthesis of metallothionein (MT) in the placenta for its retention and to avoid its transference (Frías-Espericueta *et al.*, 2014). High concentrations of MT increase the formation of Zn – MT complexes which decrease the Zn available to be transferred to the embryo (Espart *et al.*, 2018). However, higher Zn concentrations in embryos seems to indicate that the presence of Cd in maternal tissues did not affect substantially the transference of Zn to its offspring. Higher concentration of Se in pregnant female liver of elasmobranchs (Lopes *et al.*, 2019; Souza-Araujo *et al.*, 2020; current work), high concentration in yolk (Lopes *et al.*, 2019) and higher concentration of Se in embryo tissues than pregnant female in embryos feeding on yolk evidence a Se transference during vitellogenin synthesis for yolk formation and during embryo development when they are feeding on yolk (Lopes *et al.*, 2019; Souza-Araujo *et al.*, 2020; current work). Cr was barely transferred to embryo tissues compared to the pregnant female concentrations. This element can be found mainly in two forms in the organism: Cr(III) and Cr(IV) (Bradl, 2005). Cr(III) specie is an essential form that is found mainly in the liver and spleen, while Cr(IV) is a toxic form mainly found in the kidney (Ducros, 1992). Unfortunately, this work only measured total Cr, and not its form. Therefore, it cannot be established if the concentrations measured in both tissues favor embryo development or if it is a toxic form that can cause adverse effects. Mn was transferred

to embryos during gestation although tissue concentrations were not as high as the pregnant female. Transference of Mn during organogenesis is essential as this trace element plays a key role in the formation of chondroitin sulfate, an important cartilage component (Hostetler *et al.*, 2003; Sim *et al.*, 2007). In this work, Cu concentrations in embryo tissues were higher than paired pregnant female tissues, evidencing the transfer of this essential trace element during gestation, while yolk is its main nutritional input. Other studies in elasmobranch (Frías-Espericueta *et al.*, 2014; Lopes *et al.*, 2019) and marine mammals (Wagemann *et al.*, 1988; Gerpe *et al.*, 2002) found a similar tendency in maternal transfer of Cu, where concentrations in embryo tissues were higher than pregnant female and liver of embryo had a higher concentration than muscle of embryo. Co was the essential trace element with lower concentrations. Higher concentrations of Co were found in muscle than liver in both stages. These results do not agree with *Narcine brasilensis* and *Mustelus higmani* studies, where Co concentrations were higher in liver embryo than muscle (Lopes *et al.*, 2019; Souza-Araujo *et al.*, 2020). The ability of Co to cross the placenta (Ziaee *et al.*, 2007) seems to indicate that Co concentrations will be higher as the *R. longurio* embryos grow.

Maternal transfer of non-essential elements is known to be a detoxicant way for the pregnant female, where embryo works as a sink (Brookens *et al.*, 2008; Guirlet *et al.*, 2008). Three (Sb, Sb and Pb) of the eleven non-essential elements analyzed in this study had measurable concentrations in pregnant female tissues and concentrations BDL in embryo tissues. This result indicates that transference of those trace elements was found in such minimum amounts that the equipment used for this investigation could not evaluate this process. In a previous study, yolk concentrations of Pb in *N. brasilensis* embryos had really low concentrations compared to those of muscle and liver tissues of the pregnant female (0.002 µg/g w.w. in embryo yolk, 0.03 µg/g w.w. in muscle and 0.064 µg/g w.w. in liver of the pregnant females; Lopes *et al.*, 2019), which explains the low amount of Pb transferred to the embryos of this investigation, as they are feeding on yolk. Maternal transfer of Pb in elasmobranch was corroborated in *R. longurio* in muscle and liver embryos, when its nutrition is through the placental (Frías-Espericueta *et al.*, 2014). This element (Pb) can cross the placental barrier (Caserta *et al.*, 2013), which points out that probably, when *R. longurio* embryos change the type

of nutrition supply and nutrients come directly from the mother through the umbilical cord, embryos Pb concentration in muscle and liver tissue would increase significantly. Although recoveries for both SRM were higher than 115% (Table 5.2). It seems that no quantification amounts of Sn were transferred to the embryo during the first gestation period; however, it is been demonstrated that organic compounds of Sn can cross the placental barrier and accumulate in large quantities in the placental and other fetal tissues in rats (Cooke *et al.*, 2008). When *R. longurio* embryos change their nutrition input, Sn may cross the placental barrier and accumulate in the organism, which can cause embryonic death or suppress embryonic growth (Ostrakhovitch, 2015). Although maternal transfer of Sb has been evidenced in elasmobranchs, while embryos are feeding on yolk, this process could not be validated in this work. A transplacental transfer of this element can occur in later gestation stages as this process has been demonstrated in rats (Miranda *et al.*, 2006).

Arsenic was the non-essential trace element with the highest concentrations in pregnant females and embryo tissues. Those high As concentrations in pregnant female tissues could be related in the study area where samples were taken is found near a mining region. Large amounts of mine-waste material could be seen near de coast during sampling, which can be transported to the sea due to rains or by wind (Shumilin *et al.*, 2013). A high As concentration in the pregnant female muscle could be related with hydrothermal activity close to the sample area too. It has been demonstrated that the extremely high concentrations of this trace element in Conception Bay waters, approximately 50 km south of San Bruno are due to the marine hydrothermal waters discharged at the bottom (Leal-Acosta *et al.*, 2013; Villanueva-Estrada *et al.*, 2013). It has been found that 90% of As found in the body of marine fish is arsenobetaine (Zhang *et al.*, 2016), a form of As that has low toxicity (Kaise *et al.*, 1985). Different As compounds were analyzed in *Larus crassirostris* eggs and found that the percentage of arsenobetaine was between 74.7 and 97.4% of total As (Kubota *et al.*, 2002). This result gives reason to believe that most of the As transferred to *R. longurio* embryos within this first gestation period (as yolk composition of both species could be similar) might be arsenobetaine, which would reduce its toxic effects in developing embryos. Sr was transferred to *R. longurio* embryos tissues, having higher

