Lack of xenoestrogen-induced vitellogenin in male olive ridley sea turtles (*Lepidochelys olivacea*) from the Pacific coast of Costa Rica

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Abstract: Olive ridley sea turtles (*Lepidochelys olivacea*) distribute widely throughout Eastern Pacific (EP) waters. During reproduction these animals aggregate near coastal waters where they may be exposed to industrial or agrochemical environmental pollutants that may disrupt the endocrine system of these reptiles. One group of compounds of concern is the persistent organic pollutants (POPs), some of which have been detected in Costa Rican waters. Some of these POPs, such as polychlorinated biphenyls and DDT and its metabolites, exhibit estrogenic activity in reptiles. It is unknown whether olive ridleys are exposed to endocrine disruptive doses of these POPs throughout their life in EP waters. Consequently, this study was conducted to determine whether there is evidence of exposure of male olive ridleys to estrogenic compounds. During the study a total of 35 males were hand-captured while mating off the coast of Ostional, Costa Rica, a major nesting beach for this species. The captured turtles were brought aboard a small boat, and blood samples taken with a hypodermic syringe. Turtles were carefully released in the water after the blood samples were taken. In the laboratory, blood collected from these males was analyzed by Western blot using an antibody developed against red-eared slider turtle vitellogenin. Vitellogenin is a female-specific protein that is induced by estrogen during gonadal recrudescence. Estrogenic compounds have been shown to induce this protein in reptilian species. Results of this study indicate the lack of evidence of vitellogenin in the blood of olive ridley males as shown by Western blot analysis. This study demonstrates the feasibility of using the slider's vitellogenin antibody to detect the presence of the protein in the olive ridley sea turtle, *L. olivacea*. Rev. Biol. Trop. 56 (Suppl. 4): 49-57. Epub 2009 June 30.

Key words: *Lepidochelys olivacea*, olive ridley turtle, vitellogenin, marine pollution, endocrine disruption, Eastern Pacific, Costa Rica.

Many industrial and agricultural activities result in the discard of environmental pollutants that may pose a significant threat to sea turtles due to their toxicity or to their ability to behave as endocrine disruptors, i.e., as exogenous chemicals that may interfere with endocrine physiology by either mimicking or blocking endogenous hormonal signals (Crews *et al.*, 2000). Indeed a recent review on endocrine disruption in wildlife by Cheek (2006) suggests that this phenomenon, in combination with other stressors such as habitat loss, over-harvesting, and global climate change, may contribute to local wildlife extinctions. An important group of endocrine disruptors is that of the persistent organic pollutants (POPs) that, because of their hydrophobicity and persistence in the environment, tend to bioaccumulate in fatty tissues of invertebrates and vertebrates and to biomagnify through the food chain (Lindström *et al.*, 2004). Two widely distributed POPs are 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (DDE) and polychlorinated biphenyls (PCBs). DDE is a highly toxic pesticide and PCBs are industrial pollutants whose presence has been detected in aquatic ecosystems

