SEX DETERMINATION AND DETECTION IN VERTABRATES

LITERATURE REVIEW THESIS WORK

Written by: Aaron I (Ron) Korabelnik

Thesis Supervison: Prof. Zöldág, László

Budapest
2015
Table of Content

1. Summary ................................................................. 3
2. Introduction ............................................................. 4
3. Classification and Example for the Different Mechanisms ....................... 5
   3.1. ESD ................................................................. 5
   3.2. GSD ................................................................. 7
4. Evolution: .................................................................... 9
   4.1. Progress ............................................................. 9
   4.2. SSD- Hermaphrodites ........................................... 10
5. Methods ..................................................................... 11
6. Results ....................................................................... 14
7. Hermaphrodites (Few Words) ......................................... 19
8. GSD Molecular Genetics of Sex Determination (Genomic Data) ............... 20
   8.1. Molecular genetics background ............................... 20
   8.2. X- and Y-Linked Specific Sequences ......................... 22
   8.3. Sexual Characteristics and Ranking, Levels of Sexuality .................. 26
   8.4. Genomic Abnormalities and Infertility ....................... 28
9. Discussion and Conclusion ............................................. 31
10. Appendices ............................................................. 34
10.1. Methods of Investigation ........................................ 34
10.2. X chromosome Sequencing: ...................................... 37
Bibliography ................................................................... 38
Acknowledgements ......................................................... 41
Copyright Declaration ..................................................... 42
**1. Summary.**

Historical background: the question of how does the sex being determined was one of the biggest questions in the ancient time. Aristotle claimed that the determination was made by the warmth of the male's partner during the intercourse. The warmer the passion is, the more probability for male to get born, also he advised to get into pregnant during the summer in order to have a son. More ever, he claimed that the female (woman) is an undeveloped male yet to complete his development (premature) or somehow arrested in development and his development was forced the embryo to be arrested in development by the womb's coldness that overcame the warmth that was given by the male's sperm. In the 19th century, they believed that nutrition is a major factor in the energetic amount and state the parents are passing on to the embryo (Gilbert 2010). Aristotle was right in some way, as for temperature sex determination, though, not with human. There are some factors who are determine the sexual fate which lead to an intracellular chain of events which will affect the organism for the rest of its life, genetically for the gonad gland and phenotypically, for appearance and extra gonad duct and physiology. The rough classification among the scientific community is into two plus one: which combines both classifications, in my thesis, I will explain briefly about those mechanisms (because the topic is almost infinite). I will detail about the factors that influence the sex determination, the evolutionary assumption behind the idea of each mechanism and the transition from one mechanism to another, I will mention organisms under each group, the genetic role and encoding (homologic and heterologic of sex chromosomes of zygote), I will mention researches on both strategies that proves and contradict known facts. The knowledge about the sex determination mechanism can promote the world and science tremendously e.g. extinct species or species in risk that were born into a changing environment, food crisis industry and financial source (male fish grow faster can serve us for aquaculture) (Sarah B. M. Kraak, 2002).
2. Introduction.