concentrations than tissues of the pregnant female. Sr has properties resembling to Ca and its presence in the organism can inhibit Ca uptake causing hypocalcemia (Chowdhury & Blust, 2011). Sr accompanies Ca across the placental barrier in fetus of rats and Sr content correlates with increasing fetal calcification (Ruhmann *et al.*, 1963). Cartilage mineralization in elasmobranch starts during gestation (Seidel *et al.*, 2016) and together with the capacity of Sr to cross the placental barrier, it is probably that Sr concentrations in embryo tissues will increase more during the later periods of *R. longurio* embryos gestation. The concentration of V was higher in the muscle of the pregnant female than the liver and was barely transferred to the tissues of the embryo. A low transference of V was found between the mallard ducks (*Anas platyrhynchos*) and their eggs (White & Dieter, 1978) similar than this study to *R. longurio* embryos. This can be explained because *R. longurio* embryos nutrition of this study is by yolk, similar as the main constituent of mallard duck eggs, which is synthetized through the same process of vitellogenesis. When embryo nutrition of *R. longurio* shifts, concentrations of V in embryo tissue might increase as this trace element has the property to cross the placenta (Domingo, 1996). Higher concentrations of Cd were found in the pregnant female tissues than *R. longurio* embryo tissues. This tendency was also found in a study realized to the *N. brasiliensis* ray, where yolk was analyzed for Cd finding really low concentrations in this tissue too (Lopes *et al.*, 2019). In a study conducted on hens found that although high concentrations of Cd were administrated to females, follicle yolks presented a minimum concentration of this element, as Cd-binding metallothioneins were biosynthesized in the follicle walls (Kojima *et al.*, 1991). Similar processes can occur in elasmobranch when yolk is synthesized, reducing its transference during the first period of gestation. Cd-binding metallothioneins are synthesized in the placenta too, which retain most of the amount of Cd and avoid to cross the placenta and to be accumulate in embryo tissues in large concentrations (Gundacker & Hengstschläger, 2012). Li concentrations values should be taken with caution as the recovery percentage of DORM-4 was low (Table 5.2). However, Li results exhibit a tendency where concentrations are higher in embryo tissues than the pregnant female matched tissues, which indicates a high transference of this trace element during this early period of gestation. It is been found that Li has the ability to

cross the placental barrier (Newport *et al.*, 2005), which can result in higher concentrations of this trace element in embryo tissues at the end of the gestation period. For U, although recovery of DORM-4 was slightly superior than 115% (122%; Table 5.2), DOLT-5 recovery was between the limits (85% - 115%), which indicates that U values are reliable. Higher concentration of U in liver tissues of embryos and pregnant females was found in *M. higmani* (Souza-Araujo *et al.*, 2020), opposite of this study which found higher concentrations of U in muscle of *R. longurio* organisms. Embryos tissues concentration of U were lower than the pregnant female concentration, meaning that maternal transference of U during the first period of gestation is taking place but not at high rates. Embryo tissue concentrations of U can increase during gestation as this trace element can cross the placenta barrier (Bertell, 2006). Tl concentrations were higher in embryo tissues than the matched tissue of the pregnant female. Concentrations of Tl in egg content of black-tailed gulls (Agusa *et al.*, 2005) can suggest an easy transfer of Tl to yolk during vitellogenesis, which can be related to high concentrations of this trace element in *R. longurio* embryos of this study, which are still feeding on yolk. The ability of Tl to cross the placenta (Hoffman, 2000) can result in higher concentrations in embryo tissues when nutrition strategy shifts during gestation. As seen in *N. brasilensis*, higher concentrations of Ag were found in embryos liver than in muscle (Lopes *et al.*, 2019). Ag concentration in liver of embryos far exceed Ag concentrations of both tissues of the pregnant female evidencing the sink function of embryos during pregnancy.

High varieties of ratios between elements (Table 5.4) show that maternal transference is not a process regulated by the same mechanism for all elements. Each element behaves distinct from each other as they do not share the same properties (Greenwood & Earnshaw, 1998). Higher ratios values were found in liver tissues than muscle for all elements except for Se, V and Cd (Table 5.4). This may be explained as liver is related to metabolism, storage and removal procedures of compounds (Hinton *et al.*, 2001). In liver, most of the essential elements were highly transferred to embryos (> 1), except for Cr and Mn. For non-essential elements, the two extreme scenarios were found; high ratios of transference, which show an easy maternal transfer of those elements to the offspring, and low ratios of transference (< 1), which suggest a maternal

mechanism to avoid their transference to the offspring during gestation. For muscle, ratios were lower than liver for most of the elements except for Se, V and Cd. High concentration of Se in muscle might be related in a reduction of $MeHg^{2+}$ toxicity in the tissue, however this study did not analyze Hg concentrations and this hypothesis cannot be corroborated. For both tissues three elements (Sn, Sb, Pb) were not transferred to the offspring, evidencing an efficient mechanism to prevent their transference and adverse effects during the first stage of gestation.

