Assessing Population Health in the American Crocodile (*Crocodylus acutus)* using Molecular Methods

Gene sequencing of the mRNA biomarkers (CYP19, CYP1A) and mercury concentrations in *C. acutus* fetuses

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Abstract

The American crocodile (*Crocodylus acutus*) population in the Tempisque River of Guanacaste, Costa Rica, is stable but a skewed sex ratio of 3:1 towards males is indicated in previous studies. One proposed explanation to this observation, is the possibility that biochemical alterations due to pollution may have caused or contributed to the skewed sex ratio. In order to reject or confirm such a hypothesis, molecular methods need to be developed, and this study focused on developing a quantitative method for analyzing selected biomarkers and genes relevant to sex determination and pollution (CYP1A, CYP19 (aromatase), and beta-actin). Crocodile fetuses of varying developmental stages were collected from the Tempisque River, and liver and brain tissues were used in an attempt to find the mRNA sequences and the expression of the selected genes. The method for measuring the mRNA expression of the selected biomarker genes was partially developed using primers designed from evolutionary conserved areas of genome sequences from other species. The first confirmed Beta-Actin gene sequence for *Crocodylus acutus* was found.

Furthermore, mercury concentrations were measured in heart and brain tissues, and measurable amounts were found in all fetus tissues tested. The mercury concentrations were higher in heart tissues than in the brain tissues, but can be considered relatively low in comparison to concentrations measured in entire crocodile eggs from other studies. The mercury concentrations did not differ in crocodile fetuses from nests located downstream or upstream from a tentative source of pollution, indicating an even distribution (i.e. no point source) of mercury.

It is unlikely that the skewed sex ratio (if any) in the Tempisque River is due to endocrine disruption or other biochemical alterations due to pollution, and the population does not seem to be exposed to significant amounts of Mercury.

Keywords

Crocodylus acutus, CYP1A, CYP19, aromatase, mRNA, beta-actin, Costa Rica, American crocodile, mercury

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Introduction

In Costa Rica around 13 million kilos of pesticides are imported annually (2009) and distributed to a variety of crops (Ramírez Muñoz, 2011). Banana, coffee, rice and sugar cane are the most common crops, together with a smaller amount of pineapple and watermelon agriculture (Ramírez Muñoz, 2011). It is not fully known to what extent this extensive use of pesticides, agriculture practices and other anthropogenic activities affects the surrounding ecosystem. A powerful tools for discerning cause and effect in a biological system suspected to be affected by pollution is the usage of biomarkers (Bucheli and Fent, 1995). A biomarker can be defined as "*a measurable response at any level of biological organization that can be related to an impact of contaminants*". Usage of biomarkers at the molecular level can serve as an early warning system in environmental monitoring as all environmental biological responses to a toxic substance always starts with a biochemical reaction. This thesis is a part of a larger project with the title "Population ecology and ecotoxicology of *Crocodylus acutus* in Tempisque River basin" (SINAC-SE-GASP-P1-R-019-2014), realized in cooperation with the Universidad Nacional de Costa Rica and the Sistema Nacional de Áreas de Conservación (SINAC). Together with complementary data on pollution and climate it aims to provide tools to shed light on the ecosystem health of the Tempisque River in Costa Rica and examine the alleged skewedness in sex ratio in the American crocodile through a molecular approach.

This thesis is an attempt to develop methods for analyzing gene expression and concentrations of mercury as biomarkers, to be used as a part of general population health assessments. It also presents data on mercury concentrations from the crocodile fetuses. Mercury has never before been analyzed in the tissues of crocodilian fetuses. Mercury analysis of selected tissues will serve as an early indicator to whether there is an extensive mercury contamination in the area and serve as a benchmark for future studies.

Research approach

Chemical analyses of pollutant concentrations in an environment, valuable as they may be for assessing environmental health, cannot alone expose the impact they may have on a biological system, nor do they take in consideration the combined effect of a mixed xenobiotic exposure. Because molecular changes are the first measurable responses in a biological system to an environmental change or an exposure to xenobiotics, I considered it appropriate to focus on developing a molecular biomarker methodology as the first step for evaluating such a change or exposure. Therefore I chose to analyze mRNA concentrations of chosen biomarker genes, as mRNA can serve as snapshots of the biochemical processes in time and space, and are usually the first quantifiably response to environmental change regardless of the complexity of the substances that induced the gene activity. The mRNA biomarkers in this study were chosen by evaluating what likely biochemical changes to be expected in response to the exposure to the variety of pollution that exist in the Tempisque River in Costa Rica, taking into account that such changes could also affect the sex ratio in the crocodile population.

The cytochrome P450-system gene CYP1A mRNA biomarker was chosen, due to it's versatile substrate induction, especially to organic pollution and PAHs, and because it is the most likely P450 monooxygenase system (CYPs) to respond to organic pollution from agriculture and other land usage in the area (see appendix). The members of the cytochrome P450-family of proteins (CYPs) are a part of the mixed-function oxidase (MFO) system (de Rolla 2010) and metabolize their substrates through a monooxygenase reaction. There are many different types of cytochrome P450-proteins but the most

extensively studied cytochrome P450 isoenzyme is CYP1A[1] (CYP1A1, CYP1A4 *etc*.), and it has been extensively used as a biomarker. Organic pollutants such as polycyclic aromatic hydrocarbons (PAHs) are among the most common organic contaminants in the aquatic environment (Lepom *et al.* 2009, Roose and Brinkman 2005), and exposure of PAHs, coplanar PCBs, polychlorinated dibenzofurans and dibenzodioxins, many of which are persistent (POPs), induce the AH-receptor mediated transcription of CYP1A. In a recent study using water from the Tempisque River, fish showed increased levels of CYP1A upon exposure, suggesting that there are polychlorinated biphenyls (PCB's), polyaromatic hydrocarbons (PAH's), dioxins or furans in the river (Navarro *et al.* 2014). Out of the biomarkers on the molecular level, CYP1A is considered to be the most sensitive and accurate biomarker for a wide range of environmental pollutants (Bucheli and Fent 1995), with the AH-receptor mediated response being highly specific, providing quantifiable increases of CYP1A mRNA, protein, and an increased metabolic activity in response to substrate exposure. CYP1A has also been show to metabolize estrogen in rats (E_2) (Badawi 2000), and may therefore also affect the hormonal levels of testosterone and estrogen in crocodiles in response to pollution, why an analysis of CYP1A mRNA expression might shed light on the balance of sex hormones.

Most likely all invertebrates have isozymes of this enzyme (Bucheli and Fent, 1995), including turtles (CYP1A1) and birds (CYP1A4) (Watanabe et al., 2013), and crocodiles. It was indirectly shown that the CYP1A system very likely is present in crocodilians through a study on *Alligator mississippiensis* in southern Florida. The CYP1A activity was successfully measured for juveniles by measuring EROD activity (enzyme activity), and showed an EROD activity higher in those areas with lower contamination, indicating inhibition of CYP1A expression does occur in response to pollution (Gunderson *et al.* 2004) It is, however, not known which of the isozymes of CYP1A (CYP1A1, CYP1A4 etc.) that crocodiles have. Because the EROD activity could be measured for crocodiles, it can be safely assumed that crocodiles, too, have a CYP1A system in place.

Aromatase, a product of the CYP19 gene, converts androgens (testosterone) to estrogen and plays a critical role in sex development and maturation of crocodilians (Gabriel *et al.* 2001). Aromatase, the product of the CYP19 gene, is also a member of the Cytochrome P450-family, but is responsible for the irreversible conversion of androgens into estrogens in most animals (Simpson *et al.* 1994) and balances the relative amounts of estrogen and androgens in the body. It is expressed in a number of tissues in mammalian fetuses, including the liver, brain skin and placenta (Toda *et al.* 1994), but in crocodilians the expression of aromatase, at least during development, mainly occurs in the hypothalamus in both male and female embryos (Gabriel *et al.* 2001). The expression of aromatase in the brain is present from around stage 20 when the TSD period begins (TSD - temperature sex dependency, sex is determined by surrounding temperature during the TSD period), until hatching (Toda *et al.* 1994). The aromatase expression differs between the sexes in the gonadal region after the TSD period, where females show much greater expression than males (Gabriel *et al.* 2001). The aromatase expression in crocodilians isn't the trigger for sex determination, but it does, however, play an essential role in the development of ovarian differentiation in females (Gabriel *et al.* 2001, Smith and Joss, 1994). A synergistic effect to increase aromatase inhibition has been noted for structurally similar chemicals to those expected to be found in the Tempisque River (Benachour *et al.* 2007), and therefore the development of a method to measure aromatase expression in developing crocodiles is a good start, as such a method could serve as a powerful tool for indirectly assessing if these pollutants may be causing endocrine disruption in the general population of the crocodiles or in the ecosystem as a whole, as well provide a way of determining the sex of juvenile American crocodiles.

[1] Although the nomenclature of the hydrocarbon-inducible isozyme cytochrome P450 was reviewed by (Stegeman, 1992) and he concluded that the name for the cytochrome P4501A can be referred to as CYP1A, and that the term CYP1A1 and CYP1A2 only are appropriate for the isozyme for trout, and that other isozymes from other species should be referred to as CYP1A only, this thesis makes exceptions to this suggestion as it is unknown what isozymes crocodiles have, and not all other studies have followed this suggestion.

The beta-actin gene was chosen as a reference gene because of its evolutionary stability, i.e. the genetic similarity between species, and having been an appropriate reference gene for past studies. Measuring the relative abundance of a gene-product to a reference gene gives you more valuable information than simply looking at the abundance of the gene product itself – a concentration of anything wouldn't tell you much unless you have something to compare to.

Population description of the American crocodile

The American crocodile (*Crocodylus acutus*) population in the Tempisque River and its nearby wetlands in Guanacaste, Costa Rica, appears strong with its large and increasing numbers of individuals (Porras unpublished, Mauger *et al.* 2012). The population within this area is estimated to about 2300 animals (2010) and reported to be stable but increasing (Bolaños unpublished, Mauger *et al.* 2012) with 2.28 – 11.1 individuals/km in the main branch of the Tempisque River (between the years 1997 and 2001) (Mauger *et al.* 2012, Sánchez J. 2001, Sánchez J. *et al.* 1997). In comparison, the population of the nearby river Río Tárcoles is decreasing, hypothesized, due to pollution and alteration of the habitat (Abadia and Orjuela 1998, Mauger *et al.* 2012; Sánchez J. 2001, Sánchez J. *et al.* 1997). Although abundant in numbers, the population may exhibit a skewed sex ratio with males being overrepresented at a 3.3:1 ratio (Mauger *et al.* 2012). Generally, crocodile populations have a 1:1 sex ratio (Brant *et al.* 1995, Cedeño-Vázquez 2006). The cause for this observed effect is unknown, and possible explanations include; ecological factors such as climate change, habitat alterations due to anthropogenic activities, endocrine disruption caused by agriculture usage of pesticides and growth hormones used in local aquaculture, and mercury pollution. However, the most recent study (2012) on sex ratios in the area found a 2:3 males to females for captured non-hatchlings (Mauger *et al.* 2012), in contrast to the unpublished studies on which this thesis was initiated.

The controlling factor in sex-determination for crocodiles is temperature (Thorbjarnarson, 1997), but whether a change in temperature or climate is the cause for the observed skewedness is heavily debated (Bolaños, 2010, Charruau 2012). It is today common knowledge that crocodilians, including *C. acutus*, have a temperature-dependent sex determination (TSD)(Charruau 2012). There are two pivotal temperatures (31° and 32.5°C) for the female-male-female TSD (Charruau 2012), with males being produced in the middle range only. The rate of development of the crocodiles is positively correlated to temperature, which means that the post-laying development of the fetuses speeds up with an increase of the surrounding temperature. As with all invertebrates, the development of a crocodilian embryo go through a set number of developmental stages where stage one is the time following fertilization of the female gamete and for crocodiles end with stage 28 where the crocodile is fully developed and ready to hatch (Ferguson 1987a). Sex is estimated to be determined between days 25 – 45 of incubation (Lang and Andrews 1994, Piña *et al.* 2007), which corresponds to stage 17 - 22 of its development (Ferguson 1987a). A method for determining the sex ratio in hatchlings and sexually immature individuals has not yet been established, making it impossible to investigate the sex ratios in the

Figure 1. The research area in Costa Rica: The Tempisque River that divides the Nicoya Peninsula from the rest of the country, and runs from north to south, with Palo Verde National park on the eastern side of the river. The dots represent nesting locations sampled in the present study.

nests. This also makes it exceptionally difficult to establish the cause of the alleged skewedness in sex ratio observed for the population in the Tempisque River.

Tropical dry forest and agricultural crops such as sugar cane and rice fields surround the Tempisque River. Given the high level of pollution from agrochemicals in the area (IPS 2002, Ramírez Muñoz 2011), the possibility of endocrine disruption affecting the sex ratio distribution should not be excluded. It can be difficult to assess the amount and type of pollution in an area, and whether pollution is causing a measurable effect on an ecosystem or the organisms living there. In such situations the usage of biomarkers as an indirect method for establishing a relationship between pollution and environmental effects is a useful approach. Research indicates that when crocodilians are exposed to environmental contaminants the maternal burden of xenobiotics is transferred to the eggs, and exposure to xenobiotics may occur early in the lives of hatchlings (Rauschenberger *et al.* 2004a, 2004b, Wu *et al.* 2006), making the contaminant egg burden itself, as well as biochemical processes within the eggs, good indicators of environmental pollution. Crocodiles are large top predators living in diverse aquatic habitats, which make them excellent indicative species of general ecosystem health (Thorbjarnarson et al., 2006) .