This thesis project will be divided into two parts, the first part will be a review for the sex determination mechanisms and strategic for different organism and in the second part, I will explain about the genetic role and coding of the GSD\textsuperscript{1}, the common classification for the sex determination are the GSD and the ESD\textsuperscript{2} and another sub classification which floats in between those two with is the Mixed Sex Determination which combines both mechanisms, the MSD\textsuperscript{3}. ESD takes place some times after the fertilization, in a "window of opportunity" during a defined time in a specific timing dedicated for the sex determination. In some cases, the sex determination is taking place during the whole incubation time and throughout the embryonic development till a very close timing from the hatching. The GSD however, determined by intracellular event dictated by sex chromosomes (homologic and heterologic) and almost exclusively isn't affected by the environment (Gilbert 2010). The knowledge of the factors that determine the sex of the organism can help us a lot, considering that we want to interfere with nature. The usage and benefit potential that it has is huge. E.g. rescue extinct species or in danger of extinction by creating a breeding packs, diverting sexes toward increasing amount of females knowing that one male can fertile more than one female, embryo conservation which involving (embryology field). The inconclusive rule is that reptiles have ESD and mammals have GSD. Again, we can find reptiles with GSD (Tobias Uller, 2007), an additional sex determination mechanism is the "Mixed Sex Determination" which combines both genomic and environmental which every mechanism has a specific affect on the sexual determination and fate of the individual. (David O Conover, 1987) (Sarah B. M. Kraak, 2002). For some coral reef fish, sex determination is very unique and interesting, they defined as "Hermaphrodites", the individual owns two reproductive organs, male and female, which charachterized by a"sequential" system, therefore, they can not be both male and female at the same time (Robert H. Devlin, 2002) (David O. Conover, 1987) and"Simultaneous Hermaphrodites" ("Simultaneous Hermaphrodites") produce both male and female gametes at the same time, of which some species are capable of alternating between sperm or egg production, (Serranus sp. or Gobiidae) and remarkably, some (Rivulus marmoratus) that are even capable of internal self fertilization. In sequential hermaphrodites, individual fish first produce one gamete type, then reverse sex and produce the other type in a subsequent spawning cycle. Sequential hermaphrodites are classified as protandrous if they mature first as males (Sparus or Amphiprion sp. Or protogynous if development first occurs as female. (Robert H. Devlin, 2002).

\textsuperscript{1} GSD- Genetic Sex Determination
\textsuperscript{2} ESD- Environmental Sex Determination
\textsuperscript{3} MSD- Mixed Sex Determination
3. Classification and Example for the Different Mechanisms

3.1. ESD.
The sex determination is affected by the environment, by extracellular and extra genomic factors which are not encoded in the genome and are not dictated by the genome. The maintenance of the reproductive organs and their physiology, are encoded in the autosomal or sex- chromosomes and genes, the timing of the sex determination takes place during the embryonic development and not immediately (Gilbert 2010). For an "emergency" plan, the ESD organisms have a "default" system which aims for a specific sex when the sex hasn’t been determined during the embryonic development and after hatching, it could be classified as "GSD" (Tobias Uller, 2007) or ESD (Godwin, 2009) (Gilbert, 2010). It is a combination of factors influencing the sex, 1. The main environmental determinants of sex. 2. The critical period of the gonad sensitivity to these factors. 3. The interactions between genotype and the environmental effects. And 4. The molecular mechanisms involved in the modulation of sex differentiation by external factors (J. F. Baroiller, 2001).

3.1.1. Temperature:
The most common ESD is the TSD\(^4\), some species can reach 1:0 ratio between the sexes (female: male), Turtles, Alligators and Crocodiles are known to have temperature as the main factor for the sex determination. In the phylogenetic aspect, as a general rule, there is a certain correlation between reptiles and ESD mechanism. (Tobias Uller, 2007) (Sarah B. M. Kraak, 2002). This mechanism is typical for reptiles and yet to be discovered a mammal which is influenced by an external factor which exclusively determines the sexual fate (O'Connor, 2008). The timing for the sex determination is happening during the embryonic incubation (Gilbert 2010) (Sarah B. M. Kraak, 2002). The fertilized egg (zygote) is bi-potential and could develop into either male or female. (Gilbert, 2010) *Apistogramma* genus is an example for TSD (J. F. Baroiller, 2001).

3.1.2. Size:
A recent discovery shows the correlation between the size of the egg and the offspring’s sex. The size affects and diverts the sexual fate of a lizard; egg size is positively correlated with female offspring (Dolgin, 2009).

3.1.3. pH:
Temperature appears to be the main environmental determinant of sex in most sensitive species. Interestingly, in these species sensitive to temperature and/or pH, there appears to be no effect of other factors such as photoperiod, density, or salinity, and thus suggested certain specificity for the type of sensitivity to external factors.