in Costa Rica, including the Pacific coast (De la Cruz, 1994; Spongberg, 2004b; Spongberg, 2004c; Spongberg, 2004a). DDE has been detected in bivalves in Costa Rican estuaries (De la Cruz, 1994). Both DDE and PCBs have been detected in tissues of carnivorous marine turtles, including ridleys and loggerheads in the EP and in other regions (Lake *et al.*, 1994; Gardner *et al.*, 2003; Keller *et al.*, 2004). These organochlorinated compounds are known to be endocrine disruptors, having estrogenic activity in turtles and being capable of reversing the sex of turtles with temperature-dependent sex determination (Bergeron *et al.*, 1994; Palmer and Palmer, 1995; Willingham and Crews, 1999). Another sign of endocrine disruption in oviparous vertebrates by organochlorinated hydrocarbons is the ability of these compounds to induce vitellogenin, a phospholipoglycoprotein precursor of egg yolk proteins, from the liver (Heppell *et al.*, 1995). Vitellogenin is a female-specific protein normally present in the blood of females with recrudescing gonads, but not in juveniles or males. Interestingly, it has been shown that males and immature turtles are also capable of producing vitellogenin when exposed to exogenous sources of estradiol (Palmer and Palmer, 1995; Irwin *et al.*, 2001; Sifuentes-Romero *et al.*, 2006). Thus, the presence of vitellogenin in male blood has been used as evidence of exposure to estrogenic compounds, like organochlorinated chemicals (Heppell *et al.*, 1995). Such exposure may lead to negative impacts on physiological processes that may be deleterious to endangered wildlife populations such as sea turtles. A clear case of relevance of endocrine disruption at the population level is that of alligators in Lake Apopka. Exposure to high concentrations of organochlorinated compounds led to reproductive dysfunction and a decline in the alligator population (Guillette *et al.*, 1994). Declines of sea turtle populations in Costa Rica have been documented, although the specific causes have not been elucidated (Valverde *et al.*, 1998). Thus, we undertook an exploratory study searching for evidence of endocrine disruption in marine turtles in Costa Rica.

The olive ridley sea turtle (*Lepidochelys olivacea*) is an endangered species with a pantropical distribution. Its abundance is greatest on the east coast of India and on the west coast of Mexico and Central America (Cornelius, 1982). The species exhibits a mass nesting behavior, also known as *arribada*, which is characterized by the synchronous nesting of thousands of conspecifics in a relatively small section of beach (Valverde *et al.*, 1998). An extensive tagging program conducted in the early 1980s at Nancite and Ostional, the two most important *arribada* beaches on the Pacific coast of Costa Rica, revealed that these nesting assemblages distribute widely throughout the Eastern Pacific (EP), between Baja California and northern Peru (Cornelius, 1986). Tag recoveries indicate that this species distributes along the coast and also travels as far as 3,200 km off the Costa Rican coast, which is consistent with its pelagic behavior (Cornelius and Robinson, 1986). This ample distribution in the EP, including highly productive coastal and estuarine areas, provides an extensive foraging area for this carnivorous species along the coast (Reichart, 1993). It is in these coastal areas where ridleys are most impacted by anthropogenic activities including direct and incidental take in various fisheries, poaching of eggs at the nesting beaches and several industries, among other threats. The impact of environmental pollutants on sea turtle populations remains to be demonstrated.

The objective of this study is to establish whether there is evidence of endocrine disruption in olive ridley sea turtles in Costa Rica as a function of estrogenic contaminants.

MATERIALS AND METHODS

Olive ridleys were captured over a period of three years off the coast of the Ostional National Wildlife Refuge, Pacific coast of Costa Rica. Ostional beach is one of the world's most important mass nesting beaches for the olive ridley sea turtle (Fig.1a). Turtles were captured during the months of June or July over a period of three years, just before

the beginning of the heavy nesting period in Costa Rica (July-November), when mating is believed to be most frequent. All animals were captured during mating by means of the turtle rodeo technique (Limpus, 1985). Briefly, upon sighting a mating pair a diver leaps off the boat, captures the animals and brings them onboard. Each sampling trip lasted an effective time of no more than four hours. All animals were marked using a water-proof marker to avoid recapture during the same season.

Blood samples of 5 ml were collected from each individual from the cervical sinus using heparinized vacutainers with 21G, 2.5 inch

Fig. 1a. Mass nesting event (*arribada*) at Ostional National Wildlife Refuge, Costa Rica. This *arribada* took place in November of 2006, and was estimated at 64,388 ± 4,951 females. *Arribadas* like this are characteristic of this beach (photo taken by Diana Solís).