Hypotheses

i) Methods for analyzing the selected biomarkers can be successfully developed and be used for future genomic studies on *C. Acutus.*
ii) Expression of CYP19 and CYP1A mRNA occurs in brain- and liver tissue respectively during the crocodile fetus sex determining development period.
iii) The American crocodile (*Crocodylus acutus*) exhibits a P450-monooxygenaze system more similar to the CYP1A1 found in reptiles and amphibians rather than the CYP1A4 found in birds.
iv) There are measureable amounts of mercury in crocodile fetus tissues and the concentrations differ between tissue types.
v) The mercury concentrations in fetus tissues are comparable to mercury concentrations found in other crocodilians around the world.

Materials and methods

Sample collection

Crocodylus acutus eggs were collected from nests located by the Tempisque River in the Guanacaste province. The nests were found by locating crocodile entrances by the shoreline and by looking for loitering crocodiles by the sandbanks, presumed being females guarding their nests. Potential nesting sites were visited on foot, and promising sites were carefully excavated with a small shovel to a depth of about 2-3 dm. If at this depth no eggs were found, a stick was used to poke further into the ground in order to exclude the possibility that eggs were deeper than that. Once a nest had been found, the developmental stage and viability of the eggs was determined according to the methods described by Ferguson (1987). If the eggs were deemed to be too young to contain a fetus of considerable size, temperature loggers (results can be found in thesis of Ludvig Orsén 2016) were placed inside the nests and these were later revisited. If the eggs were not considered too young they were collected upon the first visit. The eggs were removed at random from different locations from within each nest, cleaned with alcohol (95%), wrapped in tin foil and were put in a -20°C freezer one to three hours following

excavation. The eggs that were not collected were carefully put back into the nest in the order and direction that they were found. All of the nests were reassembled and adjusted to look undisturbed. To prevent predation on the nests containing temperature loggers (4 out of 9 nests), these nests were covered with a metal net and the surrounding edges of the net with larger sheets of metal. These protective nets were removed upon the second visit to the nest at the time of excavation of the temperature loggers and collection of the eggs.

The eggs were taken out of the freezer and left in an ice bucket filled with water and ice and left to thaw over night. Each egg was cleaned with 95% ethanol, and weighted. A pointy scissor was used to poke a hole in the egg shell and an oval window was cut from the elongated part of the egg similarly to the methods as described by Webb and Manolis (1987), and the fetus was extracted from the rest of the egg, and the remaining egg content was put in falcon tubes. The viability of the eggs was determined from the uniform existence of opaque bands on the eggs. If the eggs were visibly damaged (cracks), but had a uniform opaque band with the other eggs in the same clutch they were assumed to be vital. The estimated time of egg lying was determined using the post-laying stages of development guide by Ferguson (1987). The estimated dates for the nests were created using *C. porosus* data (The incubation period for *C. acutus* is 85-111 days and *C. porosus* is 80 – 98 days Ferguson (1987)). The developmental stages of the fetuses were determined by using the methods described by Ferguson (1987), but because the developmental stages of crocodilians also depend on temperature, the dates presented in this thesis are only rough estimates to serve as indications of when the eggs were laid.

A section of the brain, believed to contain the hypothalamus (figure 2) and the liver were removed from the fetus. Each tissue sample was divided into two pieces, approximately 1,5 - 2 mm in diameter and the pieces were put in individual Eppendorf tubes. One of the two tissue pieces was stored in 1 mL RNA*later*® stabilization solution from *Ambion*^{TM} to stabilize and protect the cellular RNA, and the other piece in 95% alcohol for DNA stabilization. The samples were frozen in -20°C. Upon arrival in Sweden they were initially put in a -20° freezer, and 24 hours later in a -80° freezer. During the dissection the remaining brain (figure 2) and heart tissues were stored in -20°C in eppendorf tubes to allow for later analysis of mercury concentrations.

Chemical analysis

The genome of the American crocodile has not been sequenced previously, so the designing of primers for the two target genes (CYP19, CYP1A) had to be made degenerative to increase the likelihood of amplifying a PCR-product. The degenerative primers for the CYP1A were designed based on FASTA gene sequences from evolutionary similar species from the NCBI GenBank database (see appendix for exact sequences). The primers for CYP19 were designed using a FASTA file from Alligator mississippiensis, which aligned nicely with other CYP19 species' sequences in the NCBI database. Highly conserved areas in the genomes were found using the Clustal Omega tool at the EMBL-EBI website for alignment of different gene sequences, and the NCBI primer blast function was used

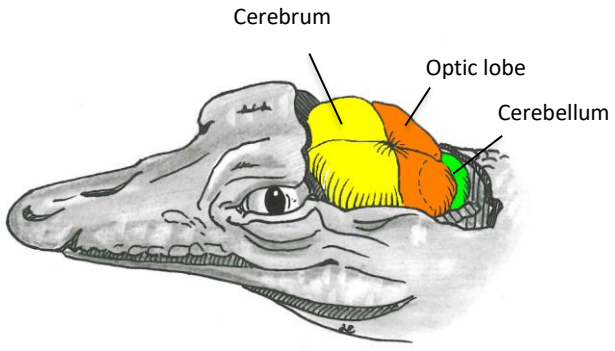
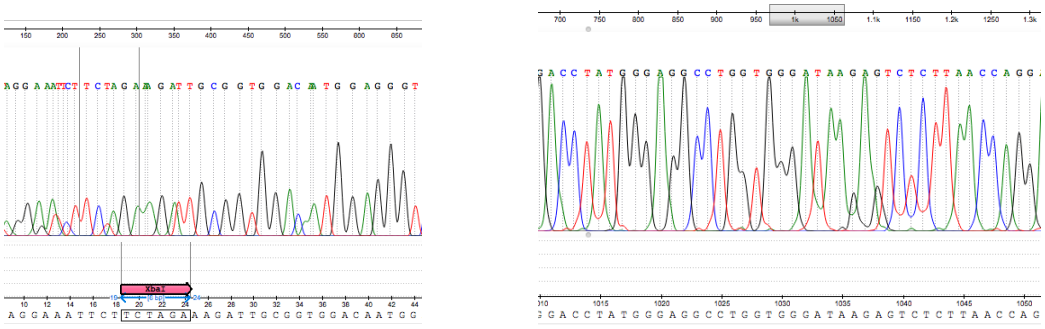


Figure 2. The crocodile fetus brain at the developmental stage of 25, once the skull bone has been removed. The colored sections were used for mercury analysis, and the remaining portion of the brain including the hypothalamus and the brain stem were used for mRNA analysis.

to design primers specific to the conserved areas of the target genes. Primers were selected so that no more than 2 bases were non-specific in a 20 bp long primer sequence.

The samples to be analyzed for mRNA concentrations were homogenized using a glass Teflon homogenizer and treated with the RNA extraction kit *Quick-RNA™ MiniPrep* from *Zymo Research*, USA, according to the manufacturer's protocol for samples stored in RNA*later*® stabilization solution from *Ambion™* (600 µl lysis buffer). Samples were treated with *DNase 1* (supplied with kit). Retro-transcription was performed using the first strand cDNA synthesis kit *qScript™ Flex cDNA Kit* from *Quanta BioSciences Inc.™*, USA, and RT-PCR was used with Phire II polymerase (initially *Phire Hot start II DNA polymerase* from *Thermo Scientific* and then *Phire® Hot Start II DNA Polymerase* from *Finnzymes* a part of *ThermoFisher Scientific*) and accompanied 5x Buffer and dNTPs. A range of different PCR-programs were tried but the PCR program generating clean products most frequently was: 50.0°C for 2 min, 95° for 15 min, (94° for 1 min, 69°C for 1 min, 72.0° for 1 min) x 40, 72°C for 7 min, and finally 4.0°C until the machine was unloaded.

PCR-products were run on a 1% agarose gel and if bands approximately matched the expected size of DNA-fragments, they were cut out and purified by using *QIAquick Gel Extraction kit* from Qiagen (Germany). Purified PCR-products were transformed with the pJet1.2/blunt Vector using the kit and then cloned into DH5α competent cells using the CloneJet PCR Kit purchased from Thermo Scientific (made in Lithuania), and following the protocol for blunt end ligation and cloning. The transformed cells were grown at 37°C at 225 rpm for an hour to allow for the ampicillin resistance gene in the vector to be transcribed, and then plated on plates with and 100 µg ampicillin/ml LB-medium overnight (>16 hours). If the positive control and negative control were both according to expected results, colonies were picked and put to grow in LB-medium and ampicillin for 16 hours. The vectors with the ligated PCR-product were extracted using *QIAEX® II Gel Extraction Kit* (Germany), and the product was sent to GATC (Germany) for Sanger SUPREME sequencing. The vector sequences received from GATC were analyzed to first find the location of the genes Xhol and Xbal (Figure3), in between which the insert should be located, using the program uGENE, and then isolated and compared to sequences in the NCBI data base for confirmation of identity. The alignment score and the product name were used to confirm that the cloning of selected genes were successful.
All gene sequence's chromatograms were analyzed and the inserted segment of the vector was identified using restriction sites and the primer sequences. The vector sequence and areas of the inserted sequence with low quality were cropped out (figure 3), leaving a sequence known to have been the PCR product. The cropped PCR product sequence was then run against the NCBI GenBank database to find previously reported matching sequences, with the goal for them to be the same gene as the primers were designed to produce. If the sequences did not match any products on the database they were compared to the initial sequence used when designing the degenerative primers.



i) ii)

Figure 3. An example for how sequence chromatograms were analyzed to detect uncertainties in reported base pairs. The figures show a beta-actin sequence from *C. acutus.* i) after the restriction site XbaI where the certainty of the sequencing being correct is high ii) the end of the sequences where this certainty decreases due to multiple peaks in the chromatogram.

Mercury analysis

For analyses of the total mercury concentrations in heart and brain tissue (all parts except the hypothalamus), samples were shipped to Canada frozen in containers that were free of mercury. About 10 mg of sample was weighed in a sample boat and placed in the auto-sampler of a Nippon MA-3000 high temperature combustion mercury analyzer. The instrument runs automatically printed.

Statistical analysis and data interpolations

All ecological data and data on mercury concentrations were compared using linear regression models designed using the program *R*. All models were first checked for normality, the distribution of residuals and Cook's distance to ensure that the models were appropriate, and then an anova was run on the models. If the anova showed significance, a post-hoc test was performed to investigate the source of variation.

Results

Ecological data

A total number of nine nests were found by the *Tempisque River* between February 18 and April 11, 2016 (figure 4), out of which eight bordered the national park *Palo Verde* (figure 5). The ninth nest was found south of the river mouth, by one of the creeks supplying water to one of the main cultivation basins of a shrimp farm. Spatial analysis of the surrounding land usage showed that rice and sugar cane cultivation lay upstream of the nests bordering the national park (figure 6).

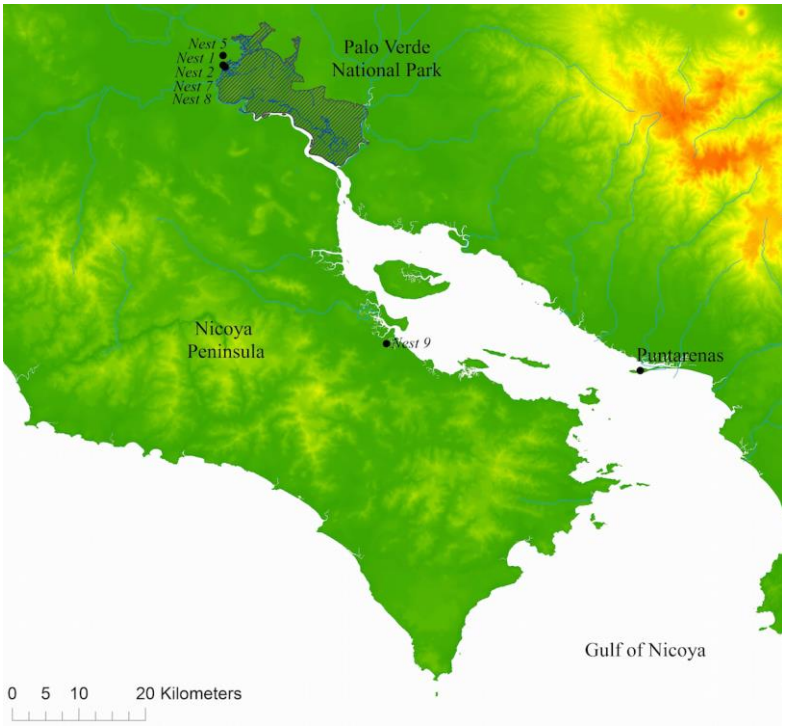


Figure 4. Map showing the locations of the found nests of *Crocodylus acutus* in Costa Rica. All but one of the nests were located within or at the border of the Palo Verde National park in the Guanacaste providence

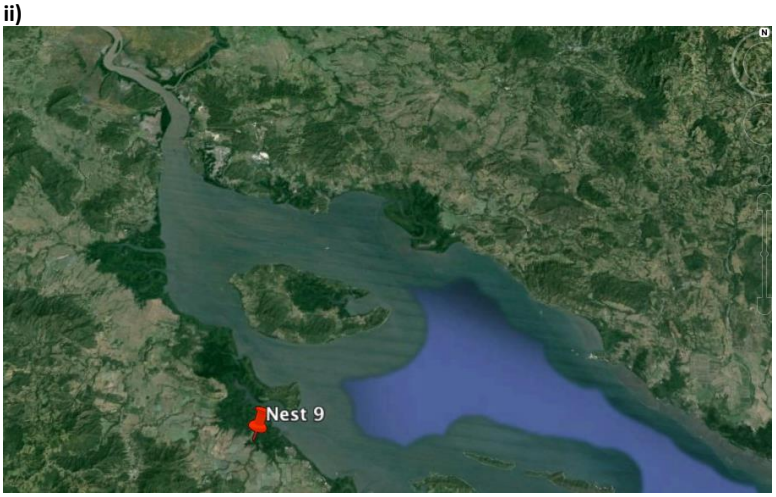
The clutch sizes varied between 8 and 42 eggs, with the average number being 28 eggs (SD = 12.11). In one of the nests (No. 6) all of the eggs were decomposing. The youngest clutch was laid February 3rd – 11th 2016, and the oldest clutch February 22nd – 24th 2016. The average weight of the eggs was 107.48 g and ranged from 98.30 to 121.42 g for the different clutches (SD = 5.4). The heaviest clutch weight average was 114.5 g and was also one of the older ones (developmental stage 25), and the lightest clutch weighed an average of 98.4g (SD within clutches = 1.62 - 5.94). The average length of all of the fetuses within their clutches ranged from 11.1 to 22.8 cm (SD within clutches = 0.27 – 1.78). The weight of the eggs within each clutch varied to a greater extent than that of the length between the embryos within clutches. These data are summarized in table 1 and 2.