---

\(^{4}\) TSD- Temperature Sex Determination
For the genus *Apistogramma*, TSD is the major environmental determinant of sex. However, progenies of *A. caetei* are not sensitive to temperature but their sex ratios are strongly influenced by pH. Influence of pH in all the pH sensitive *Apistogramma* species, the proportion of males is higher at an acid pH (4.5) than at a more neutral value (6.5). (J. F. Baroiller, 2001).

### 3.1.4. SSD:

The most interesting to my opinion is the Social Sex Determination (SSD⁵). In a relation to "hermaphroditism", the idea behind SSD is to ensure reproduction and sexual mate for reproduction and by that, thriving of the pack, the mechanism of the SSD is that the pack is grouped in a certain area and being dominated by alpha male and/or alpha female (Godwin, 2009) (Robert H. Devlin, 2002). There is hierarchy in the pack and each individual will be, when time is right and the environment allows to, turn to the reproducing individual. E.g. a pack of *Pseudanthias squamipinnis* will live in the coral reef and the population will be assembled by one dominant male, one dominant female and the rest are female fish, the alpha female will change its sex into a male when the male is gone, dead or removed from the pack. The assumption is, that the hermaphrodite has to be followed with a multi-female polygamy and one male or a multi-male and one female, (J. F. Baroiller, 2001) (Robert H. Devlin, 2002).

![Image 2. ESD Main factors and their influence on the sex ratio in fish.](J. F. Baroiller, 2001)

---

⁵ SSD- Social Sex Determination
3.2. GSD.

The sex determination is influenced by chromosomes which mostly, determined immediately or very close to the fertilization time and the embryo develops according to the genetic encoding in the sex chromosomes, chromosomes which are specific and specially encoding for the sexual fate of the individual, (O’Connor, 2008), worth mentioning, that the fertilized egg is bi-potential, could turn into a male zygote or a female zygote (Gilbert, 2010). Immediately after the fertilization, intracellular events are starting which are crucial for the sex determination and the sexual fate is determined (mostly) due to the genes and GSD (Sarah B. M. Kraak, 2002). This mechanism can be divided into two, XX-XY system and WW-WZ system (Ellegren, 2000) (O’Connor, 2008). A diverse and different with each individual and different factors can be affected in different amount of each factor and subjectively to each individual, even if they are evolutionary related. E.g. polychromosomes for sex determination, sub classification of GSD, when there are no specific sex chromosomes or more than a pair of chromosomes, which determine the sex but a scattered in different loci and together, activating number of chain reactions and series of events that lead to an enzymatic activities in the fertilized egg. Each locus has a different affect, creating series of actions and also, forming an enzymatic trigger for the following gene in order and for a sequence of activities, this phenomenon is less frequent and more rare, (Sarah B. M. Kraak, 2002) (Tariq Ezaz, 2006). The platypus has 26 pairs of chromosomes in total and it is recently discovered of five separate pairs, which join together in a chain during cell division, determine an individual’s sex. (Roxanne Khamsi, 2004).

3.2.1. XX-XY:

The investigated GSD (Ellegren, 2000) more typical to mammals (Gilbert, 2010), there are fish and reptiles with XX-XY chromosomes (Tariq Ezaz, 2006) (Sarah B. M. Kraak, 2002), usually, the female is homogametic (XX) while on the first X chromosome founds the code for ovary formation (primary development) and in order to complete the development and reach for final "tubing" and ducts for the ovaries, germ cells and extra-ovarian phenotype, the second X chromosome is needed "XX", (secondary development) (Gilbert, 2010), any abnormal number of x-sex chromosomes, will be consider as female because the body tolerances an excess X-chromosomes by mute or rather silencing the excess X-chromosome(s) and only one of the X-chromosomes will be expressed (O'Connor, 2008). The female has to have two X chromosomes in order to complete the development. For the GSD, we have a "default" mechanism for the sexual fate of the individual, toward female formation, as mentioned, for ovary formation, only one X chromosome is needed, (Gilbert, 2010), while the male in mammals is heterogametic (Jill Kent, 1996) (O'Connor, 2008).