Fig. 1b. Blood sampling of a male olive ridley captured by means of the turtle rodeo technique during mating off the coast of Ostional Beach, Costa Rica (photo taken by J. Sibaja).

needles (Fig. 1b). The turtles were released to the sea after the blood samples were taken. Blood samples were placed on ice and centrifuged shortly after collection while still on the boat using a portable 12 V centrifuge, or shortly after landing. Before aliquoting into cryovials all plasma samples were supplemented with approximately 50 µl of the wide spectrum protease inhibitor aprotinin to prevent vitellogenin degradation. Samples were frozen on dry ice and then taken to the CIMAR laboratory were they were kept frozen at -80ºC until they were transported frozen to the SLU laboratory for analysis.

***Trachemys* vitellogenin induction:** *T. scripta* vitellogenin antibody used in this study was originally characterized by Selcer and Palmer (Selcer and Palmer, 1995). To validate the immunological identity of olive ridley vitellogenin as determined by the red-eared slider (*Trachemys scripta*) vitellogenin antibody we used a source of native protein. For this, eggs were purchased from Kleibert's Turtle Farm in Hammond, LA, transported to the lab and incubated. Hatchlings were placed in tanks with water and artificial lighting on a 12:12 photoperiod, and fed commercial turtle chow once a day. Turtles used in this part of the study were three years old. The average length and mass of the turtles were 139mm ± 11.3 and 442.7g ± 83.3, respectively. Estradiol powder was first dissolved in 100 µl of 95% ethanol and diluted in corn oil to a concentration of 25 mg/ml. All three turtles were given a series of four intraperitoneal injections at a dose of 1 mg/Kg of body mass. The second injection was given six days after the first injection, whereas the last two injections were given at two day intervals from the second. Turtles were bled 15 days after the first injection. Blood was kept on ice, supplemented with aprotinin and centrifuged. Plasma was aliquoted and stored at -80ºC until analyzed.

Western Blot: Plasma samples were thawed on ice and a Bradford protein assay performed. Equal amounts of protein were fractionated for each sample on a 4 % to 12% gradient precast (Invitrogen) polyacrylamide gel under reducing conditions. A molecular weight marker (BlueRanger; Pierce) was also included as a signal reference. After fractionation, proteins were transferred to Immobilon-P (Millipore) polyvinylidene fluoride (PVDF) membranes using a semi-dry transfer cell (Bio-Rad), with a solution of NuPage transfer buffer (Invitrogen) and 20% methanol. Membranes were incubated in a weight boat for 1 hour at room temperature in a phosphate buffer-saline solution (PBS) supplemented with 4% casein and 10% normal goat serum. Blots were then incubated with primary anti-turtle vitellogenin antibody (Palmer and Palmer, 1995) at a dilution of 1:30,000, diluted in an antibody diluent solution (PBS supplemented with 1% casein and 0.04% Tween-20) at room temperature for an hour with gentle shaking. Blots were then rinsed briefly with PBS one time and then three times with PBS for five minutes each. Membranes were then incubated at room temperature for 30 minutes with a peroxidase-conjugated secondary antibody solution (goat anti-rabbit; Pierce) at a dilution of 1:10,000 in antibody diluent solution. Membranes were washed in PBS as after the primary antibody incubation. Chemiluminescent signal was detected using a commercial kit (Pierce) according to manufacturers' specifications and visualized with a CCD camera (Chemidoc; Bio-Rad)

RESULTS

The turtle rodeo technique proved an efficient method for capturing olive ridley males. Using this technique a total of 35 males and 19 females were captured over the study period. It does seem that the months of June and July are adequate to capture males, a time when many females are reaching the proximity of the coast to breed.

The vitellogenin induction regime used on lab-raised red-eared sliders proved effective. The estradiol dose used (1 mg/Kg) effectively induced vitellogenin in the sliders raised under

lab conditions 15 days after the first injection. Plasma samples assayed by western blot for these same turtles before injection (data not shown) did not have detectable vitellogenin.

The vitellogenin antibody developed against the *Trachemys scripta* protein detected a single band of about 210.1 kDa in olive ridley female plasma. Both the red-eared slider and the olive ridley turtle proteins are virtually the same size. The blot shows virtually no background and minimal or no vitellogenin degradation (Fig. 2).