The only correlation is between the lengths of the embryos and their developmental stages (linear regression model, p-value = 0.0002)(Figure 7-9). There was no correlation between the weight of an egg and it's length or it's developmental stage, nor any indicators that the clutch size or weight of the eggs would be affected by other ecological factors such as nest location. Weight did also not affect the survival of the eggs during development. The developmental stage can be extrapolated to time of egg-laying, and the time of egg laying did not seem to be a factor for any other ecological factor such as weight, clutch size or survival. Predation had with certainty occurred in 2, and possibly in 4 of the 9 nine nests. There was no measurable significant effect of opening the nests (4 in total), moving the eggs and reclosing the nests, based on the ratio between dead and viable eggs between first and second time opening a nest (Welch Two Sample t-test p-value of 0.08145, p-value = 0.08145). All different factors were combined in a linear regression models, but no combinations showed any significant relationships between for example the clutch size and the weight of the eggs, or other factors, except

for the relationship between length and developmental stage. The different factors examined include; clutch size, weights of eggs both within clutches and average weight of clutches, lengths of embryos, upstream vs. downstream positioning, and developmental stage.

i)

ii)



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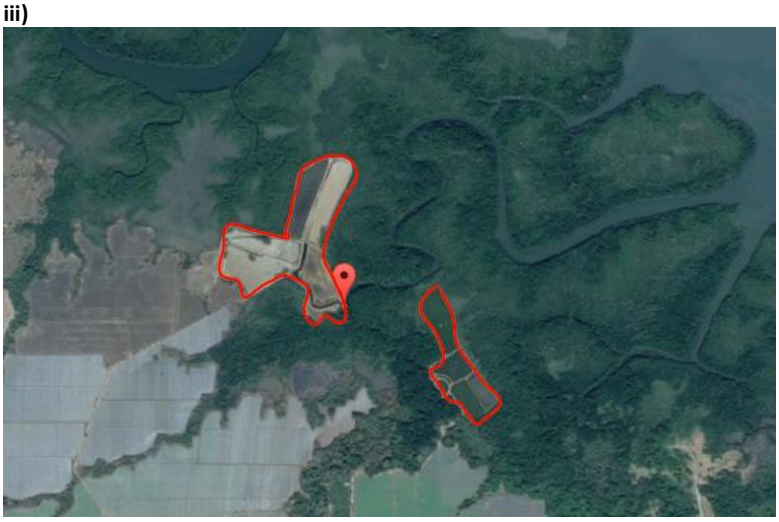

Figure 5. Satellite images of nest locations. i) Nests found bordering the Palo Verde National park, ii) Nest number 9 found in the river mouth of the Tempisque River iii) Showing the exact location of nest number 9 and the boundaries of the shrimp farm basins. (Photos: Google Earth)

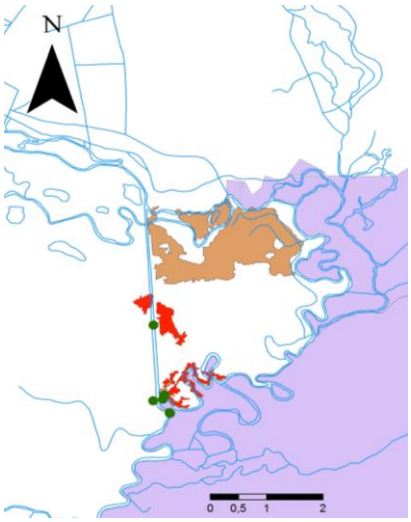

Figure 6. Spatial analysis using ArcGIS for landsat images from 2005 over the land usages. The figure illustrates the nest locations (green dots) in relation to rice fields (red) and sugar cane fields (orange). The purple field represents the national park Palo Verde, in Guanacaste (scale in km).

Table 1 The table summarizes the biologically relevant data taken from the field collection of *Crocodylus acutus* eggs from the Tempisque River, Guanacaste, Costa Rica. The viability of the eggs were determined from the uniform existence of opaque bands on the eggs, some of the eggs were visibly damaged (cracks, and indents) but if they had bands uniform to those around they were counted as vital.

Nest No.	Dev. stage	Average weight (g) (SD)	Average length (cm) (SD)	Date found (d/m) 2015	Estimated date of egg laying earliest – latest (d/m) 2015
1	24	106.02 (1.62)	20.6 (0.5)	18/2	6/2 – 13/2
2	25	114.63 (5.94)	23.2 (0.5)	22/2	4/2 – 19/2
3	24	91.97 (N/A)	18.5 (N/A)	25/2	7/2 – 14/2
4	23	106.05 (2.75)	14.2 (1.8)	25/2	3/2 – 11/2
5	20	110.96 (4.39)	15.8 (0.3)	6/4	22/2 – 24/2
6	N/A	109.41 (0.21)	N/A	7/4	Unknown – all eggs rotten
7	23	110.96 (4.38)	15.8 (0.4)	11/4	8/2 – 16/2
8	25	107.90 (3.31)	21.8 (1.2)	11/4	5/2 – 20/2
9	25	98.40 (3.22)	22.3 (0.4)	10/4	4/2 – 19/2

Table 2 The table shows the number of eggs found in each nest upon the first and second visit, how many eggs that were removed upon each visit, whether predation had occurred on the nests (i.e. eggs missing) and the number of eggs that were damaged due to the finding or excavation of the eggs.

Nest No.	Number of eggs in nest		Number of vital eggs in nest % (No.)		Number of eggs removed		Predation on nest (No.)	Eggs damaged by error
Visit	**1st**	**2nd**	**1st**	**2nd**	**1st**	**2nd**	**Last**	**Total**
1	41	35	100%	88.6%	6	4	No	0
2	36	34	97%	85.2%	0	4[4]	No	3
3	26	2	100 %	50%	3	1	Yes	1
4	42	31	100%	71.0 %	3	4	Yes	0
5	32		96.9%		5		No	N/A
6	8		0%		5		Maybe	N/A
7	29		96.6%		4		No	N/A
8	-		-		3		No	N/A
9	10		100%		4		Maybe	N/A
AVG	28	25.5	98.81%	73.7%	**TOTAL:** 46			4

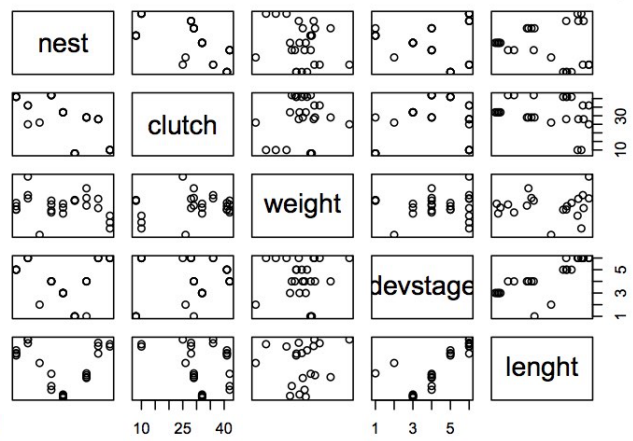

Figure 7. A plot generated by the statistics program R showing all of the ecological data (nest location, size of clutch, weight of eggs within each clutch, the developmental stage of the clutch, and the length of the embryos within each clutch) and how they relate to one another. The only obvious correlation that can be seen is that of the developmental stage "devstage" and length of the embryos "length".

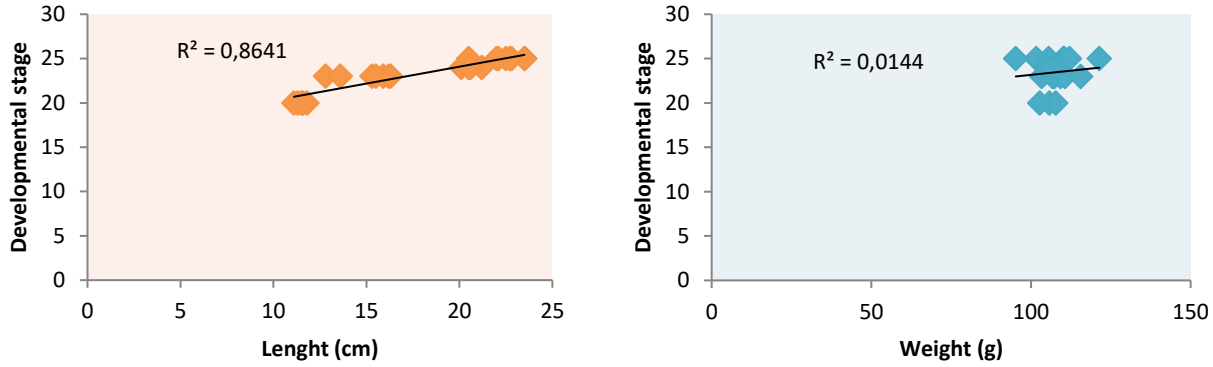

Figure 8. The developmental stages of the embryos plotted against the length and the weight of the embryos. There is a correlation between the developmental stage and the length of the embryos, but not with their weights.

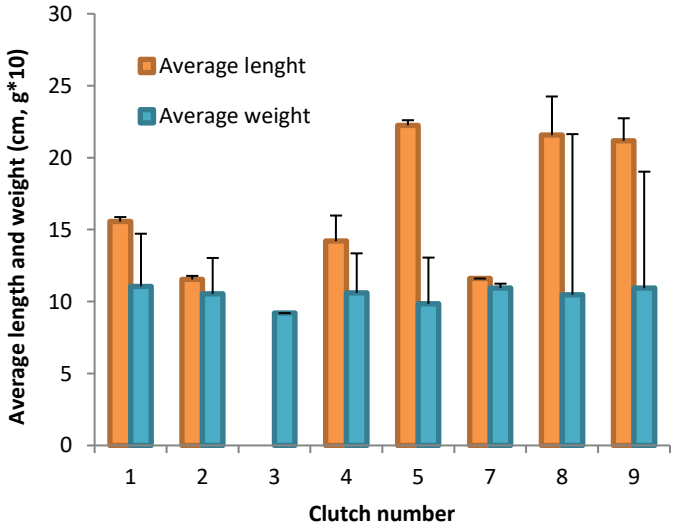

Figure 9. Average weight and length in different clutches. Error bar = standard deviation.

Mercury concentrations

The mean mercury concentrations for the brain and heart samples (0.01 ppm and 0.03 ppm respectively) are the lowest mercury concentrations ever detected from crocodilian fetuses or eggs (table 3, figure 9). The mercury concentrations in heart tissue were significantly higher than those in brain tissue (anova, p-value = 0.039), and also had a greater variance of the concentrations than the brain mercury concentrations did (figure 10). None of the ecological factors (weight of eggs, nest location, clutch size, lenghts of embryos) noted in this study affected the concentrations in the two tissue types. The mercury concentrations were similar within clutches (figure 11).

Table 3. A summary of the mercury data collected from American crocodile fetus tissues in this study and other mercury concentration data from crocodile species worldwide (ppm wet mass). Table modified from Rainwater (2001).