---

6 A locus (plural loci), in genetics, is the specific location or position of a gene, DNA sequence, on a chromosome.
7 Primary Development - Ovary and gonads formation.
8 Secondary Development - an extra-ovarian phenotype.
3.2.2. ZZ-ZW:
Typical to the avian and unlikely for mammals, the male in this case is the homogametic for the sex chromosomes while the female is the heterogametic (Justin Chue, 2011). The W chromosome, like the mammalian Y chromosome, is relatively small comparing to the Z chromosome and carries lower amount of genetic code because of its size (Ellegren, 2000). Relatively to the XX-XY, little is known about the ZZ-ZW system and still, many questions remained unclear, e.g. if the absence of the W chromosome is triggering the male formation or is it the ratio between the Z chromosomes to the autosomal chromosomes, (O’Connor, 2008) (Ellegren, 2000).

3.3. MSD:
A combined mechanism of both, GSD and ESD (Tobias Uller, 2007), we can think about it as an alternative mechanism for the sex determination for the relevant population, when the conditions are changing and geographic location is different. (David O. Conover, 1987) Different organisms are located and changing in-between the sex determination mechanisms without difficulties (Bull, 1980), reptiles with ESD are presenting heterogametes signs for sex chromosomes (Sarah B. M. Kraak, 2002) and fish with GSD, presented influence by the environmental temperature (David O. Conover, 1987).

3.3.1. Hormones:
For the MSD species, hormones have the ability to reverse the sex for some certain vertebrates (Godwin, 2009) and take an important role for the MSDs.
4. Evolution:

4.1. Progress.
Most likely, there was a progress of the reproduction strategy from ESD to GSD (Ellegren, 2000) (Sarah B. M. Kraak, 2002), throughout this evolutionary progress as we approaching to a more complex organism, the sex(es) ratio is shifting to 1:1 males to females, it was statistically proven that developed organisms with GSD, have ratio of 1:1 male to female. (Eshel, 2011) (Miller, 2004) (Bull, 1980) while the ESD which characterizes as "less developed" vertebrates (according to our perception) show different ratio with the ability to change this ratio up to 100% males or females, e.g. reptiles (Gilbert, 2010). Platypus chromosomes provide clues to the relationship between mammal and reptile chromosomes, and to the origins of mammal sex chromosomes and dosage compensation. The platypus's 52 chromosomes show no correlation between the position of orthologous genes on the small platypus chromosomes and chicken micro chromosomes, for the unique 5-X chromosomes of platypus we reveal considerable sequence alignment similarity to chicken Z and no orthologous gene alignments to human X, implying that the platypus X chromosome evolved directly from a bird-like ancestral reptilian system. The Platypus seems to be the missing link between the avian and the mammalian at least reproductively, they have in one hand of the 10 sex-chromosomes an orthologous X (mammalian) like chromosome and with some homology to the bird Z chromosome orthologous (avian) like chromosome and with a few large and many small chromosomes, reminiscent of reptilian macro- and micro chromosomes. (Wesley C. Warren, 2008).
4.2. SSD- Hermaphrodites.
A situation which the organism owns both reproductive organs, male and female and it changes its sex throughout life (Robert H. Devlin, 2002) and some do it more than once and some may live as both sexes (male and female) in order to create a successful reproduction and continuation every meeting (J. F. Baroiller, 2001). The hermaphrodites phenomena is quite frequent among the coral reef fish population especially in the "Perciformes" family, we can divide the hermaphrodites in to two groups; The Simultaneously and the Consecutive, the fish of the deep water usually live as Simultaneously hermaphrodites due to low frequent and low chances of meeting other individual, therefore, the need to increase probability and chances for success reproduction. As for the Sequential hermaphrodites, the strategic is Protandrous and protogynous for Social Sex Determination (David O. Conover, 1987). Hermaphrodite species that can initially mature either as males (protandrous) or females (protogynous), (Robert H. Devlin, 2002).