Western blot analysis conducted on all 35 male plasma samples captured off Ostional Beach revealed no trace of vitellogenin (Fig 3). However, vitellogenin was present in the blood of all females (only 6 representative samples shown for each sex). Treatment of plasma samples with the protease inhibitor aprotonin indicate that this chemical is effective in preventing denaturation of vitellogenin, even under field conditions.

DISCUSSION

Coastal areas are well known developmental and adult habitats for a wealth of marine life. In recent years there has been an increased interest in the study of aquatic contamination in the Pacific and Caribbean coasts of Costa Rica and on the impact of pollutants on marine life (e.g., Gravel *et al.*, 2006). Sources of pollution include industrial, urban and agricultural discards associated with those anthropogenic activities (Whelan, 1989). Some pollutants may represent a serious risk to marine vertebrates and invertebrates. To our knowledge this is the first study exploring the possibility of sea turtle exposure to estrogenic compounds in the EP based on the induction of vitellogenin. The Costa Rican Pacific coast comprises a series of important nesting beaches for the olive ridley sea turtle (Cornelius, 1982), a species considered endangered. The detection of PCBs and DDE in Costa Rican coastal waters (De

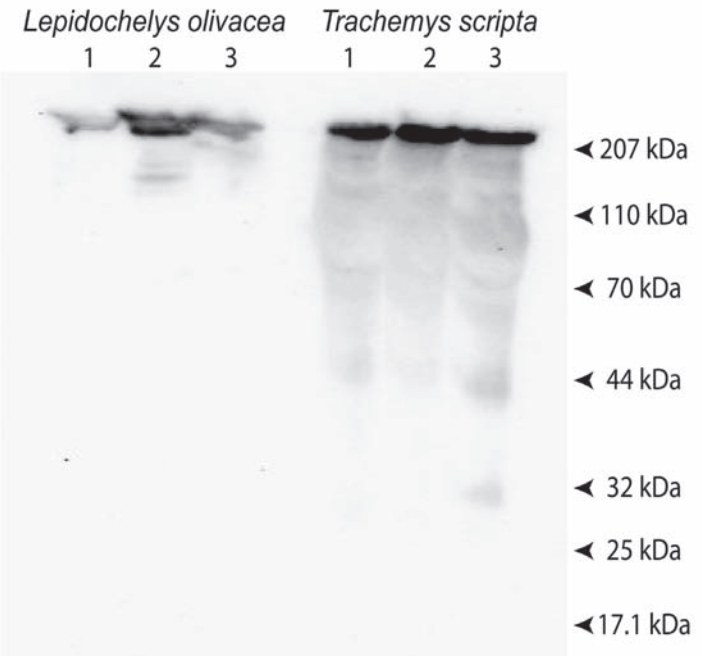

Fig. 2. Western blot of female olive ridley and induced red-eared slider turtles plasma samples. Blots were probed with antibody raised against *Trachemys scripta* vitellogenin.

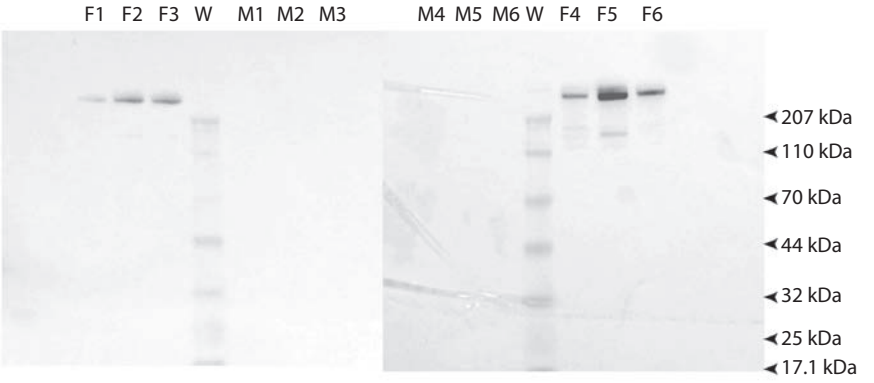

Fig. 3. Representative Western blots of male (M1-M6) and female (F1-F6) olive ridley sea turtles captured off the coast of Ostional Beach, Costa Rica. Blots for the remaining 29 male and 13 female plasma samples follow the same pattern shown here, i.e. all females have detectable levels of vitellogenin while no males had traces of the protein.