Species	Tissue	Location	n	Mean (± SE)	Range	Ref.
American crocodile (*Crocodylus acutus*)	**Brain (fetus)**	**Costa Rica**	**20**	**0.01 ± 0.001**	**0.003 - 0.024**	**This study**
	Heart (fetus)	**Costa Rica**	**20**	**0.03 ± 0.002**	**0.008 - 0.045**	**This study**
	Egg	Florida, USA	5	0.09 ± 0.01	0.07 – 0.14	(Ogden *et al. 1974)*
	Egg	Florida, USA	9	0.13 ± 0.01	NR	(Stoneburner and Kushlan, 1984)
	Scutes	Costa Rica	6	0.09 ± 0.03	NR	(Rainwater *et al.* 2007)
Morelet's crocodile (*Crocodylus moreletii)*	Egg	Belize	31	0.07 ± 0.01	<0.02 – 0.023	(Rainwater *et al.* 2007) (Wu *et al.* 2000)
	Scutes	Belize	9	0.10 ± 0.02	NR	(Rainwater *et al.* 2007)
	Scutes	Belize	10	0.07 ± 0.02	NR	(Rainwater *et al.* 2007)
Nile crocodile (*Crocodylus niloticus*)	Egg	Zimbabwe	26	0.23 ± 0.02*	0.02 – 0.54	(Phelps *et al.* 1986)
American alligator (*Alligator mississippiensis*)	Egg	Florida, USA	4	0.54 ± 0.06	0.41 – 0.71	(Ogden *et al.* 1974)
	Egg	Florida, USA	34	ND	ND	(Heinz *et al.* 1991)
	Egg	South Carolina, USA	10	NR	0.01 – 0.02	(Bowles 1996)

NR = Not reported, ND = Not detected, * = Based on dry mass

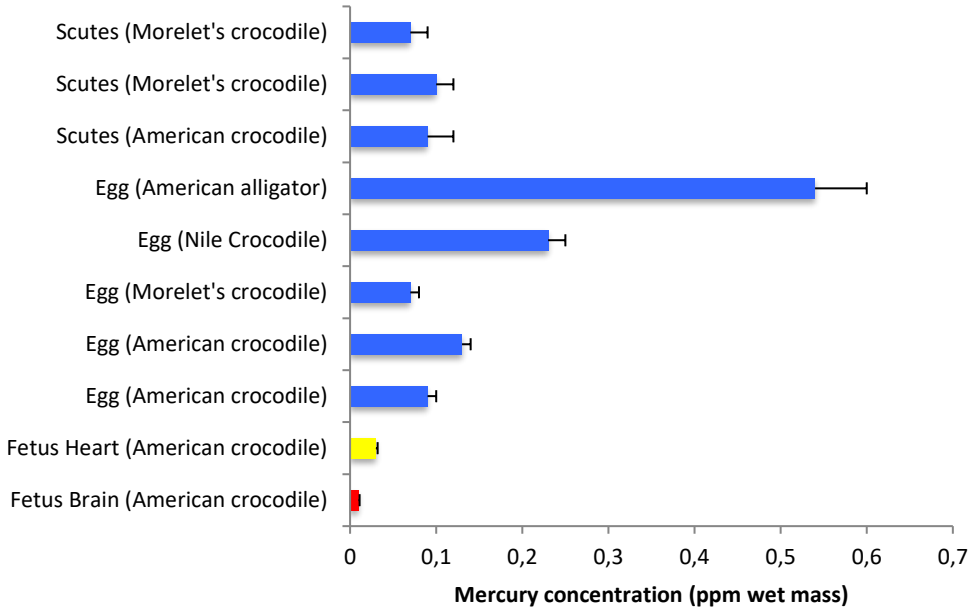

Figure 9. Mercury concentrations (± SE) found in crocodilian tissues around the world. The red and yellow represents mercury concentrations found in fetus heart and fetus brain, respectively, from this study. The blue bars represent the data collected in other studies where mercury was measured in scutes and eggs (For data references see table 3).

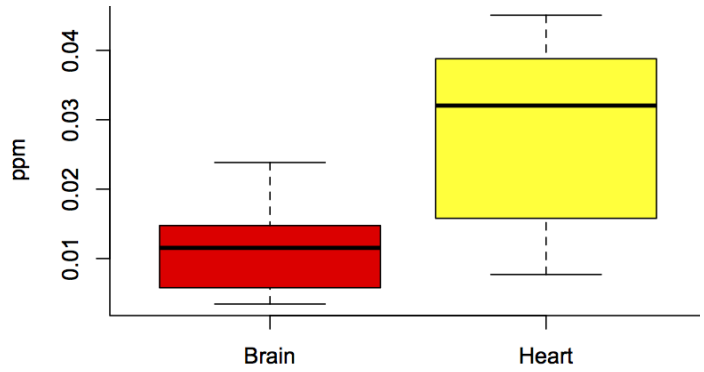

Figure 10. Mercury concentrations (ppm) from brain and liver tissues from crocodile fetuses collected.

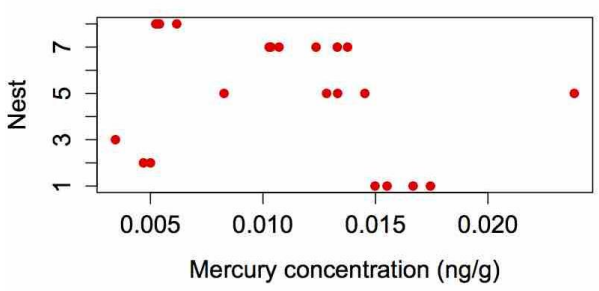

Figure 11. Mercury concentrations (ng/g) from brain tissues from crocodile fetuses collected, demonstrating how mercury concentrations differ between clutches.

mRNA biomarkers

Out of the primers designed, a few consistently showed products (table 4) following PCR and gel electrophoresis. None of the PCRs had only a single product, and multiple bands were always present, if any product. The only sequence product identity that could be confirmed was the reference gene (Beta-Actin).

The primer pairs designed for Beta-actin were consistent, showing bands on all samples when used (if any bands), and all of the bands of expected length that were sequenced always aligned with other Beta-actin sequences from other species on the NCBI database, providing good proof of the sequence's identity. The extracted mRNA beta-actin sequences matched with other partial beta-actin cds sequences made from mRNA in the NCBI database. The closest match in the database was a partial mRNA cds sequences from *Alligator sinensis* (accession id. NM_001286847.1), and the match had a query coverage of 38% and an identity similarity of 99%. The top 50 sequence matches in the NCBI database were all beta-actin sequences and they all had a minimum of 93% identity similarity to my sequence, and a query coverage ranging from 15 to 38%. When beta-actin cds sequences from the database with high query coverage (>95%) were compared to the sequence segment obtained from *C. acutus*, they aligned in different places within the sequence (figure 11), indicating that the sections between these confirmed segments most probably is beta-actin. When these sections in between the confirmed beta-actin sections were examined and aligned with BLAST, they did not match with any relevant products or got high query coverage scores, validating this conclusion.

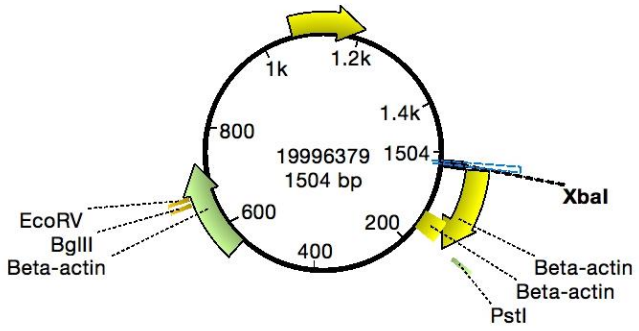

Figure 11. Blast alignment with the NCBI database and the placements of the portions of the sequences that match with >95% to reported partial cds mRNA Beta-Actin genes and restriction sites. The yellow and green sections indicate different matches within the database, and the blue dotted line indicates the beginning of the sequence, next to the restriction site XbaI.

The identity of the other mRNA cds sequences (CYP1A and aromatase) could not be confirmed. The primers designed to generate portions of the CYP1A gene did not produce products with convincing query coverage or identity scores with any gene related to CYP1A in the NCBI GenBank database. The mRNA cds sequences obtained are therefore unlikely the right sequence for the gene. None of the degenerative primers designed for CYP1A4 produced a product after PCR during the pilot study.

The mRNA cds product designed to be CYP19 gene did not match other aromatase sequences within the NCBI database. It did however match exclusively with crocodilian species, and the hit with the highest query coverage on the NCBI database using BLAST, was an MHC class II beta chain, with 43% query coverage and 82% identity similarity (accession number KP11841.1). MCH (major histocompatibility complex) class II family consists of proteins that exclusively are present on antigen-presenting cells. When the sequences for two different cloning products using CYP19 primers were aligned (multiple sequence alignment), they showed almost identical resemblance to one another (figure 12), and when one of these sequences were compared to a confirmed aromatase sequence it showed some similarity (figure 13). See appendix for exact mRNA products.

Table 4. The degenerate primers used that consistently produced products

Gene	Forward primer	Reverse Primer	Expected product length	Confirmed product	Single band
CYP1A	GCCCTCAAAACCTTTGCTAC	GATTMACTTGCCACTGGTTG	720	No	No
CYP19	TGGRAGCAAGCTTGGRTTAC	GCACCGTCTCAGAAGWGTCA	233	No	No
Beta-Actin	AGCAAAAGAGGTATCCTGAC	TGCGGTGGACAATGGAGGGT	900	Yes	No

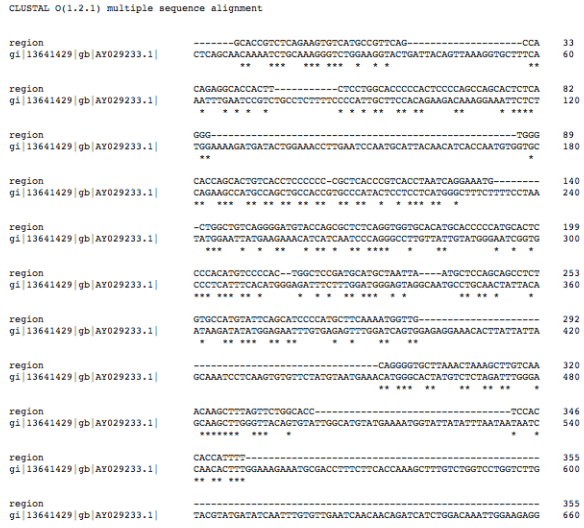

Figure 13. The alignment of one of the sequenced cloning products designed to be CYP19 showed some resemblance to a confirmed CYP19 sequence.

Figure 12. When the sequences for two different cloning products with the suspected product CYP19 were aligned they showed almost identical resemblance to one another.

Discussion

This thesis has shown that it is possible to design primers for PCR using gene sequences that are not known, but that it is tedious work doing so, and that there might be better solutions for designing molecular biomarkers, and that knowing the genome is a key to doing it efficiently – why it would have been more time efficient to just sequence the genome instead of designing degenerative primers. However, this thesis has successfully produced a sequence for a reference gene that can be used for future genomic studies on this species. It also provides valuable benchmark values for future studies on the American crocodile population in the Tempisque River as well as for other populations of the same group of species worldwide. It is unlikely that there is a mercury contamination problem in the area based on the low mercury concentrations in heart and brain tissue, and the total mercury present is probably not methyl-mercury. It is also very unlikely that the observed skewedness in sex ratio in the crocodile population is caused by pollution or endocrine disruption but is more likely being caused by ecological factors pertaining to the increasing population size. This thesis provides a first step in the process of developing molecular methods for linking ecological and chemical observations for the *C. acutus* population in Costa Rica.

Hypotheses

i) Methods for analyzing the selected biomarkers be successfully developed and be used for future genomic studies on *C. acutus*

The methods for analysis of selected biomarkers were not completely successfully developed for future genomic studies; only the reference gene sequence could be confirmed, and the optimization of a qPCR program or specific primers for any of the sequences were not developed as time became a limiting factor. The method was however developed a good length of the way, and the obtained sequences might still prove to be useful and valuable – they just need to be confirmed. There are no actual reasons to believe that a qPCR could not be preformed once a specific DNA sequence has been found and confirmed, and with that in mind this thesis could serve as very valuable for those who wish to continue on with the development of mRNA markers for *Crocodylus acutus*. The methods developed in this thesis are in need of improvement before they can be used for future genomic studies on the skewedness of the sex ratio for the crocodile population in Costa Rica, but if more time is invested in finding the sequences for the genes, the author strongly believes that they can be applied for linking environmental effects to their causes. Perhaps the most time efficient way to do so would be to sequence the genome as a whole and then look for the selected genes, instead of using degenerative primers.

ii) Expression of CYP19 and CYP1A mRNA occurs in brain- and liver fetus tissue respectively during the sex determining development period.

Although bands were present after gel-electrophoresis following the RT-PCR for all of the designed degenerative primers, it cannot be determined whether the expression of CYP19 and CYP1A was present in brain- and liver tissue respectively, because none of the mRNA products could be confirmed.

iii) The American crocodile (*Crocodylus acutus*) exhibits a P450-monooxygenaze system more similar to the CYP1A1 found in reptiles and amphibians rather than the CYP1A4 found in birds

The sequence generated to match the CYP1A1 gene through the usage of degenerative primers did not match with any cytochrome P450-systems in the NCBI database, and the primers designed to match CYP1A4 did not yield any product at all. It can therefore, through this study, not be concluded whether the American crocodile exhibits a P450-monooxygenaze system more similar to the CYP1A1 found in reptiles and amphibians rather than the CYP1A4 found in birds.

iv) There are measurable amounts of mercury in crocodile fetus tissues and the concentrations differ between tissue types

Yes, there were measurable amounts of mercury in both heart and brain crocodile fetus tissues and they differed between tissue types. Mercury concentrations were higher in heart tissue than in liver tissue, most likely due to the type of mercury that the crocodiles are exposed to.

v) The mercury concentrations in fetus tissues are comparable to mercury concentrations found in other crocodilians around the world.

The mercury concentrations found in the fetuses were relatively low in comparison to other reported mercury concentrations in different tissues from other crocodile species. In order to be completely sure one should do a complete analysis of the eggs as a whole.

Ecological data and mercury concentrations

The most notable observation made was that the average clutch size for the Tempisque population seems to be smaller compared to the average clutch size reported on other sites in the world (Ferguson 1987b). Differences in clutch size can be attributed to the age of the female (Ferguson 1987b), which means that the clutch sizes in the Tempisque River can function as a demographic measure of mating females. If this holds true for the population in the Tempisque River, then one must wonder whether it is because the females are younger on average than the males, or if the population as a whole is younger compared to other populations worldwide. Furthermore, it raises the questions why the females would have a lower survival rate than the males, or why the population would be filled with young individuals. The collection of demographic data from this study may function as benchmark value for future studies, as there is very little information available about the nests in Costa Rica. The lightest clutch average weight was of the same developmental stage as the clutch of the heaviest average weight, confirming that weight is not a measurement of the development of the fetus. (It should however be noted that the heaviest clutch average was from a clutch residing very close to pools of water with cultivated shrimp treated with testosterone and this may have affected the weight of the eggs.)