It is necessary to maintain the trace elements at acceptable levels in the interests of the public health (Commission of the European Communities, 2008). For that reason, countries stablish limits for different contaminants in different kinds of food. In this study, trace element concentrations in muscle tissue of the pregnant female, except for As, were below the limits of international standards (Table 5.5). High As concentrations in muscle can affect the health of the organism and their consumers, such as humans. Elevated concentrations of this trace elements in the pregnant female muscle could be related to a nearby hydrothermal activity (Leal-Acosta *et al.*, 2013; Villanueva-Estrada *et al.*, 2013). Although organisms were caught near a Cu mining area, Cu concentration in muscle of the pregnant female and its embryos were below the international standards. Low concentrations of Cu were found in the brown seaweed *Padina durvillaei* and the scorpionfish *Scorpaena mystes* of Santa Rosalía too (Rodríguez-Figueroa *et al.*, 2009; Piñón-Gimate *et al.*, 2020), although Cu concentrations in Santa Rosalía sediments were found to be enriched (Shumilin *et al.*, 2013). Low concentrations in the pregnant female studied could be due to the fact that Cu is not highly transmitted through the trophic levels, since organisms studied presented low concentrations compared to Santa Rosalía sediments or, since *R. longurio* is a migratory species (Márquez-Farias *et al.*, 2005), trace elements concentrations could be the reflection of a diet outside the Santa Rosalia area. Although essential trace elements are necessary for the performance of essential functions (Santamaria & Sulsky, 2010), they can be toxic if exceeded (Mertz, 1995). In this study, only Se concentrations in muscle embryos exceeded the limit set for this element. High maternal transference and concentrations of Se could be related in high demand during this early stage for an optimum organism development.

5.6 CONCLUSION

All essential elements analyzed in this study were transferred during gestation, which will ensure a correct development of the embryos as long as elements are not found in excess in the organism. Elements such as Sn, Sb and Pb were not detected in *R. longurio* embryos tissues, which suggests that those trace elements are barely transferred during the early stage of *R. longurio* embryo gestation. The remaining non-essential trace elements were transferred maternally to embryos, which plays a role as a sink for the pregnant female. Transfer of non-essential elements was especially efficient for Sr, Li, Tl and Ag, with concentrations that were higher in embryo tissues than the pregnant female. Liver presented higher element ratios than muscle except for Se, V and Cd. Exposure to non-essential elements during gestation in embryos should not be overlooked, as the transference beyond minimum concentrations could cause health problems.

6. GENERAL CONCLUSIONS

This dissertation presents evidence of maternal transfer of elements during gestation of both, essential and non-essential. Nitrogen fractionation and mercury transfer from pregnant females to embryos was studied for more than one species and variation between species was observed. Differences seem to be related to biological factors as the reproductive strategy, the nutrition type during the gestation period, the maternal tissue concentration or embryo total length. Moreover, maternal transfer of thirteen essential elements (K, S, P, Na, Ca, Mg, Fe, Zn, Se, Cu, Mn, Cr and Co) and eleven non-essential elements (As, Sr, Cd, V, Li, U, Tl, Ag, Sn, Sb and Pb) in *R. longurio* show different patterns of transference depending on the element; as Sn, Sb and Pb, which were not detected in embryos tissues or Sr, Li, Tl and Ag, which concentrations were higher in embryo tissues than the pregnant female. Maternal transference of essential elements is necessary for embryo development, instead transference of non-essential elements is a product of maternal detoxification and can cause health problems on the offspring.

7. RECOMMENDATIONS

It is necessary to perform more studies regarding the maternal transfer of elements in elasmobranch to know which are the most relevant factors that influence the concentration in embryo tissues. Those factors to be considered should include more reproductive strategies as histotrophy or oviparity; more tissues as blood, kidney, cartilage, histotroph (or uterine milk) or placenta; a wide range of embryos lengths and more species with different duration of the gestation period. Moreover, the existence of oxidative stress should be investigated, as well as micronucleus or malformations in embryos, which can be related to element concentration. Those correlations can give an idea of which elements can be the cause of health problems in embryos. Laboratory experiments would be complicated; however, with a controlled environment, element concentrations delimitating embryo damage thresholds could be established more accurately. Also, maternal transfer of organochlorine contaminants and microplastic should be further investigated.

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ANNEX I

Essential elements

- Calcium

Most of the calcium (Ca) found in the ocean is as a carbonate form (calcium carbonate). The ocean is not running out of Ca as it is continuously recycled; calcium carbonate dissolves when calcifying organisms die, its deposition forms calcareous deposits as evaporites or dolomites which can be worn and remobilized into the ocean also, hydrothermal fluid release Ca which reacts with seawater sulfate depositing anhydrite and forming the “chimney” (Libes, 2009). Ca concentrations in the upper continental crust is 29450 µg/g (Wedepohl, 1995) and in sea sediments is 29000 µg/g (Chester, 2000). Ca is basic for building strong bones, in muscle contraction, regulating heart beat and fluid balance within cells (Pravina *et al.*, 2013). Its deficiency can result in osteoporosis and weaking of bones (Pravina *et al.*, 2013) and an excess in the organism can lead to zinc deficiency through competitive exclusion (Cline, 2012).

- Chromium

Natural sources of chromium (Cr) in the marine environment are riverine and atmospheric input and mineral weathering processes. Discharges of tanning industry and textile dyeing are anthropological sources of Cr (Geisler & Schmidt, 1991). Cr concentration in the upper continental crust is 35 µg/g (Wedepohl, 1995). Concentration of Cr in marine sediments is 60 µg/g and mean ocean concentration is 5 nmol/L (Chester, 2000; Chester & Jickells, 2012). Trivalent Cr (Cr(III)) is considered an important nutrient for metabolize and stabilize glucose levels and helps the body lose fat and keep muscle (Geisler & Schmidt, 1991; Prasad, 2008). Deficiency of this element can alter glucose metabolism, cholesterol and protein synthesis and decrease in reproductive functions. An excess of this trace element can cause skin rashes. Hexavalent Cr (Cr(VI)) is a toxic substance carcinogenic for human and animals, as is a strong oxidant agent (Bradl, 2005; Prasad, 2008).