5.1. TSD.
Incubation was performed in extreme condition (Temperatures) and resulted with 100% hatching of the same sex by changing the incubation temperature of the nest, another experiment was performed with the same relation in an alligator hatchery nest and during this trail, the eggs were incubated in different temperatures and the variety of the sex ratio, after many repetitions they discover an amazing finding which I will discuss about it in the discussion and results chapter, (Yaron Tikochinski, 2010) (Gilbert, 2010).

5.2. Hormones.
With the enzyme "Aromatase" which converts Testosterone to Estrogen, can result as external environmental factor influencing the sex determination in avian and fish (Gilbert, 2010), moreover, a hormonal experiments were taken and the phenotype was the exact opposite than the genotype with cases of GSD, (Robert H. Devlin, 2002). Research was taken on the "Blue head wrasses" (Thalassoma bifasciatum), in a restricted social environment (with high competition) (Godwin, 2009) a sex inversion with steroid was made successfully in presence of a dominant male, the alpha-female was taken as the control female and another female with lower hierarchy was treated with steroids.

5.3. Size and sex correlation.
A relation and connection between size of incubated egg and the sexual fate of the individual was investigated, after many surveys, they discover a found with high statistical significance and high reliability of the sex and the size of the egg of the Three-lined skink lizard (Bassiana duperreyi). A causal effect of yolk allocation (and thus, egg mass) on offspring sex: If eggs are incubated at low temperatures, offspring sex can be changed by adding or removing yolk from the newly laid egg. (Rajkumar S. Radder, 2009).

5.4. pH.
The relation between environmental pH and the sex of the individual, they are evidences that the ratio between the sexes is increasing in different water acidity in the Apistogramma species fish, (J. F. Baroiller, 2001).

5.5. Polygenicity for Sex Determination.
The platypus (Ornithorhynchus anatinus) has ten sex chromosomes and several series of X and a single Y chromosome (males), the polygenicity system is typical to many vertebrates groups, throughout the evolution, we could see this mechanism is appearing repeatedly and in many forms both with XX-XY and ZZ-ZW (Tariq Ezaz, 2006).
5.6. Heterochromes.
This discovery took place in 1959 during research on "Turner's syndrome" and "Klinefelter's syndrome" by karyotype dyeing of the chromosomes in the cell. A difficulty for heterochromes recognition was dyeing with binding color which bind to known sequences in the sex chromosomes as snakes. (Sarah B. M. Kraak, 2002) (Tariq Ezaz, 2006).

5.7. The discovery of the SRY.
Encoded on the Y chromosome, (SRY- Sex Determination Region on Y), was discovered first in 1959 during the research of "Turner’s syndrome" and "Klinefelter’s syndrome", which has a critical role in the male's phenotype. (ncbi, 1998)

5.8. MSD discovery.
Evidence for sex chromosomes were discovered in vertebrates of many groups and different species which were known to have the GSD (Testudines) and the presence of heterogametes for sex chromosomes (Emys orbicularis) (N. Mrosovsky, 1991) (Girondot, 1999), fish (Manidia manidia) (David O. Conover, 1987). An experiment was performed on the manidia, which lives in a very specific habitat temperature (16°C-21°C) and has sex chromosomes (David O. Conover, 1987). Interestingly, the Manidia manidia, has both GSD and ESD/TSD, can hinder for adaptation for external stable and suitable temperature and development of reproduction independent on the changing environment when the external stress is too radical.

---

9 Turner’s syndrome - Monosomy X, female phenotype, one X chromosome and no Y chromosome.
10 Klinefelter syndrome - Males with Klinefelter syndrome carry two or more X chromosomes.
5.9. **Newborn Fish Investigation.**
They have been investigating the coral reef fish, histological investigation, in order to find the origin of the sex inversion and the physiological expression of it (Godwin, 2009).