It is to date unknown whether excavating eggs from nests and then re-placing them affects the hatching success or the survival rate of the hatchlings. My study could not show any statistical significance that opening and reclosing the nests would affect the survival rate of the eggs, but with a sample size smaller than what's needed for running reliable statistical testing and an inability to take into account the effect of predation, it might still be very possible that this handling of the eggs would affect the hatchlings negatively. There is to the author's knowledge only one other study where this has been

done and where the hatching success has been noted (Charruau, 2012). Charruau's study (2012) did not find any relationship between opening the nests and the hatching success and the proportions of crocodiles born alive, but offers no explanations to why only 73.1 % of the variability in the proportion of hatchings reaching a complete development could be explained with multiple regression analysis based on environmental data. Crocodilians have on multiple occasions been removed from their native nesting site to a controlled laboratory environment with complete hatching success, but this doesn't necessarily mean that it can be done in the field where conditions cannot be controlled. Upon excavation of about half of the nests in this study, an air pocket above the eggs was clearly present, but impossible to restore when the nest was recovered after egg handling. The function and importance of the air pocket is unknown.

The total mercury concentrations in the brain and heart fetus tissues correlated with no ecological factor (egg weight, clutch size, date laid etc.). Because the relative egg weight represents the hatchling size of the crocodile when born, and larger individuals would have a higher survival probability in terms of competition, this indicates that the observed mercury concentrations most likely do not affect the survival of the hatchlings. Furthermore, the mercury concentrations found in the crocodile tissues are the lowest ones observed for crocodilians, although it should be noted that this study only analyzed specific tissues whereas the other studies have examined the mercury concentrations in either entire eggs or in the scutes, and that if the eggs as a whole had been analyzed that assumption might not hold true as the relative mercury concentrations between tissues will depend on the type of mercury the organism is exposed to. The fact that mercury levels were significantly higher in heart tissue than in brain tissue gives a clue to what type of mercury that the crocodiles in the Tempisque are exposed to. Methylated mercury can penetrate the brain blood barrier, whereas non-methylated mercury cannot, indicating that methylmercury is less abundant than Mercury in other types chemical states. Similarly to a study done on elasmobranchs on the pacific coast of Costa Rica (Sandoval-Herrera et al., 2016), it is probable that the total mercury contamination in the area is minimal.
Metals and organochlorine pesticides have however been found in measurable quantities in crocodiles scutes from the Tempisque and Tarcoles rivers (Rainwater *et al.* 2007). This shows that crocodiles accumulate environmental contaminants that are found in the Central Pacific region (Fuller *et al.* 1990). In a study including 6 captured individuals of the American crocodile from the Tárcoles River, Costa Rica, mercury was found in scutes with a mean concentration of 93.5 ± 27.0 ng/g wet mass (Rainwater *et al.* 2007). The mean concentrations of mercury from scutes found in Morelet's crocodiles from Belize ranged from 72.7 – 98.7 (same study), indicating similar level of mercury contamination in the two studied areas.

Messenger RNA biomarkers

The development of mRNA biomarkers was not fully completed due to a lack of time and the inability to confirm the gene sequences intended to function as the biomarkers. The only mRNA sequence that could be confirmed was for the reference gene beta-actin. As both the end and the beginning of the sequence matches with confirmed beta-actin sequences from other species, it can be concluded that I have found a larger piece of the beta-actin gene than what has been reported for any previous crocodilian, which may prove very useful for future genomic studies on this species. It is also noteworthy that it matched without exception to birds, turtles and alligators, indicating that the beta-actin gene for crocodiles might be significantly different to that of more distant vertebrates such as mammals. Although the development of a method for using CYP19 and CYP1A mRNA concentrations as biomarkers was not completed, the author still considers the continued development of this approach to be of great value. The sequence of aromatase (CYP19) could maybe be used as a new tool for establishing the sex ratios, as there is yet no developed method for sexing American

crocodile juveniles. Sexing on hatchlings can be performed on both *C. johnsoni* and *C. porosus* by examination of the cliteropenis as well as through histological examination of the gonads (Webb *et al.* 1987), but whether sexing can be done on the American crocodile is not known. If aromatase concentration values are divergent from the expected concentrations based on logged temperatures in the nest and what is known about aromatase expression and sex-development, it can also be used as an indicator of whether endocrine disruption on the pathways of aromatase expression is occurring or not. An analysis of the aromatase expression of the crocodile fetuses in the Tempisque River could help answer the question if the sex ratio is due to fewer females than males being hatched, or if something else is causing the shift, i.e. temperature or other ecological factor such as habitat change. It could also help rule out endocrine disruption as a cause for sex ratio skewedness. Unfortunately, a complete verification of the sex ratios within a clutch would probably require sacrificing the entire clutch, as random selection and single sampling might not be sufficiently random when the sex distribution in the nest itself is far from random and might differ between clutches (Charruau 2012).

Further molecular analysis of the CYP1A gene (qPCR of mRNA concentrations) should ideally be combined with molecular analysis including measurements of protein abundance and enzyme activity (EROD, ECOD, AHH, MROD) to get gain a more complete picture for monitoring environmental organic pollution in the area. Likewise, the analysis of CYP19 mRNA expression would also ideally need to be complemented with hormone concentration data. The results from only analyzing an mRNA expression, cannot alone provide evidence for causality, but must rather be combined with data through the range from a molecular level spanning all the way to an environmental level. Nevertheless, this thesis has developed a tool that can be applied for such a study in the future, and used properly together with other observations, it may help serve as a red flag for environmental contamination that in the future might have a negative effect on the ecosystem as a whole.

For future attempts at developing mRNA biomarkers, a creation of a cDNA library from start or a sequencing of the entire genome of the crocodile is suggested. It would have been more time efficient and would be about equally costly as trying to develop degenerative primers and go through the testing thereof and optimizing PCR-programs. However, this thesis shows that it is possible to find products whose genome sequences are unknown, using degenerative primers, and that the idea as a whole to use mRNA markers as biomarkers could work in the future if the method is further developed. Another comment should be made on the difficulty of finding and using positive and negative controls, as permits did not allow for a negative control site for this study and no samples existed that could be used as positive controls. A design ensuring both negative and positive controls should have been given more thought ahead of the initiation of this project.

The observed skewedness in sex ratio

If the observation that males are overrepresented in the Tempisque River is representative of actual sex distributions in the area, it is still to be determined what is the cause of this shift in sex ratio. With the TSD in mind, the most plausible cause would of course be an ecological change in temperature, affecting sex distribution through hatchling sex ratios, or perhaps an ecological change causing a higher survival of males. With the extensive use of pesticides in today's agricultural practices, and with more occurring evidence indicating endocrine disruption in wild populations (Bergman *et al.* 2013), the possibility of pollution as a cause for a skewedness in the sex ratio should be explored. Generally, there are two different explanations to consider for reptiles with a temperature-dependent sex determination (Bergman *et al.* 2013); One explanation is that chemicals could interfere directly with the biochemical processes of sex determination, which leads to early death or to poor conception of one of the sexes (Svensson *et al.* 2007), and the other explanation is that an exposure to a chemical may induce sex maturation of one of the sexes more than the other (Bergman et al., 2013). For reptiles

with temperature-based sex determination (TSD), changing temperature conditions, such as local or global climate change, could also present a plausible third explanation for a skewed sex ratio. There are relatively few examples where sex ratios are skewed towards an overrepresentation of males in natural animal populations (Allner *et al.* 2010; Erikstad *et al.* 2011, Larsson and Förlin 2002, Larsson *et al.* 2002). For unknown reasons, the most commonly observed cause for a skewed sex ratio biased towards males is the presence of a pulp mill, although the exact mechanism behind this is not known (Kovacs *et al.* 1995, Mower *et al.* 2011, Örn *et al.* 2006). Experimentally tested methods for shifting the sex ratio to benefit males include studies where exposure to androgens, and aromatase inhibitors in the laboratory have shown to shift the sex ratios to benefit males in both fish and birds (Kinnberg *et al.* 2007, Morthorst *et al.* 2010; Yang *et al.* 2011; Zerulla *et al.* 2002). Research on birds also suggests that species that are sexually size dimorphic can shift the offspring sex ratios if the mother is in poor body condition, often due to contaminants (organochlorines) (Erikstad *et al.* 2009, 2011). Although the ovulatory patterns differ between birds and crocodilians, these studies may still be relevant for crocodiles because of the anatomically homologous physiological reproductive system of crocodilians and birds (Guillette and Milnes 2001). Aromatase inhibitors can also serve as means of changing the sex ratio to benefit males in animal populations, but in crocodilians with a TSD, the expression of aromatase isn't the trigger for sex determination. It does, however, play an essential role in the development of the ovarian differentiation (Gabriel *et al.* 2001, Smith and Joss 1994).

All evidence points in the direction that the temperature-based sex-determination in crocodiles cannot be overridden by exposure to chemicals. All laboratory attempts to override the TSD has failed, and although low doses of embryonic *in ovum* topical exposure to atrazine and endosulfan (two known aromatase inhibitors) (0.02ppm and 0.2ppm respectively) to *Caiman latirostris* eggs during the sex-determination period altered the testicular histoarchitecture in the hatchlings, as well as the balance between proliferation and apoptosis of their testicular cells, it did not over-ride any sex-determination temperatures (Rey *et al.* 2009). These doses represent environmentally relevant concentrations in South America (Laabs *et al.* 2002, Menone *et al.* 2000; Wu *et al.* 2000), and possibly also the Tempisque River. The differentiation of the female ovaries during fetal development in crocodilians is highly dependent on the presence of aromatase (Gabriel *et al.* 2001, Smith and Joss 1994), and aromatase expression in the eggs can be affected by environmentally relevant concentrations of aromatase inhibitors (Rey *et al.* 2009, Laabs *et al.* 2002, Menone *et al.* 2000, Wu *et al.* 2000). If an exposure to xenobiotics is to explain an observed sex-ratio skewedness originating from the biochemical processes during fetus development, the only theoretical explanation would be that an inhibition of aromatase causes an inability for developing of the female sex organs, which may or may not rendering a masculinization of these organs if there is not a negative feedback preventing testosterone to be produced, which would make adult females exhibit protrusions similar to that of the males. Considering all data on sex ratios for *C. acutus* is based on cloaca examination, *i.e.* based on the presence or lack of a male sex organ, rather than histological samples of their reproductive organs, it's something to keep in mind when talking about sex ratios in polluted area with exposure to aromatase inhibitors. But it is evident that if the observed sex ratio skewedness is caused by xenobiotics or pollution, and not by an ecological factor, it is unlikely that the sex determination process itself is affected – the TSD cannot be changed.

The observed change in sex ratios might very well simply be a product of incorrect surveying, temporal variation, or ecological factors. This thesis was initiated with the postulation that there is a skewed sex ratio in the river. It should be noted that this conclusion is partially based on unpublished papers, and that it is contradicted by opposite results from a newer but smaller study (Mauger 2012). Although skewedness in sex ratio in the same species has been observed in other locations as well, at no instance has any strong evidence been presented to offer explanations for the observed effect, and considering the sex ratio often varies between seasons and depending on the ages and sizes of the examined

crocodiles (Ferguson 1987a), many surveys would be required to be sure that the observed sex ratios correspond to actual field conditions. The author is very doubtful to the reported skewedness in sex ratio observed in the Tempisque River as the surveys are few and does not take in consideration enough ecological factors to be deemed trustworthy. Nevertheless, if males are indeed more common than females in the river, it is more likely that the observed sex ratio is due to the steadily increasing numbers of crocodiles in the rivers. There would be more competition for food and territories, and with females being smaller and less aggressive than males, they would have a disadvantage during non-breeding season and more difficulties competing.

Acknowledgments

The journey of completing this thesis has been a roller coaster of excitement and seemingly impossible challenges (figure 14). What an amazing journey it has been, realized with much help and support along the way - why proper acknowledgements are most definitely in order; A great and many thanks to Michael Tedengren, my primary advisor for this project; Your ability to step in and solve problems when things were difficult have been immensely valuable, as well as your invaluable input on the scientific process. Likewise, Denis Warshan, the savior of all things laboratory related, who taught me all of the techniques used in the lab and provided support in solving problems with the chemical and statistical analysis. Also, thank you to Dr. Luisa Castillo, Clemens Ruepert, Laura Porras, and especially Freylan Mena for your time and support. Thank you Sergio & Davinia, Ulises Chavarria Garcia, the staff of OET and Palo Verde National Park and Chema Tijerino. Ludvig Orsén, my partner in crime - the only person I would ever feel comfortable launching an inflatable boat with into a river full of crocodiles – thank you. A final thank you goes to those individuals who have supported me throughout my long and tedious student years. And lastly, thank you coffee – if I could list you as a co-author I would.