- Cobalt

Natural ocean sources of cobalt (Co) are rivers, coastal sediments and eolian and hydrothermal inputs (Bundy *et al.*, 2020). Emissions from coal burning, emissions from metal use, other combustion processes and fertilizers are different types of anthropological sources of this element (Merian, 1984). Co is found in the upper continental crust in concentrations of 11.6 µg/g (Wedepohl, 1995). Concentration of Co in marine sediments is 13 µg/g and mean ocean concentration is 0.02 nmol/L (Chester, 2000; Chester & Jickells, 2012). Co is an essential element for fatty acid and folate metabolism as is an integral part of vitamin B_{12}. A deficiency can cause anemia to the organism and an excess can cause cartiomyopathy, with damage to the heart muscle due to anoxia (Prasad, 2008).

- Copper

Copper (Cu) is emitted by coal combustion and metallurgical processing of iron, copper, and steel production. It is used as Cu fertilizers too (Bradl, 2005). Concentration of Cu in the upper continental crust is of 14.3 µg/g (Wedepohl, 1995). In the sea, Cu concentration in the sediment is 56 µg/g, 8.9 µg/g of particulate material and 3 nmol/L dissolved in water (Chester, 2000; Chester & Jickells, 2012). This element is crucial for the function of metalloproteins and enzymes and is important for regulate the gene expression. It is necessary for blood cell production, iron transport and bone strength. A deficiency of Cu can direct to a decrease in white blood cells, anemia, osteoporosis, and neurological diseases. An excess in the body can cause gastrointestinal problems (Prasad, 2008).

- Iron

Ocean sources of iron (Fe) can be coastal and shallow sediments, hydrothermal fluids and dust (Boyd & Ellwood, 2010). Fe^{2+} in oxic seawater, reacts with O_2 and iron oxyhydroxides are formed. Other trace metals and phosphate are likely to become incorporated into the oxyhydroxides, which will precipitate to the ocean floor (Libes, 2009). In the upper continental coast, Fe concentrations are 30890 µg/g (Wedepohl, 1995). In the sea, Fe concentration in sediment is 65000 µg/g and 0.5 nmol/L for the

dissolved form (Chester, 2000; Chester & Jickells, 2012). In the organism, Fe is a structural component in heme proteins like myoglobin and hemoglobin and for this reason, a deficiency leads to severe anemia. Instead, an excess can give diarrhea, gastrointestinal problems, vomiting and can be carcinogenic (Prasad, 2008).

- Magnesium

An important natural source of magnesium (Mg) into the ocean is the hydrothermal activity, which is a major net sink. Evaporites, a sedimentary rock, is also a significant sink of Mg. In sediments, calcium can be exchanged for Mg, which converts calcite into the mineral dolomite. This exchange have the capacity of remove a significant amount of Mg from seawater, which will decline the seawater ratio of Mg/Ca (Libes, 2009). The upper continental crust concentration of Mg is 13510 µg/g (Wedepohl, 1995). In the sea sediments, Mg concentration is 21000 µg/g and 53 mmol/L in the ocean water (Chester, 2000; Chester & Jickells, 2012). This essential nutrient is important to improve enzyme activity, helps converting blood sugar to energy, and for effective muscle and nerve function (Prasad, 2008). Mg is known to be an antagonist of Ca, competing for the same binding sites on plasma protein molecules (Jahnen-Dechent & Ketteler, 2012). A deficiency of Mg can cause muscle contractions, abnormal heart beat and high blood pressure (Prasad, 2008; Jahnen-Dechent & Ketteler, 2012). High concentrations of it can result in neuromuscular dysfunction, cardiac diseases and coma (Jahnen-Dechent & Ketteler, 2012).

- Manganese

Manganese (Mn) is an essential element which its main anthropogenic sources to the environment are mining, wastewater discharges and mineral processing (Howe *et al.*, 2004). The major natural source of Mn to the ocean are the hydrothermal emissions. Once in the water Mn^{2+} is transformed into manganese oxides, which will precipitate onto the sediments and be removed from seawater before it is transported into surface ocean water (Libes, 2009). Mn is found in the upper continental crust in concentrations of 527 µg/g (Wedepohl, 1995). In the sea, Mn is found in concentrations of 850 µg/g in sediments, 27 µg/g of oceanic total suspended material and its mean ocean concentration is 0.3 nmol/L (Chester, 2000; Chester & Jickells, 2012). It is essential for

body function and a normal development. Mn can cross the blood - brain barrier and an overexposure can result in neurodegenerative damage in brain that can develop into a parkinsonian syndrome. Moreover, a Mn excess can cause an increase of fetal abnormalities and a decrease of fertility (Crossgrove & Zheng, 2004).

- Phosphorus

Phosphorus (P) is a macronutrient which main ocean sources are via river run off and atmospheric (Libes, 2009; Ni *et al.*, 2015). The main anthropogenic inputs of P to the ocean via river run off are phosphorite mining, fertilize use and sewage discharges. Once in the ocean, P can be removed when reacts with Fe present in overlying sediments and hydrothermal plumes (Libes, 2009). P concentration in the upper continental crust is 665 µg/g (Wedepohl, 1995). Concentration in marine sediments of P is 550 µg/g (Chester, 2000). It can be found everywhere of the cell and it is essential to life as it has a key role in numerous molecules, including ATP, ADP, AMP, ADN and RNA (Elser, 2012). Deficiency of P results in skeletal deformities, poor bone mineralization and a decline of growth performance (Chen *et al.*, 2017) and an excess in the body can cause vascular calcification, renal failure and bone loss (Calvo & Uribarri, 2013).