la Cruz, 1994; Spongberg, 2004c; Spongberg, 2004a) raised the possibility that ridleys might be exposed to endocrine disruptive doses of these persistent pollutants. Moreover, the detection of DDE and PCBs in marine invertebrates in Costa Rican coastal waters (De la Cruz, 1994; Spongberg, 2006) provide evidence that these compounds are present in the lower food chain and that have the potential to biomagnify and, thus, impact sea turtles and other organisms. Significant amounts of DDE and PCBs have been found in tissues of ridleys and other sea turtles in the EP and in other regions of the world (Lake *et al.*, 1994; Gardner *et al.*, 2003; Keller *et al.*, 2004). This suggests the possibility that olive ridleys in the EP may also have body burden levels of these compounds that may disrupt endocrine processes. Consequently, we collected and analyzed olive ridley blood samples for physiological evidence of endocrine disruption.

Analysis of olive ridley plasma samples by Western blot proved to be an excellent method for the detection of circulating vitellogenin. Indeed, the antibody raised against *Trachemys scripta* vitellogenin (Palmer and Palmer, 1995) cross-reacted well with and detected olive ridley vitellogenin with great specificity. The Western blot revealed the presence of a single estradiol-induced protein in red-eared slider blood and of a native protein in olive ridley blood that approximates 210.1 kDa as predicted by the regression analysis of the electrophoretic mobility of the single high-molecular weight protein (r^2=0.97). The estimated size of these vitellogenins is in agreement with published data for this protein that ranges between 200-214kDa for emydid and chelonid reptiles (Heck *et al.*, 1997; Irwin *et al.*, 2001; Herbst *et al.*, 2003). In addition, the Western blot demonstrated that our method of blood collection and sample handling methodology was appropriate as it resulted in little or no degradation of olive ridley vitellogenin in the field.

Vitellogenin is an estrogen-inducible protein produced by the liver of ovoviviparous and oviparous vertebrates that is believed to be a good biomarker of xenoestrogenic exposure (Denslow *et al.*, 1999; Cheek *et al.*, 2001; Irwin *et al.*, 2001). Potential endocrine disruptive effects of estrogenic compounds may pose a significant environmental stress on free-ranging populations of endangered or threatened olive ridley sea turtles (Colborn *et al.*, 1993). PCBs and DDE are persistent environmental pollutants that have been shown to have powerful endocrine disruptive effects on wildlife, including reptiles. For instance, exposure to endocrine disruptive chemicals resulted in reproductive abnormalities in a population

of alligators in Lake Apopka (Guillette *et al.*, 1994). In the red-eared slider, a turtle with temperature-dependent sex determination, PCBs proved effective at reversing the sex of embryos at male producing temperatures (Bergeron *et al.*, 1994). In this same turtle, DDE was able to induce vitellogenin (Palmer and Palmer, 1995). Application of the Western blot analysis to male olive ridley plasma in the present study revealed the absence of circulating vitellogenin in these animals, which suggests that endocrine disruption is not a problem for this population. It is important to point out that no study has ever shown that PCBs or DDE can effectively induce vitellogenin in free-ranging reptiles. For instance, Irwin et al (2001) failed to detect vitellogenin in the painted turtle (*Chrysemys picta*) living in cattle ponds that contained estrogen. In the alligator Matter et al (1998) did not observe vitellogenin induction in hatchlings exposed to various POPs, even at the high exposure doses of DDE during incubation that led to sex reversal in this reptile with temperature-dependent sex determination. It may be that vitellogenin in reptilian species is not inducible at ecologically relevant concentrations. In addition, it is also important to keep in mind that the Western blot methodology used in this study cannot distinguish between endogenous vitellogenin and the exogenously-induced protein in females. Although the vitellogenin detected in females is likely related to normal reproductive function the possibility remains that it may be the result of exposure to estrogenic compounds.