Sincerely,
Linda Eckardt, Kalmar, April 2017

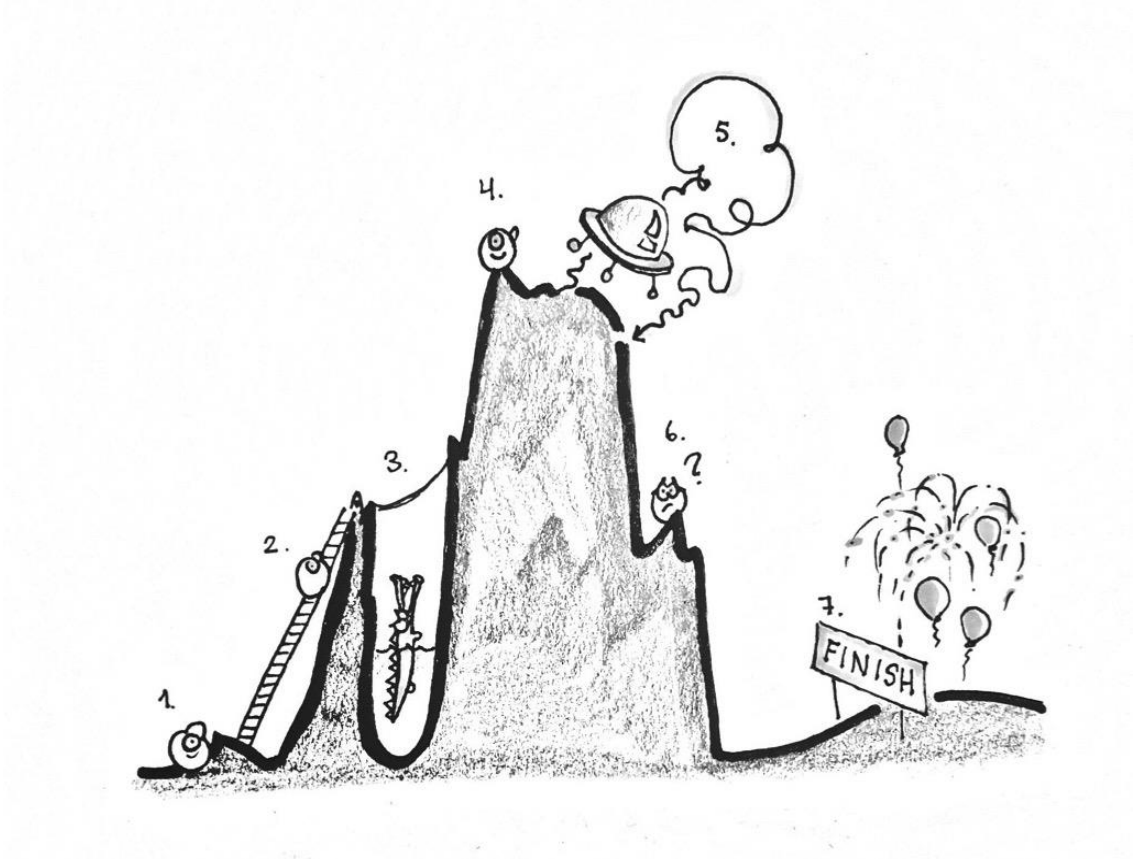

Figure 14. The journey of completing a thesis. 1. The student begins her journey full of enthusiasm 2. With great determination she starts to climb upward in the name of science 3. She almost falls down into the pit of despair and deadly crocodiles 4. She reaches the top of the monster-mountain, and thinks that the worst is over 5. Gets abducted by aliens and fails to do any work whatsoever 6. Finds herself a year later being very confused by as to why she still hasn't finished her thesis 7. After much debate with herself she decides to pull an all-nighter and finish it up.

References

Abadia G, and Orjuela M (1998) Crocodylus acutus in the Tarcoles River, Costa Rica. Page 378 in Proceedings of the fourteenth working meeting of Crocodile Specialist Group. IUCN - Gland, Switzerland.

Abass K, Turpeinen M, and Pelkonen O (2009) An evaluation of the cytochrome P450 inhibition potential of pesticides in human hepatic microsomes. J. Environ. Sci. *44*:553–563

Allner B, von der Gönna S, Griebeler, E, Nikutowski N, Weltin A, and Stahlschmidt-Allner P (2010) Reproductive functions of wild fish as bioindicators of reproductive toxicants in the aquatic environment. Environ. Sci. Pollut. *17*:505–518

Badawi A (2000) Effect of chlorinated hydrocarbons on expression of cytochrome P450 1A1, 1A2 and 1B1 and 2- and 4-hydroxylation of 17beta-estradiol in female Sprague-Dawley rats. Carcinogenesis *21*:1593–1599

Benachour N, Moslemi S, Sipahutar H, and Seralini G (2007) Cytotoxic effects and aromatase inhibition by xenobiotic endocrine disrupters alone and in combination. Toxicol. Appl. Pharmacol. *222*:129–140

Bergman A, Heindel J, Jobling S, Kidd K, Zoeller R, World Health Organization, and United Nations Environment Programme (2013) State of the science of endocrine disrupting chemicals - 2012: an assessment of the state of the science of endocrine disruptors prepared by a group of experts for the United Nations Environment Programme (UNEP) and WHO (Geneva, Switzerland: United National Environment Programme : World Health Organization)

Bolaños J (2010) Population Status of the American Crocodile (*Crocodylus acutus*) in the Tempisque Great Wetland, Guanacaste, Costa Rica, 2009-2010. Unpublished

Bowles M (1996) The comparison of mercury concentrations in American alligator (*Alligator mississippiensis*) eggs from two sites in coastal South Carolina. Bull SC Acad. Sci. *58*:29

Brant L, Mazzotti F, Wilcox P, Barker G, Hasty G, and Wasilewski J (1995) Status of the American crocodile (Crocodylus acutus) at a power plant site in Florida, USA. Herpetol. Nat. Hist. *3*:29–36

Bucheli T, and Fent K (1995) Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. Crit. Rev. Environ. Sci. Technol. *25*:201–268

Cedeño-Vázquez J (2006) Population status and distribution of *Crocodylus acutus* and *C. moreletii* in southeastern Quintana Roo, México. Herpetol. Nat. Hist. *10*:53–66

Charruau P (2012) Microclimate of American crocodile nests in Banco Chinchorro biosphere reserve, Mexico: Effect on incubation length, embryos survival and hatchlings sex. J. Therm. Biol. *37*:6–14

De la Cruz E, and Castillo L (2002) The pesticide use in Costa Rica and their impact on coastal ecosystems. In Pesticide Residues in Coastal Tropical Ecosystems,(London, Routledge), pp. 338 - 374

Erikstad K, Bustnes J, Lorentsen, S-H, and Reiertsen T (2009). Sex ratio in lesser black-backed gull in relation to environmental pollutants. Behav. Ecol. Sociobiol. *63*:931–938

Erikstad K, Moum T, Bustnes J, and Reiertsen T (2011) High levels of organochlorines may affect hatching sex ratio and hatchling body mass in arctic glaucous gulls: Sex ratio and organochlorines in glaucous gulls. Funct. Ecol. *25*:289–296

Ferguson M (1987a) Post-laying stages of embryonic development for crocodilian. In Wildlife Management: Crocodiles and Alligators, (Chipping Norton, N.S.W: Surrey Beatty & Sons Pty Limited), pp. 427–444

Ferguson M (1987b) Reproductive Biology and the Embryology of the Crocodilians. In Wildlife Management: Crocodiles and Alligators, pp. 330–491

Gabriel W, Blumberg B, Sutton S, Place A, and Lance V (2001) Alligator aromatase cDNA sequence and its expression in embryos at male and female incubation temperatures. J. Exp. Zool. *290*:439–448

Guillette L, and Milnes M (2001) Recent observations on the reproductive physiology and toxicology of crocodilians. In Crocodilian Biology and Evolution, (Chipping Norton, N.S.W: Surrey Beatty & Sons), pp. 199–213

Gunderson M, Oberdörster E, and Guillette L (2004) Phase I and II liver enzyme activities in juvenile alligators (*Alligator mississippiensis*) collected from three sites in the Kissimmee–Everglades drainage, Florida (USA). Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. *139*:39–46

Heinz G, Percival H, and Jennings M (1991) Contaminants in American alligator eggs from Lake Apopka, Lake Griffin, and Lake Okeechobee, Florida. Environ. Monit. Assess. *16*:277–285

Hernández-Moreno D, Soler-Rodríguez F, Míguez-Santiyán M, and Pérez-López M (2008) Hepatic monooxygenase (CYP1A and CYP3A) and UDPGT enzymatic activities as biomarkers for long-term carbofuran exposure in tench (*Tinca tinca*). J. Environ. Sci. Health Part B *43*:395–404

IPS (2002) Evalucaión del daño ambiental ocasionado por la descarga de vinaza, en septiembre del 2001, en la parte baja de los ríos Tempisque y Bebedero. Instituto de Políticas para la Sostenibilidad

Kinnberg K, Holbech H, Petersen G, and Bjerregaard P (2007). Effects of the fungicide prochloraz on the sexual development of zebrafish (Danio rerio). Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. *145*: 165–170

Kovacs T, Gibbons J, Tremblay L, Oconnor B, Martel P, and Voss R (1995) The Effects of a Secondary-Treated Bleached Kraft Mill Effluent on Aquatic Organisms as Assessed by Short-Term and Long-Term Laboratory Tests. Ecotoxicol. Environ. Saf. *31*:7–22

Krüger M, Schledorn P, Schrödl W, Hoppe H-W, Lutz W, and Shehata A (2014) Detection of Glyphosate Residues in Animals and Humans. J. Environ. Anal. Toxicol. *04*:210

Laabs V, Amelung W, Pinto A, Wantzen M, da Silva C, and Zech W (2002) Pesticides in Surface Water, Sediment, and Rainfall of the Northeastern Pantanal Basin, Brazil. J. Environ. Qual. *31*:1636

Lang J, and Andrews H (1994).Temperature-dependent sex determination in crocodilians. J. Exp. Zool. *270*:28–44

Larsson F, and Förlin L (2002). Male-Biased Sex Ratios of Fish Embryos near a Pulp Mill: Temporary Recovery after a Short-Term Shutdown. Environ. Health Perspect. *110*:739–742

Larsson D, Hallman H, and Förlin L (2002). More male fish embryos near a pulp mill. Environ. Toxicol. Chem. *19*:2911–2917

Lepom P, Brown B, Hanke G, Loos R, Quevauviller P, and Wollgast J (2009) Needs for reliable analytical methods for monitoring chemical pollutants in surface water under the European Water Framework Directive. J. Chromatogr. A *1216*:302–315

Ma Y, Han J, Guo Y, Lam P, Wu S, Giesy J, Zhang X, and Zhou B (2012) Disruption of endocrine function in in vitro H295R cell-based and in in vivo assay in zebrafish by 2,4-dichlorophenol. Aquat. Toxicol. *106-107*:173–181

Mauger L, Velez E, Cherkiss M, Brien M, Boston M, Mazzotti F, and Spotila J (2012) Population assessment of the American crocodile, *Crocodylus acutus* (Crocodilia: Crocodylidae) on the Pacific coast of Costa Rica. Rev. Biol. Trop. *60*:1889–1901

Mena F, Fernández San Juan M, Campos B, Sánchez-Avila J, Faria M, Pinnock M, de la Cruz E, Lacorte S, Soares A, and Barata C (2014) Pesticide residue analyses and biomarker responses of native Costa Rican fish of the Poeciliidae and Cichlidae families to assess environmental impacts of pesticides in Palo Verde National Park. J. Environ. Biol. Acad. Environ. Biol. India *35*:19–27

Menone M, Aizpún de Moreno V, Moreno, V, Lanfranchi A, Metcalfe T, and Metcalfe C (2000). PCBs and Organochlorines in Tissues of Silverside (*Odontesthes bonariensis*) from a Coastal Lagoon in Argentina. Arch. Environ. Contam. Toxicol. *38*:202–208

Morthorst J, Holbech H, and Bjerregaard P (2010) Trenbolone causes irreversible masculinization of zebrafish at environmentally relevant concentrations. Aquat. Toxicol. *98*:336–343

Mower B, Munkittrick K, McMaster M, and Van Beneden R (2011) Response of white sucker (*Catostomus commersoni*) to pulp and paper mill effluent in the Androscoggin River, Maine, USA. Environ. Toxicol. Chem. *30*:142–153

Navarro A, Mena F, Casado M, Pinnock M, de la Cruz E, Barata C, and Piña B (2014) Development of quantitative RNA biomarkers for detecting dioxin-like and estrogenic pollutants in Costa Rican native fish species. J. Environ. Biol. *35*:99–105