- Potassium

Ocean natural sources of potassium (K) are from the breakdown of silicate minerals during continental weathering and hydrothermal inputs (Libes, 2009; Santiago Ramos *et al.*, 2020). Anthropogenic sources of K are fertilizers and industrial sewage discharges (Skowron *et al.*, 2018). The upper continental crust has a K concentration of 25650 µg/g (Wedepohl, 1995). K concentration in sea sediment is 25000 µg/g and mean ocean concentration is 10.2nmol/L (Chester, 2000; Chester & Jickells, 2012). This element is important to prevent stroke, for healthy nervous system and a steady heart rate. A deficiency of K can result to impairment of heart and nervous functions as dysrhythmias, risk of hypertension or heart failure (Schaefer & Wolford, 2005; Prasad, 2008). An excess of K can lead to a risk of sudden death from asystole or ventricular fibrillation (Schaefer & Wolford, 2005).

- Selenium

Aquatic ecosystems can reach high concentrations of selenium (Se) due to different anthropogenic sources such as: coal mining, metal smelting, agricultural irrigation and gold, silver, nickel and phosphate mining (May *et al.*, 2008). Se concentration in the upper continental crust is 0.083 µg/g (Wedepohl, 1995). Mean concentration of Se dissolved in the sea is 1.7 nmol/L and in sea sediment is 1.69 µg/g (Cutter & Bruland, 1984; Chester & Jickells, 2012). In the organism, Se main functions are oxidative damage protection, defense against infections and grow and development regulation. Another important function is protection against Hg toxicity. Demethylation of methylmercury ($MeHg^{+}$) occurs in the liver, where HgSe granules are from as a final product of detoxification (Lahaye *et al.*, 2007; Habran *et al.*, 2011). Se deficiency can cause los of immunocompetence, increased virulence of viral diseases, early loss of the embryo, cardiovascular diseases, and depression. Instead, Se excess leads to gastrointestinal disturbance and hair loss (Prasad, 2008).

- Sodium

Sodium (Na) is an essential nutrient and is one of the major ions components of seawater (Prasad, 2008; Libes, 2009; Chester & Jickells, 2012). Na ions were mobilized from volcanic rocks by attrition and were transported into the ocean through rivers and hydrothermal emissions. Evaporites are an important sedimentary sink of Na (Libes, 2009). Residence of Na ions in seawater have longer residence time in the ocean than ocean water itself (Chester & Jickells, 2012). This element is known to be biounlimited as is found in high concentrations in seawater and is not growth limiting (Libes, 2009). Anthropogenic source of Na is agricultural drainage water (Boyacioglu & Boyacioglu, 2008). Concentration of Na in the upper continental crust is 25670 µg/g (Wedepohl, 1995). Sediment concentration of Na in the sea is 40000 µg/g (Chester, 2000) and the mean ocean concentration of Na is 468 mmol/L (Chester & Jickells, 2012). Na, through the sodium/potassium pump, is essential for controlling the fluid balance of the organism, a deficiency of this trace element can cause heart diseases (Prasad, 2008).

- Sulfur

Sulfur (S) is a biounlimited element too. In the ocean, evaporites and pyrites work as S sink (Libes, 2009). Some anthropogenic sources of S are combustion, crude oil, emissions from ocean-going ships and terrestrial run off (Komarnisky *et al.*, 2003; Brimblecombe, 2014). The upper continental crust have a concentration of 953 µg/g (Wedepohl, 1995). Mean concentration of S in ocean water is 28 mmol/L (Chester & Jickells, 2012). S is an important constituent of enzymes, proteins, vitamins and amino acids (methionine and cysteine). A deficiency of S in the body is rare but its toxicity of prevalence excess can cause asthma and occasionally anaphylactic shock (Komarnisky *et al.*, 2003).

- Zinc

Zinc (Zn) is an essential nutrient which can be found naturally in different deposits, like Cu for example. Anthropogenic sources of Zn are mining, smelting, Zn fertilizers and sewage sludges (Bradl, 2005). Zn concentration in the upper continental crust is 52 µg/g (Wedepohl, 1995). In the sea, Zn is found in average concentrations of 92 µg/g in the sediment, of 43 µg/g of oceanic total suspended material and 5 nmol/L dissolved in water (Chester, 2000; Chester & Jickells, 2012). This trace element is important for the metabolism of all molecules, such as acid nucleic, and the synthesis of transcription factors. Thus, maternal transfer of Zn is essential for offspring growth and development (Guirlet *et al.*, 2008; Sinaei & Bolouki, 2017). A deficiency of Zn in the organisms can impact the reproduction, immune system and growth. On the other hand, an excess can cause nausea, vomiting, diarrhea and fever (Prasad, 2008).

Non-essential elements

- Antimony

Sources of antimony (Sb) in the aquatic environment are rock weathering, soil runoff and anthropogenic activities. Anthropogenic emissions of Sb to the atmosphere are mainly from oil and coal combustion, metal smelting and refining activities (Filella *et al.*,

2002; Reimann *et al.*, 2010). The upper continental crust has a concentration of 0.31 µg/g of Sb (Wedepohl, 1995). Mean ocean concentration of Sb is around 1.64 nmol/L (Filella *et al.*, 2002). Antimony exposure can cause gastrointestinal problems, pneumoconiosis, respiratory irritation and is carcinogenic (Sundar & Chakravarty, 2010). Sb it is been used for leishmaniasis treatment (Sundar & Chakravarty, 2010).