One caveat about this study is the identification of the sampled animals. During each sampling trip captured males were distinguished by marking their carapace with a water-proof marker that lasted for at least a week. Because every sampling trip was restricted to a single week it is unlikely that we captured the same male any given year. However, the possibility exists that we captured and sampled the same male over different years, as males followed the large aggregations of females on their nesting migration to Ostional Beach. Two main lines of evidence suggest that this was not the case. First, satellite tagged males tend to disperse far and widely in the EP after mating (Plotkin, 1996). Although they may reproduce every year it is not very likely that we encountered the same animal in the vastness of the ocean. Second, microsatellite analysis of olive ridley DNA indicates that females practice polyandry even in the face of a highly female-biased ratio (Jensen *et al.*, 2006). This suggests that a high number of male olive ridleys appear to visit our study area to mate, making it less likely to encounter the same male during the short sampling periods. To completely rule out the possibility of sampling the same animals in future studies sampled individuals should be tagged.

In this study evidence of endocrine disruption in olive ridley sea turtles in the EP as indicated by the absence of vitellogenin in males was not detected. However, it is important to point out that lack of evidence does not imply lack of exposure. It may be that the amount of POPs in the environment represents a non-significant dose of the compounds to impact the physiology of vitellogenin production in these animals. We suggest that more sensitive biomarkers need to be developed to detect exposure to estrogenic pollutants in reptiles.

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RESUMEN

La tortuga lora (*Lepidochelys olivacea*) se distribuye ampliamente en aguas del Pacífico del Este (PE). Estos animales se congregan cerca de aguas costeras durante la reproducción, en donde pueden ser expuestas a contaminantes de origen industrial o agroquímico que pueden distorsionar el sistema endocrino de estos reptiles. Uno de los grupos de compuestos de interés son los contaminantes orgánicos persistentes (POPs), algunos de los cuales han sido detectados en aguas de Costa Rica. Algunos de estos POPs, como los bifenilos policlorados y el DDT y sus metabolitos, exhiben actividad estrogénica en reptiles. No se conoce si estas tortugas están expuestas a dosis disruptoras endocrinas de esos POPs a lo largo de su ciclo de vida en aguas del PE. Consecuentemente, este estudio fue conducido para determinar si hay evidencia de exposición de tortugas macho a compuestos estrogénicos. Durante el estudio un total de 35 machos fueron capturados a mano cuando se reproducían en aguas del Refugio Ostional, Costa Rica, uno de los mayores sitios de anidación de esta especie. Las tortugas capturadas fueron traídas a bordo de una embarcación y se les extrajo muestras de sangre con una jeringa hipodérmica. Las tortugas fueron liberadas cuidadosamente después de la toma de muestras. En el laboratorio las muestras de sangre fueron analizadas mediante la técnica de Western blot utilizando un anticuerpo desarrollado para vitelogenina de otra especie de tortuga (tortuga *red-eared slider*). La vitelogenina es una proteína específica de las hembras que es inducida por estrógeno durante el recrudecimiento gonadal. Compuestos estrogénicos han sido conocidos por inducir esta proteína en especies de reptiles. Los resultados del estudio indican la falta de evidencia de la presencia de vitelogenina en tortugas macho según las pruebas por Western blot. Este estudio demuestra la factibilidad de usar el anticuerpo contra la vitelogenina de la otra especie para detectar la presencia de la proteína en la tortuga *L. olivacea*.

Palabras clave: *Lepidochelys olivacea*, tortuga lora, vitelogenina, contaminación marina, disrupción endocrina, Pacífico del Este, Costa Rica.

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