Ogden J, Robertson W, Davis G, and Schmidt T (1974) Pesticides, polychlorinated biphenyls, and heavy metals in upper food chain levels, Everglades National Park and vicinity. Final report. (Division of Natural Science and Research Management Studies, Everglades National Park)
Örn S, Svenson A, Viktor T, Holbech H, and Norrgren L (2006) Male-Biased Sex Ratios and Vitellogenin Induction in Zebrafish Exposed to Effluent Water from a Swedish Pulp Mill. Arch. Environ. Contam. Toxicol. *51*:445–451
Phelps R, Focardi S, Fossi C, Leonzio C, and Renzoni A (1986) Chlorinated hydrocarbons and heavy metals in crocodile eggs from Zimbabwe. Trans Zimbabwe Scient Assoc *63*:8–15
Piña C, Siroski P, Larriera A, Lance V, and Verdade L (2007) The temperature-sensitive period (TSP) during incubation of broad-snouted caiman (*Caiman latirostris*) eggs. Amphib.-Reptil. *28*:123–128
Poletta G, Kleinsorge E, Paonessa A, Mudry M, Larriera A, and Siroski P (2011) Genetic, enzymatic and developmental alterations observed in *Caiman latirostris* exposed in ovo to pesticide formulations and mixtures in an experiment simulating environmental exposure. Ecotoxicol. Environ. Saf. *74*:852–859
Rainwater T, Wu T, Finger A, Cañas J, Yu L, Reynolds K, Coimbatore G, Brady B, Platt S, Cobb G. (2007) Metals and organochlorine pesticides in caudal scutes of crocodiles from Belize and Costa Rica. Sci. Total Environ. *373*:146–156
Ramirez Muñoz F, Chaverri F, de la Cruz E, Wesseling C, Castillo L, and Bravo V (2009) Importación de Plaguicidas en Costa Rica; Periodo 1977 - 2006 (Heredia, Costa Rica: IRET)
Ramírez Muñoz F (2011) Importación de Plaugicidas en Costa Rica: Periodo 2007 - 2009 (Heredia, Costa Rica: IRET)
Rauschenberger R, Sepúlveda M, Wiebe J, Szabo N, and Gross T (2004a) Predicting maternal body burdens of organochlorine pesticides from eggs and evidence of maternal transfer in *Alligator mississippiensis*. Environ. Toxicol. Chem. *23*:2906–2915
Rauschenberger R, Wiebe J, Buckland J, Smith J, Sepúlveda M, and Gross T (2004b) Achieving environmentally relevant organochlorine pesticide concentrations in eggs through maternal exposure in Alligator mississippiensis. Mar. Environ. Res. *58*:851–856
Rey F, González M, Zayas M, Stoker C, Durando M, Luque E, and Muñoz-de-Toro M (2009) Prenatal exposure to pesticides disrupts testicular histoarchitecture and alters testosterone levels in male Caiman latirostris. Gen. Comp. Endocrinol. *162*:286–292
Richard S, Moslemi S, Sipahutar H, Benachour N, and Seralini G-E (2005) Differential effects of Glyphosate and Roundup on human placental cells and aromatase. Environ. Health Perspect. *113*:716–720
De Rolla S (2010) 10 Organic Contaminants in Reptiles. In Ecotoxicology of Ambhibians and Reptiles: 289
Roose P, and Brinkman U (2005) Monitoring organic microcontaminants in the marine environment: principles, programmes and progress. TrAC Trends Anal. Chem. *24*:897–926
Sánchez J (2001) Estado de la población de cocodrilos (Crocodylus acutus) en el río Tempisque, Guanacaste, Costa Rica. (Heredia, Costa Rica: Área de Conservación Tempisque-Instituto Nacional de Biodeversidad (iNBio))
Sánchez J, Bolaños J, and Piedra L (1997) Población de Crocodylus acutus (Crocodylia: Crocodylidae) en dos rios de Costa Rica. Rev. Biol. Trop *44*:835–840
Sandoval-Herrera N, Vargas-Soto J, Espinoza M, Clarke T, Fisk A, and Wehrtmann I (2016) Mercury levels in muscle tissue of four common elasmobranch species from the Pacific coast of Costa Rica, Central America. Reg. Stud. Mar. Sci. *3*:254–261
Simpson E, Mahendroo M, Means G, Kilgore M, Hinshelwood M, Graham-Lorence S, Amarneh B, Ito Y, Fisher C, and Michael M (1994) Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocr. Rev. *15*:342–355
Smith C, and Joss J (1994) Steroidogenic enzyme activity and ovarian differentiation in the saltwater crocodile, Crocodylus porosus. Gen. Comp. Endocrinol. *93*:232–245
Stegeman J (1992) Nomenclature for hydrocarbon-inducible cytochrome P450 in fish. Mar. Environ. Res. *34*:133–138
Stoneburner D, and Kushlan J (1984) Heavy metal burdens in American crocodile eggs from Florida Bay, Florida, USA. J. Herpetol. *18*:192–193
Svensson M, Rintamäki P, Birkhead T, Griffith S, and Lundberg A (2007) Impaired hatching success and male-biased embryo mortality in Tree Sparrows. J. Ornithol. *148*:117–122
Szekacs A, and Darvas B (2012) Forty Years with Glyphosate. In Herbicides - Properties, Synthesis and Control of Weeds, M.N. Hasaneen, ed. (InTech)

Thorbjarnarson J (1997) Are Crocodilian Sex Ratios Female Biased? The Data Are Equivocal. Copeia *1997*:451

Thorbjarnarson J, Mazzotti F, Sanderson E, Buitrago F, Lazcano M, Minkowski K, Muñiz M, Ponce P, Sigler L, Soberon R, *et al.* (2006) Regional habitat conservation priorities for the American crocodile. Biol. Conserv. *128*:25–36

Toda K, Simpson E, Mendelson C, Shizuta Y, and Kilgore M (1994) Expression of the gene encoding aromatase cytochrome P450 (CYP19) in fetal tissues. Mol. Endocrinol. Baltim. Md *8*:210–217

Watanabe K, Kawai Y, Ikenaka Y, Kawata M, Ikushiro S-I, Sakaki T, and Ishizuka M (2013) Avian Cytochrome P450 (CYP) 1-3 Family Genes: Isoforms, Evolutionary Relationships, and mRNA Expression in Chicken Liver. PLoS ONE *8*:e75689

Webb G, Manolis S, and Dempsey K (1987) Effects of temperature on sex determination. In Wildlife Management: Crocodiles and Alligators, (Chipping Norton, N.S.W: Surrey Beatty & Sons Pty Limited) pp. 507–531

Wu T, Rainwater T, Platt S, McMurry S, and Anderson T (2000) Organochlorine contaminants in Morelet's crocodile (*Crocodylus moreletii*) eggs from Belize. Chemosphere *40*:671–678

Wu T, Cañas J, Rainwater T, Platt S, McMurry S, and Anderson T (2006) Organochlorine contaminants in complete clutches of Morelet's crocodile (*Crocodylus moreletii*) eggs from Belize. Environ. Pollut. *144*:151–157

Yang X, Zheng J, Qu L, Chen S, Li J, Xu G, and Yang N (2011) Methylation Status of cMHM and Expression of Sex-Specific Genes in Adult Sex-Reversed Female Chickens. Sex. Dev. *5*:147–154

Zerulla M, Länge R, Steger-Hartmann T, Panter G, Hutchinson T, and Dietrich D (2002) Morphological sex reversal upon short-term exposure to endocrine modulators in juvenile fathead minnow (Pimephales promelas). Toxicol. Lett. *131*:51–63

Appendix

The appendix contains supportive tables, figures and information that may be valuable to the reader for validating information provided in the thesis.

Pollution and enzymes as biomarkers

The amount of imported pesticides in Costa Rica has increased rapidly in recent years and doubled between the years 1996 to 2009 (Ramirez *et al.* 2009). Although governmental regulations are in place to protect the environment from pollution from pesticide use, representative models on the behavior of pesticide residues in the environment and their effects on the ecosystem are lacking (De la Cruz and Castillo 2002), and the amount of pesticides, other types of pollution and agricultural waste that end up in the river is under examination. With no actual governmental control of what is being put in the rivers and used on the lands (Ulices C, personal communication, November 2014), it is hypothesized that the Tempisque River may contain a significant amount of contaminants (Clemens Ruepert, personal communication, November 4, 2014). So far research has found contaminants such as Ametryne, Hexachlobenzene, Bromacil, Epoxiconazole, Phorate, Alachlor, Chlorfenvinphos, Cyprodinil, Myclobutanil, Pyrimiphos-Me, Spinosin, and Terbutryn in the river (Mena *et al.* 2014). Many of the contaminants suspected to be in the river are difficult to measure due to technical limitations of gas and liquid chromatography. Out of the pesticides used extensively around the area in the Tempisque River, *2,4-Dichlorophenoxyacetic acid* (2,4-D) and glyphosate exhibit chemical properties with the potential of affecting the expression of aromatase. 2,4-D with 824 000 kilos imported yearly (2009), is the second most imported pesticide in the country (1997 – 2006) (Ramirez *et al.* 2009), and glyphosate is the most imported herbicide with little over one million kilos imported yearly (2009)(Ramírez Muñoz 2011). Mancozeb, Ethoprophos, and Diazinon are also common. Out of these pesticides, Mancozeb, glyphosate and 2,4-D are most likely to be the ones the crocodile population in the Tempisque River is exposed to as they are all applied to rice and pasture crops. A synergistic effect to increase aromatase inhibition has been noted for structurally similar chemicals to those expected to be found in the Tempisque River (Benachour et al., 2007). American crocodiles that live in the Tárcoles river are exposed to and accumulate multiple different environmental contaminants including p,p'-DDE, p,p'-DDT, dieldrin, endrin, methoxychlor, cadmium, copper, lead, mercury and zinc at significant levels (Rainwater et al., 2007). Aldrin, heptachlor, and lindane have, however, have not been detected in crocodile tissues.

Table 1. Pesticides used in Costa Rica, the crops they are applied to (2009) (Ramírez Muñoz, 2011), and the affected cytochrome P450 system for those pesticides likely to be used in the area based in their usage and analysis of the land usage using GIS.

Active ingredient	Biological action	Crops used for	Affected P450 system (induced or inhibited)	Amount imported (kg)	Ref.
Mancozeb	Fungicide	Banana, Plantain, Melon, ornamental plants, rice, fruit, beans	CYP1A1, CYP3A		(Hernández-Moreno et al., 2008) (Ramirez et al., 2009),
Glyphosate	Herbicide	Banana, Plantain, Coffee, Sugarcane, Fruits, Palm trees, Woodlands, cotton, pasture	CYP2C9, CYP2C8		(Abass et al., 2009) (Ramirez et al., 2009),
2,4-D	Herbicide	Sugarcane, rice, pasture, maize, palm trees	CYP1A1, CYP1A2, CYP1B1,		(Badawi, 2000) (Ramirez et al., 2009),

Primer design

FASTA files used for designing degenerative primers for the different target genes used for this study.

Table 2. FASTA files used for primer design for aromatase (CYP19)

Sequence name	Sequenced organism	GenBank ID	Type of sequence
Alligator mississippiensis aromatase mRNA, complete cds	*Alligator mississippiensis*	AY029233.1	Complete cds
Alligator mississippiensis cytochrome P450 19A1-like (LOC102566432), mRNA	*Alligator mississippiensis*	NM_001287266.1	Unknown
CHKCYAR Chicken cytochrome P-450 aromatase gene, complete cds	*Gallus gallus*	J04047.1	Complete cds
Xenopus laevis cyp19a mRNA for p450 aromatase A, complete cds, brain specific 5'UTR	*Xenopus laevis*	AB272088.1	Complete cds
Xenopus laevis cyp19a mRNA for p450 aromatase A, complete cds, gonad specific 5'UTR-2	*Xenopus laevis*	AB272087.1	Complete cds
Trachemys scripta aromatase mRNA, complete cds	*Trachemys scripta*	AF178949.1	Complete cds
Lepidochelys olivacea CYP19 aromatase mRNA, complete cds	*Lepidochelys olivacea*	KF411436.1	Complete cds
Lepidochelys olivacea aromatase mRNA, complete cds, alternatively spliced	*Lepidochelys olivacea*	KF268021.1	Complete cds
Chrysemys picta aromatase mRNA, complete cds	*Chrysemys picta*	FJ195593.1	Complete cds
ZFIAROMATA *Poephila guttata* aromatase (AROM) mRNA, complete cds	*Poephila guttata*	L81143.1	Complete cds
Danio rerio brain cytochrome P450 aromatase (cyp19b) mRNA, complete cds	*Danio rerio*	AF226619.1	Complete cds

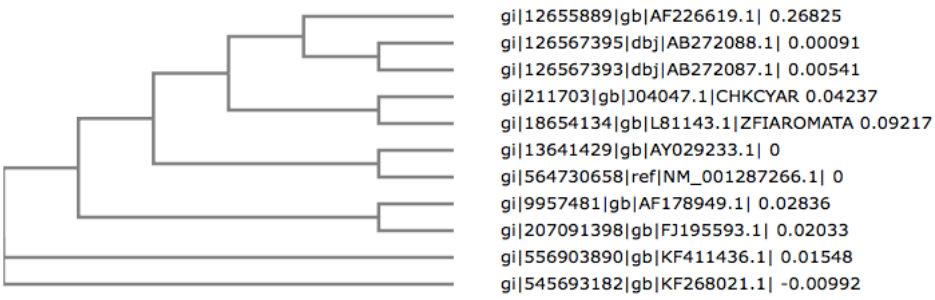

Figure 1. Phylogenetic tree for the CYP19 FASTA genome sequences.

Table 3. FASTA files used for primer design for CYP1A1

Sequence name	Sequenced organism	GenBank ID	Type of sequence
Acanthopagrus schlegelii cytochrome P450	*Acanthopagrus schlegelii*	DQ898145.1	Partial cds
Danio rerio cytochrome P450 1A1 (cyp1A1) mRNA, complete cds	*Danio rerio*	AF210727.2	Complete cds
Liza saliens cytochrome P450 1A1 (CYP1A1) mRNA, complete cds	*Liza saliens*	AF072899.1	Complete cds
Ovis aries cytochrome P4501A1 (CYP1A1) mRNA, complete cds	*Ovis aries*	S79795.1	Complete cds
Felis catus CYP1A1 mRNA for cytochrome P450 1A1, complete cds	*Felis catus*	AB199730.1	Complete cds
Phoca groenlandica mRNA for cytochrome P450 1A1 (CYP1A1 gene)	*Phoca groenlandica*	AJ621380.1	Complete cds
Xenopus laevis cytochrome P450, family 1, subfamily A, polypeptide 1 (cyp1a1), mRNA	*Xenopus laevis*	NM_001097072.1	Complete cds
Catla catla cytochrome P450 1A1 (cyp1A1) mRNA, complete cds	*Catla catla*	JX480500.2	Complete cds
Xenopus (Silurana) tropicalis cytochrome P450, family 1, subfamily A, polypeptide 1 (cyp1a1), mRNA	*Xenopus tropicalis*	NM_001097344.1	Complete cds
Gallus gallus cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1), mRNA	*Gallus gallus*	NM_205146.2|	Complete cds
Oncorhynchus mykiss cytochrome P450 1A1 (CYP1A1) mRNA, complete cds	*Oncorhynchus mykiss*	OMU62796	Complete cds

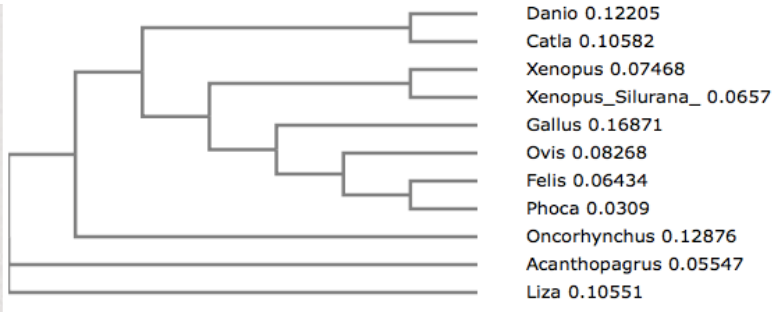

Figure 2. Phylogenetic tree for the CY1A1 FASTA genome sequences.