- Arsenic

Although arsenic (As) is known for its toxicity, there are evidences it might have metabolic functions in low concentrations in the organism, suggesting that may be essential for man (Prasad, 2008; Aliasgharpour & Farzami, 2013). As can be released to the environment through erosion and leaching from geological formations (Chung *et al.*, 2014). Important anthropogenic sources of As are combustion of coal and disposal of fly ash, mining activities and pesticides and fertilizers (Bradl, 2005; Chung *et al.*, 2014). As is found in concentrations of 2 µg/g in the upper continental crust (Wedepohl, 1995). In the sea, it is found in concentrations of 23 nmol/l dissolved in water and 5 µg/g in sediments near the coast (Chester, 2000; Chester & Jickells, 2012). Chronical exposure of As can cause reduction in growth, sexual disfunction in males and cancer. Moreover, maternal exposure of As can cause spontaneous abortions and infant mortality (Yamauchi & Sun, 2019).

- Cadmium

Cadmium (Cd) can be found naturally in areas with volcanic activity, on weathering of rocks (specially phosphate rocks) and in windblow dust. It can also enter the environment for anthropic causes through the use of phosphate fertilizers, mining and smelting of Zn, lead and Cu ores, disposal of nickel-cadmium batteries and from anti-corrosion coatings (Wood *et al.*, 2012; Ross *et al.*, 2016). Concentration of Cd in the upper continental crust is 0.102 µg/g (Wedepohl, 1995). In the sea, mean concentration of dissolved Cd is 0.6 nmol/L and 15 µg/g of the oceanic total suspended material (Chester, 2000; Chester & Jickells, 2012). A chronical exposition of Cd can disrupt the immune system and endocrine, reduce growth and survival rate and be carcinogenic (Guirlet *et al.*, 2008; Wood *et al.*, 2012).

- Lead

Natural sources of lead (Pb) in the environment are wild forest fires, volcanoes, and weathering. Anthropogenic input of Pb can be from the production of batteries, disposal of Pb-containing products such paints, Pb mining, industrial processing or the combustion of leaded gasoline (still in Africa and Asia) (Wood *et al.*, 2012; Ross *et al.*, 2016). In the upper continental crust, Pb is found in concentrations of 17 μg/g (Wedepohl, 1995). Sea sediment concentration of Pb is 22 μg/g and due to is low solubility, mean concentration of Pb in the sea is really low; 0.01 nmol/L (Chester, 2000; Chester & Jickells, 2012). Low concentrations of Pb can cause toxicity in the organism. This trace element is an antagonist of Ca, which provokes hypocalcemia in the individual as Ca is not efficiently absorbed by the body (Poma, 2008; Wood *et al.*, 2012). During pregnancy, Pb freely enters the fetal compartment, which affects fetal viability and child development during extrauterine life. The presence of Pb in the organism can cause anemia as the heme nucleus production decrease and affects the ability of the body to produce hemoglobin. Moreover, can affect the central and peripheral nervous system (Poma, 2008).

- Lithium

The main sources of lithium (Li) to the ocean are hydrothermal emissions, continental run off and seafloor weathering of the upper oceanic crust where upper volcanic rocks are found (Chan *et al.*, 2002; Libes, 2009). During last decades, an increasing attention in Li has occurred for its use in lithium batteries. An inappropriate disposal of the batteries can result in ground and surface water contamination which eventually will flow to the oceans as river input (Ewuzie *et al.*, 2020). Mean concentration of Li in the upper continental crust is 22 μg/g (Wedepohl, 1995). In the sea, sediment concentration of Li is 79 μg/g and mean ocean concentration is 26 μmol/L (Chester, 2000; Chester & Jickells, 2012). Lithium poisoning can lead to renal failure, hypothyroidism, nausea and coma among others (Timmer & Sands, 1999).

- Mercury

Mercury (Hg) toxicity even at low concentrations in the organism generates a great concern and makes it one of the most studied elements. Natural sources of Hg in the aquatic environment are geothermal releases, volcanic eruptions and weathering of soils and rocks. On the other hand, Hg can be released by anthropogenic sources as, fuel combustion, mining, intentional or accidental releases of effluents from chlor-alkali plants and mines, and was used as pesticide for pest control (Wood *et al.*, 2012; Noël *et al.*, 2015; Ross *et al.*, 2016). Hg concentration in the upper continental crust is 0.056 µg/g (Wedepohl, 1995). Hg concentrations in sea sediments can vary between 0.01 to 0.2 µg/g and can vary between 0.002 a 0.05 µg/L in open oceans (Cox & McMurtry, 1981; Boszke *et al.*, 2003).

Methylmercury ($MeHg^+$) in the organic form of Hg and is known to be the most toxic and bioavailable of all forms. It can cause neurotoxic, genotoxic and immunotoxic effects in the organism. During gestation, embryos work as a total mercury (THg) sink and have a higher percentage of $MeHg^+$ regard adult THg. Taking into account that embryos are in a continuous developing stage, effects of Hg create a great concern (Brookens *et al.*, 2008; Guirlet *et al.*, 2008).

- Silver

Silver (Ag) inputs to the ocean are mainly atmospheric which are originate anthropogenically from industrial emissions such as coal-burning and Zn and Cu metal refining and by wastewater discharges (Sañudo-Wilhelmy & Flegal, 1992; Ranville & Flegal, 2005). Ag concentration in the upper continental crust is 0.055 µg/g (Wedepohl, 1995). Ag concentration in seawater increase with depth. In the surface, concentration is around 1pmol/kg and in 2440m depth concentration is 23 pmol/kg (Martin *et al.*, 1983). Ag is a toxic non-essential trac element which can cause liver and kidney damage, irritations and permanent bluish-gray discoloration in the eyes or skin, and adverse changes in blood cells (Prabhu & Poulose, 2012).