5.10. **Sex Inverting Steroids Factors.**
A pack of *Wrass* fish (*Thalassoma bifasciatum*) in a competitive environment with the presence of a dominant male, the alpha female was used as the control individual and a female from lower hierarchy in the pack that was treated with steroids (Godwin, 2009).

5.11. **Endocrinology of Sex Inversion.**
With the help of special markers and illustration imaging during sex inversion of the Wrass fish, with special binders which bind to special substances which indicate for a certain function or properties. In order to detect activity related to physiological-sexual function, it has used for detection for the MSD and SSD (Godwin, 2009).

5.12. **Histological investigation.**
With Wrass female during the sex invert (Robert H. Devlin, 2002). The gonad changes of the protandrous hermaphrodite fish *Sparus auratus*. At first, testicular tissue predominates, but after a transition period during which both testicular and ovarian tissues are seen, the ovarian cells take over. (Gilbert, 2013)
6. Results.

6.1. Turtles with TSD.
Only within a temperature specific range we can have ratio of the sexes male to female 1:1, for the turtle *Trachemys scripta* is 28.5°C and only the temperature will dictate the final fate of the individual, (Gilbert, 2010). The temperature triggers enzymatic reaction which will induce Transcription toward a certain sex male or female, for every species and genus, a different range of temperature for 1:1 results between the species, another example for TSD, is the Alligator (*Alligator mississippiensis*) which in the temperature ranges between 32°C to 33°C we can observe 100% males while in temperature of 31.5°C and also 34°C we can observe 1:1 ratio male to female and when the temperature is lower or higher than those ranges, a female dominancy will be observed up to 100% among the offspring (Gilbert, 2010) (Yaron Tikochinski, 2010).

Image. 6 ESD in TSD for different reptiles male ratio, male relatively to temperature (Gilbert, 2010)

6.2. Hormones.
Hormones were proven as sex inverting factors and not only to sex determine factors before hatching or during life as an individual. There are many uses in hormones in aquaculture fish farming for many purposes, alpha female, sustained her sex in the presence of a dominant male while hormones treated female was changing sex even though it had a lower hierarchy rank, in the presence of the dominant male, which induces behavioral dominancy in sex inverting for the rest of the females in the pack (Godwin, 2009). They proved that sex invert is possible externally induced by hormones, we can induce sex inverting or sex reversing.
6.3. Egg size.
Statistically eggs with female offspring were significantly bigger (three-lined skink lizard *Bassiana duperreyi*) (Dolgin, 2009), a mathematic-statistical correlation was found between (fertilized) egg size and its sex. The bigger the egg the more chances to receive a female hatching.

6.4. pH.
The results of this research discovered that the sex ratio was diverted toward “males” in acidic water (environment) pH lower than 6.5 (approx. 4.5) for the *Apistogramma* species acidic environment will result with more males (J. F. Baroiller, 2001).

6.5. Poligenicity For Sex Chromosome.
It was done by kariotype dye and polygenicity was discovered for several organisms for sex chromosomes, a multiple number of chromosomes which responsible for the sexual phenotype, in addition, number of several loci in autosomal chromosomes which connecting sex hormones formation and sex phenotype, polygenicity is related to any additional number of sex chromosomes which is more than the pair sex chromosomes (e.g. XX/XY) for more details, go to GSD abnormalities chapter 8.4.

6.6. Heterochromes.
Results of a special dye research, was discovered the Y and W chromosomes, which led to the new terminology of hetero/homo gameted for sex chromosome, in addition, with the help of flurocents substrates which attached to GATA sequences ¹¹ which were typically to W and Y chromosom, the heterochrom for MSD and in suspected TSD and in cases that were difficult to recognise and to be distinguished, factor which emplies for an additional factor in the sex determination in addition to snakes and avian that were documented in researches