Table 4. FASTA files used for primer design for Beta-Actin

Sequence name	Sequenced organism	GenBank ID	Type of sequence
Crocodylus acutus isolate LSUMZ_H-6982 beta-actin (ACTB) gene, exons 3, 4 and partial cds	*Crocodylus acutus*	JF315005.1	Partial cds
Crocodylus acutus isolate LSUMZ_H-21709 beta-actin (ACTB) gene, exons 3, 4 and partial cds.	*Crocodylus acutus*	JF315001.1	Partial cds
Crocodylus porosus beta-actin mRNA, partial cds.	*Crocodylus porosus*	GQ847622.1	Partial cds
Crocodylus johnsoni isolate LSUMZ_H-21725 beta-actin (ACTB) gene, exons 3, 4 and partial cds	*Crocodylus johnsoni*	JF314969.1	Partial cds
Crocodylus siamensis beta-actin mRNA, partial cds	*Crocodylus siamensis*	GU454801.1	Partial cds
Crocodylus siamensis beta-actin mRNA, partial cds	*Crocodylus siamensis*	GU017734.1	Partial cds
Crocodylus porosus isolate LSUMZ_H-6758 beta-actin (ACTB) gene, exons 3, 4 and partial cds	*Crocodylus porosus*	JF315006.1	Partial cds
Alligator sinensis beta-actin mRNA, complete cds	*Alligator sinensis*	KC286488.1	Complete cds
Alligator sinensis beta-actin mRNA, complete cds	*Alligator sinensis*	JX183067.1	Complete cds
Alligator mississippiensis isolate LSUMZ_H-18733 beta-actin (ACTB) gene, exons 3, 4 and partial cds	*Alligator mississippiensis*	JF314980.1	Partial cds
CHKBACTN Gallus gallus beta-actin mRNA, complete cds	*Gallus gallus*	L08165.1	Complete cds
Corvus macrorhynchos ACTB mRNA for beta-actin, complete cds	*Corvus macrorhynchos*	AB561857.1	Complete cds
Mauremys mutica beta-actin mRNA, complete cds	*Mauremys mutica*	HQ244396.1	Complete cds

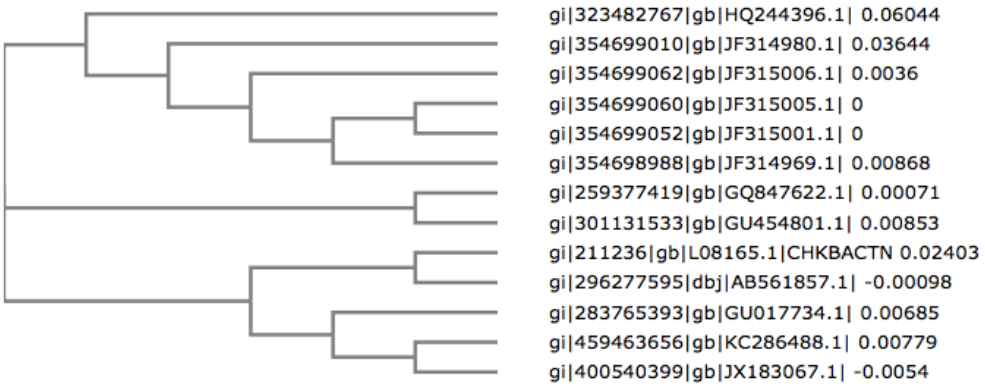

Figure 3. Phylogenetic tree for the Beta-Actin FASTA genome sequences.

Table 5. FASTA files used for primer design for CYP1A2

Sequence name	Sequenced organism	GenBank ID	Type of sequence
Gallus gallus cytochrome P450 1A4 (CYP1A4), mRNA	Gallus gallus	NM_205147.1	?
Lonchura striata cytochrome P450 1A4 (CYP1A4) mRNA, complete cds	Lonchura striata	KM515884.1	Complete cds
Phalacrocorax carbo cytochrome P450 1A4 (CYP1A4), mRNA	Phalacrocorax carbo	NM_001302368.1	Complete cds
Coturnix japonica cytochrome P450 1A4 (CYP1A4) mRNA, complete cds	Coturnix japonica	GQ906939.1	Complete cds

Genome sequences

Table 6. The sequences produced through Sanger sequencing

Target Gene	Product length	Highest query Coverage	Matches with
CYP1A1			
CCAGTGGCAAGTGAATCATCTTGCTGAAAAACTCGAGCCATCCGGAAGATCTGGCGGCCGCTCTCCCTATAGTGAGTCGTATTACGCCGGATGGATATGGTGTTCAGGCACAA GTGTTAAAGCAGTTGATTTTATTCACTATGATGAAAAAAACAATGAATGGAACCTGCTCCAAGTTAAAAATAGAGATAATACCGAAAACTCATCGAGTAGTAAGATTAGAGATAAT ACAACAATAAAAAAATGGTTTAGAACTTACTCACAGCGTGATGCTACTAATTGGGACAATTTTCCAGATGAAGTATCATCTAAGAATTTAAATGAAGAAGACTTCAGAGCTTTTGT TAAAAATTATTTGGCAAAAATAATATAATTCGGCTGCAGGGGCGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTCAGGTGGCACTT TTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCCTGATAAATGCTTCAATAATATTGAAAAAGGAA GAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTGCCTTCCTGTTTTGCTCACCCAGAACGCTGGTGAAAGTAAAGATGCTGA			
CYP1A1	628	95%	MCH class I
AAGATGATTCACTTGCCACTGGTTGCACCACAGAATCATAGAAGTAGGGTCGGAAGGGACCTTGTAGATCTTCAAGTCCGAGCCCCTGCCTGGGCAGGAGAAAAACTGGGCT CAAATGACTCCAGCCAGGTAGGCATCAAGCCTCTTCTTAAAGAACCCCAGGGTAGGAGCCAGCACCACTTCCCTTGGAAGTTGGTTCCAGATCCTAGCCGCCCTAACTGTGAA GTAGTTCTTATGGACGTCTAATCTAAATCTACTCTCCAACAACTTATGGCTGTTACTCCTTGTTATCCTGGGAGGTGCTAGGGGAAACAGGGTCTCTCCCAAACCCTTCTAGTCC CCCCTAGTGAGTTTATAGATGGTCACCAGGTCCCCCCTCAGGCTTCTCTTGTGAAGGCTGAACAGGTTCAGGTCCCGTAGTCTCTCATTGTAGGGTCTGCCCTGTTGTCTCCG GATCATGCGGGTGGCCCTCCTCTGGACCCTCTCAATGTTGTCCACATCCCTCCTGAAGTGGGGCACACAGAACTGGACACAGTATTCCAGCTGTGGCCTGACCAGTGTCGCA AAGAGGAGGAGGATCACCTCCTTGGCCCTACTTGACATGCACCTGTGGATGCACGACAAGGTC			
CYP19	401 bp	-	-
GCACCGTTCTCAGAAGAGTCATGCCGATTCGGCCACAGAGGCACCACTTCTCCTGGCACCCCCACTCCCCAGCCAGCACTCTCAGGGTAGGCACCAGCACTGTCACCTCCCC CCCGCTCACCCGTCACCTAAGCAGGTAATGCTGGCTGTCAGGGGATGTACCAGCGCTCTCAGGTGGTGCACGTGCACCCCCATGCACTCCCCACATGTCCCCACTGGCTCTG ATGCATGCTAATTAATGCTCCAGCAGCCTCTGTGCCATGTATTCAGCATCCCCATGCTTCAAAATGGTTGCAGGGGTGCTTAAACTAAAGCTTGTCAAACAAGCTTTAGTTCTGG CACCTCCACCACCATTTTGAAGCATAAGATGCTGAATACACATGACACTTCTGAGACGGTGC			
CYP19	405 bp	-	-
GCACCGTCTCAGAAGTGTCATGCCGTTCAGCCACAGAGGCACCACTTCTCCTGGCACCCCCACTCCCCAGCCAGCACTCTCAGGGTGGGCACCAGCACTGTCACCTCCCCCC CGCTCACCCGTCACCTAATCAGGAAATGCTGGCTGTCAGGGGATGTACCAGCGCTCTCAGGTGGTGCACATGCACCCCCATGCACTCCCCACATGTCCCCACTGGCTCCGAT GCATGCTAATTAATGCTCCAGCAGCCTCTGTGCCATGTATTCAGCATCCCCATGCTTCAAAATGGTTGCAGGGGTGCTTAAACTAAAGCTTGTCAAACAAGCTTTAGTTCTGGC ACCTCCACCACCATTTTGAAGCATAAGATGCTGAATACACATGACTCTTCTGAGACGGGGCATCTTT			
CYP19	271 bp	14% QC	-
AGATGCACCGTTCTCAGAAGAGTCAGCCTTCGGGGGGTGCTGGCCCCATGGGGGGGTACATACACGCCTGTGCACCCCATACCAGTCACCTATGTTTCCCATCTTGGACTAA CCAGATTTGTGTCAACCTTACCAAGAGAGAAAGCTCTTTAGGCCACTTAAGAGCCTACCACTAGTGTCCTGTACTACCACCCTTGCTGCCTCTACTGCAACTCTGAGACAGCCT CTAGGATTGATCACCCTGACACTTCTGAGACGGTGCATCTTTCTA			
CYP19	415 bp	41% QC	MCH class II
AGGATGCACCGTCTCAGAAGTGTCATGTGTATTCAGCATCTTATGCTTCAAAATGGTGGTGGAGGTGCCAGAACTAAAGCTTGTTTGACAAGCTTTAGTTTAAGCACCCCTGCA ACCATTTTGAAGCATGGGGATGCTGAATACATGGCACAGAGGCTGCTGGAGCATTAATTAGCATGCATCAGAGCCAGTGGGGACATGTGGGGAGTGCATGGGGGTGCACGTG CACCACCTGAGAGCGCTGGTACATCCCCTGACAGCCAGCATTACCTGCTTAGGTGACGGGTGAGCGGGGGGGAGGTGACAGTGCTGGTGCCCACCCTGAGAGTGCTGGCT GGGGAGTGGGGGTGCCAGGAGAAGTGGTGCCTCTGTGGCTGAACGGCATGACTCTTCTGAGACGGTGCATCTTTCTAGA			
CYP19	419 bp	41% QC	MCH class II
AAGATGCACCGTCTCAGAAGTGTCATGCCGTTCAGCCACAGAGGCACCACTTCTCCTGGCACCCCCACTCCCCAGCCAGCACTCTCAGGGTGGGCACCAGCACTGTCACCTC CCCCCCGCTCACCCGTCACCTAAGCAGGTAATGCTGGCTGTCAGGGGATGTACCAGCGCTCTCAGGTGGTGCACGTGCACCCCCATGCACTCCCCACATGTCCCCACTGGCT CTGATGCATGCTAATTAATGCTCCAGCAGCCTCTGTGCCATGTATTCAGCATCCCCATGCTTCAAAATGGTTGCAGGGGTGCTTAAACTAAAGCTTGTCAAACAAGCTTTAGTTC TGGCACCTCCACCACCATTTTGAAGCATAAGATGCTGAATACACATGACACTTCTGAGACGGTGCATCTTGCTGAAAAAC			
Beta-Actin	1181 bp	99% QC	Beta-Actin
TTGCGGTGGACAATGGAGGGTCCAGATTCATCGTACTCCTGCTTGCTGATCCACATCTGCTGGAAGGTGGACAGGGAGGCCAGGATGGAGCCACCAATCCAGACAGAGTATT TGCGCTCAGGCGGGGCAATGATCTGCAGGAAACAGGAGAGAGGGTTAGAGTTGAGGCCTGTACAAAGTGCCTACAGTTCCCAGCTGCATCTCCTACCCCTGATGTTTCTATC GTCAGGACCAAGCTACTAGGCTTGCCATTGAAGTGAGCATCTTGTCTAACAATCCCACAAGTGTTCCTGGATTTGGTCAGCCATTCTACCCAGAAAAAGACCACTCTTGGGACA ACGCTGGCCTTGCCTGCACCACCTACACCAGTGCAAGTGCCAAGACCTTGCGCTACCCTTATGGCGCGAATTACCCAGAATTTGCCAATTCCTTCCTAGGTGAATCATTAAGTC TGGGCACCACCAACCCTTGTTGGGACAGGGCAGATGTTGTATTTCAAGACTCCAAAAAATATACACACACACCTTGATTTTCATTGTGCTGGGTGCCAGGGCTGTGATCTCCTT CTGCATTCTGTCAGCGATGCCTGGATACATGGTGGTACCACCAGACAGCACAGTGTTGGCATACAGATCTTTACGGATATCAACATCGCACTTCATGATGGAGTTGAAGGTAGT TTCATGGATACCACAGGATTCCATACCTGCCAGAAGAGACGGACAGAAACAGGATCAGTTAAGGAGTTGGCCAGCTAGCCAGTCAGTCACATACGTGAGAAAGCCTGGTGTAT AGGTATAATGGAAACAGCGAAGACTTCAGCAGAAGTCCCACTCTGTAAAAGGCTCTCCTCACCCCACCACGTAGAGCCCCTCAGCCACTCCTGTATGGGGAGGGAGGTACCA CTGGGCAGTGACACCAAGATAGTCCTGTTATCCAGTCACTTCTGGAAAGGAAGCATCGCCTACCCCCCCTCCCCAAGGACCTATGGGAGGCCTGGTGGGATAAGAGTCTCTT AACCAGGATGGGAGCGGACTTACCTAAGAAAGAGGGCTGGAAGAGGGCTTCAGGGCACCTGAACCTTTCATTGCCAATGGTGATCACCTGACCATCAGGGAGTTCGTAGCTC TTCTCCAGGGAAGAGCGGGAGGCTGCGGTGGCCATCTCCTGTTCAAAATCCA			