- Strontium

Ocean inputs of strontium (Sr) to the ocean are via groundwater transport, via dissolved riverine transport and Sr exchange from mid-ocean ridge hydrothermal activity (Jones *et al.*, 2012), moreover it can be found as celestite and strontianite (Chowdhury & Blust, 2011). Anthropogenic sources are nuclear fuel processing plants, nuclear power plants and nuclear armament production facilities (Chowdhury & Blust, 2011). The concentration of Sr in the upper continental crust is 316 µg/g (Wedepohl, 1995). In the sea, concentration in marine sediments of Sr is 160 µg/g and mean concentration in seawater is 92-114 µmol/L (Chester, 2000; Chowdhury & Blust, 2011). Sr is a non-essential element analogue of Ca, and acute toxicity of it can cause hypocalcemia as it replace Ca in bone and increase renal excretion of Ca (Chowdhury & Blust, 2011).

- Thallium

Thallium (Tl) is a non-essential trace element extremely toxic, more than Hg. Natural sources of Tl to the ocean are hydrothermal fluids, volcanic emanations and rivers (Rehkämper & Nielsen, 2004; Li *et al.*, 2018). Anthropogenic sources of Tl are from coal combustion and the manufacture of Hg vapor lamps, highly-refractive optical glasses and deep temperature thermometers (Bradl, 2005; Cvjetko *et al.*, 2010). Tl concentration in the upper continental crust is 0.75 µg/g (Wedepohl, 1995). Mean sweater concentration of Tl is 65 pmol/L (Rehkämper & Nielsen, 2004). The presence of Tl in the organism affects the nervous system and cause degenerative changes in organs. Tl can inhibit enzyme reactions and interfere with vital metabolic processes and replace K in the Na+/K+ -ATPase (Cvjetko *et al.*, 2010). Symptoms related to thallotoxicosis can be hair loss, weakness, disturbance of vision and muscle pain (Li *et al.*, 2018).

- Tin

The main input of tin (Sn) to the ocean is via the atmosphere, which is manly anthropogenic. Some sources of Sn into the atmosphere are coal burning, waste incineration, herbicides, tin production, volcanoes, and forest fires. Moreover, leaching of tin-based marine paints, used to prevent biofouling of ships hulls, into the seawater

creates toxic conditions (Byrd & Andreae, 1982; Libes, 2009). The upper continental crust concentration of Sn is 2.5 µg/g (Wedepohl, 1995). Sn concentration in marine sediments is 2 µg/g and mean concentration in the ocean is less dan 50 pmol/L (Byrd & Andreae, 1982; Chester, 2000). Although in some species is an essential element (like rats) in human its role in the organism is still disputed (Prasad, 2008). Sn toxicity in humans is mainly caused by consumption of canned food and result in gastrointestinal problems as nausea, abdominal cramps and vomiting (Schäfer & Femfert, 1984; Prasad, 2008).

- Uranium

The main source of uranium (U) in the ocean is by river runoff (Palmer & Edmond, 1993). Anthropogenic sources of U into the environment are as phosphate fertilizers, where U is present, and mainly through the nuclear fuel cycle. U demand is basically by the requirement of electricity produced by nuclear reactors (Wood *et al.*, 2012). U concentration in the upper continental crust is 2.5 µg/g. Mean concentrations in marine sediments range around 1 – 3 µg/g (Suttle & Sackett, 1973), concentrations of particulate U are around 0.44 – 1.45 µg/g in seawater (Anderson, 1982) and mean concentration of dissolves U in the sea is 0.014 µmol/L (Das *et al.*, 2015). U exposure can result in changes of the renal function and renal failure in very high levels, can cause hepatotoxicity and lung toxicity too (Prasad, 2008; Yue *et al.*, 2018).

- Vanadium

Vanadium input to the ocean comes mainly from atmospheric and river runoff. Major natural sources of V are volcanic debris and crustal weathering. Anthropogenic sources are mining of V ores and combustion of fossil fuels (Duce & Hoffman, 1976; Schlesinger *et al.*, 2017). V concentration in the upper continental crust is 53 µg/g (Wedepohl, 1995). Marine sediment concentration of V is 145 µg/g and mean ocean concentration of V is 36 nmol/L (Chester, 2000; Chester & Jickells, 2012). It is an essential trace element for small animals and it is considered that might have possible beneficial effects in human body. Deficiency in small animals can affect lipid metabolism and cause higher abortion and death rates (Prasad, 2008). The major route of exposure of

V is by inhalation which cause rhinitis, nasal hemorrhage, cough, conjunctivitis and chest pain (Barceloux, 1999).

ANNEX II

Suppl. Table 2.1**:** Dunn's test results for $\delta^{15}N$ values comparison between (a) *R. longurio* and (b) *P. glaucostigmus* tissues of embryos and pregnant females. Values used for this study are in bold.

(a)

	Embryo Liver	**Embryo Muscle**	**Embryo Yolk sac**	**Embryo Blood**	**Pregnant female Liver**	**Pregnant female Muscle**
Embryo Muscle	**-0.713** **0.238**					
Embryo Yolk sac	**2.167** **0.015***	2.517 0.006*				
Embryo Blood	5.735 0.000*	6.295 0.000*	1.404 0.080			
Pregnant Female Liver	7.114 0.000*	7.641 0.000*	2.451 0.007*	1.494 0.068		
Pregnant female Muscle	4.138 0.000*	4.656 0.000*	**0.609** **0.271**	**-1.051** **0.147**	-2.437 0.007*	
Pregnant female Blood	4.461 0.000*	4.814 0.000*	1.729 0.042	**0.714** **0.238**	**-0.385** **0.350**	1.458 0.073

*p < 0.05

(b)

	Embryo Liver	**Embryo Muscle**	**Embryo Yolk sac**	**Pregnant female Liver**
Embryo Muscle	**2.319** **0.010***			
Embryo Yolk Sac	4.889 0.000*	**2.676** **0.004***		
Pregnant female Liver	4.426 0.000*	2.800 0.003*	**0.855** **0.196**	
Pregnant female Muscle	**-1.942** **0.024***	-3.641 0.000*	-5.513 0.000*	-5.259 0.000*

*p < 0.05

Suppl. Table 2.2: Statistical summary of multiple linear regression models used for *Rhizoprionodon longurio* and *Pseudobatos glaucostigmus*. Response variable: $\Delta\delta^{15}N$, Predictor variables: Total length (L_t), month and tissue. SE = Standard error.

	Coefficients	SE	t-value	p
R. longurio				
Intercept	-0.157	0.499	-0.314	0.754
L_t	0.107	0.035	3.069	0.003*
November	-0.128	0.333	-0.383	0.702
April	-0.858	0.672	-1.277	0.204
Liver	2.193	0.115	19.131	0.000*
Blood	-1.419	0.156	-9.069	0.000*
P. glaucostigmus				
Intercept	-2.795	0.524	-5.333	0.000*
L_t	0.128	0.042	3.051	0.004*
Liver	2.413	0.123	19.623	0.000*

* $p < 0.05$

Suppl. Table 2.3**:** Dunn's test results for (a) $\Delta\delta^{15}N$ and (b) $\delta^{15}N$ values comparison between *R. longurio* and *P. glaucostigmus* tissues. Values used for this study are in bold.

(a)

		P. glaucostigmus		***R. longurio***
		Liver	**Muscle**	**Liver**
P. glaucostigmus	**Muscle**	2.366 0.009*		
R. longurio	**Liver**	**-7.155** **0.000***	-10.115 0.000*	
	Muscle	-2.718 0.003*	**-5.561** **0.000***	5.477 0.000*

*$p < 0.05$

(b)

		P. glaucostigmus					***R. longurio***			
		Embryo Liver	**Embryo Muscle**	**Embryo Yolk sac**	**Pregnant female Liver**	**Pregnant female Muscle**	**Embryo Liver**	**Embryo Muscle**	**Embryo Yolk sac**	**Pregnant female Liver**
P. glaucostigmus	**Embryo**	1.060								
	Muscle	0.145								
	Embryo	2.154	1.141							
	Yolk sac	0.016*	0.127							
	Pregnant female	1.926	1.181	0.353						
	Liver	0.027*	0.1190	0.362						
	Pregnant female	-0.900	-1.677	-2.473	-2.334					
	Muscle	0.184	0.047*	0.007*	0.010*					
R. longurio	**Embryo**	**-6.226**	-7.639	-8.713	-6.298	-3.262				
	Liver	**0.000***	0.000*	0.000*	0.000*	0.001*				
	Embryo	-6.664	**-8.096**	-9.160	-6.590	-3.549	-0.509			
	Muscle	0.000*	**0.000***	0.000*	0.000*	0.000*	0.306			
	Embryo	-2.075	-2.779	**-3.485**	-3.242	-1.081	1.540	1.790		
	Yolk sac	0.019*	0.002*	**0.000***	0.001*	0.140	0.062	0.037*		
	Pregnant female	-0.872	-1.837	-2.812	**-2.483**	0.182	4.461	4.835	1.374	
	Liver	0.191	0.033*	0.003*	**0.007***	0.428	0.000*	0.000*	0.085	
	Pregnant female	-2.327	-3.318	-4.267	-3.596	**-1.851**	2.831	3.201	0.366	-1.334
	Muscle	0.010*	0.000*	0.000*	0.000*	**0.031***	0.002*	0.001*	0.358	0.091

*$p < 0.05$

Suppl. Table 3.1: Statistical summary of multiple linear regression models used for *Rhizoprionodon longurio, Mustelus henlei* and *Pseudobatos glaucostigmus*. Response variable: embryo [THg], Predictor variables: Total length (T_L), pregnant female [THg] and embryo tissue. SE = Standard error.

	Coefficients	**SE**	**t-value**	**p**
R. longurio				
Intercept	0.033	0.008	4.167	0.000*
T_L	-0.001	0.000	-2.951	0.004*
Pregnant female [THg]	0.090	0.015	6.036	0.000*
Muscle	-0.095	0.011	-8.398	0.000*
L_t: Muscle	0.003	0.001	6.235	0.000*
Pregnant female [THg]: Muscle	0.006	0.016	0.377	0.707
M. henlei				
Intercept	0.009	0.018	0.494	0.624
T_L	0.000	0.001	-0.181	0.857
Pregnant female [THg]	-0.007	0.055	-0.119	0.905
Muscle	0.035	0.020	1.727	0.090
L_t: Muscle	-0.003	0.001	-2.531	0.014*
Pregnant female [THg]: Muscle	0.296	0.060	4.967	0.000*
P. glaucostigmus				
Intercept	0.010	0.003	3.276	0.004*
T_L	-0.001	0.000	-2.300	0.034*
Pregnant female [THg]	0.017	0.007	2.349	0.031*

* $p < 0.05$