¹¹ GATA transcription factors are a family of transcription factors characterized by their ability to bind to the DNA sequence.
6.7. SRY.
SRY is the gene code responsible for both, genital organs and phenotype in the male formation. The SRY is encoding for testis with the locus "Sox9\textsuperscript{12n}" which initiates two important reactions the first, testosterone production (which starts in the testicle) and second, the AMH\textsuperscript{13} reactions, that responsible as an antagonist of any extra gonad female phenotype (tubing and ducts, which can erase any remnants for male existence). As mentioned, male has XY and the transcription of the SRY, are causing a crucial chain reaction and are highly essential for the male development (Jill Kent, 1996) (Gilbert, 2010). The SOX9 responsible among the rest, for the male's gametes formation and development and hormones production (a human embryo has both reproduction systems and carries it till the age of seven weeks, until the testis formation and additional actions), in order to cancel the expression of the additional X chromosome (Miller, 2004), there are evidence for XX men and XY women (O'Connor, 2008) (Gilbert, 2010) due to translocation of genes or fragments of those genes which came in to expression subjectively in that individual. (Gilbert, 2010). On the Y chromosome there are loci which encoding with the SRY, which the transcription and translation for this loci, are critical and essential in this chain of events for male development (Jill Kent, 1996) (Gilbert, 2010). The SRY is acting directly on the genital ridge and converts the somatic epithelial of the bi-potential zygote, into a male tactical (Gilbert, 2010) (ncbi, 1998).

6.8. MSD.
For the manidia, that has a natural habitat in the temperature range of 16°C-21°C however, when removed out of this range of temperature, a change from the sex ratio was noticed and the manidia are known to have sex chromosomes. (David O. Conover, 1987) which led to suspicion that there is more than one factor for the sex determination, in addition, an avian species- Megapode, was discovered with changing sex ratio in different temperature range 31°C- 36°C (34°C) (Australian brush-turkey Alectura lathami) in different embryonic developmental stages during a specific timing (Ann Goth, 2005), birds known to have the GSD mechanism of ZZ-ZW (Justin Chue, 2011). The moment the sex determination is affected by genetic factor and external factor it is classified as MSD.

\textsuperscript{12} - SOX-9 also plays a pivotal role in male sexual development.
\textsuperscript{13} - AMH gene.] It inhibits the development of the Müllerian ducts (paramesonephric ducts) in the male embryo.
6.9. Histology.

A section during sex change was taken and the conclusions for the Blue head wrass fish. Sex changes from female to male, left to right, sex inversion, histological follow up in chosen and known times from a fertile female, into a "sex change stage" with cell for follicles formation atretic follicles which have a role of ending the ovarian activity and for the coral reef fish it is either a sign for a stress or a sex change. On the right side, we can see spermatogenetic cell, fully functioning and producing sperm with arrows pointing on hormones producing cells in the testis (Robert H. Devlin, 2002).

Paradoxical nuclear sex chromatin findings that were widely thought to indicate that "Turner syndrome " and "Klinefelter syndrome" were sex reversed male and female respectively. An early chromatin-positive Klinefelter patient was observed to have XY sex bivalents and sperm in a single seminiferous tubule (Ferguson-Smith, 2006). The answer was found by karyotype analysis. The 47-XXY and 45 X karyotypes in Klinefelter and Turner
Sex Determination in Vertebrates

syndromes were reported. The discovery of SRY happened much later, the sex determining region on the Y becoming isolated during paternal meiosis so that recombination with the X chromosome was prevented. Attrition of the Y and accumulation of sequences involved in male gametogenesis led to a distinctive Y chromosome in which only small terminal parts were homologous with the X chromosome. These parts are necessary for synapsis and segregation of the X and Y chromosomes during male meiosis, with the Y chromosome at the small pairing (pseudoautosomal) regions, leaving a large differential segment containing the strictly X-linked genes. One of SRY’s functions is to activate SOX9 and thus the development of Sertoli cells. SRY is absent in some mole voles (Ellobius lutescens), therefore, X0 (Ferguson-Smith, 2006).

6.10. Endocrinology

A massive brain activity in the area that responsible for the homeostasis of the wrass fish during the sex change and we can detect a massive hormonal activity for male phenotype formation, on the expense of "converting hormones" which convert male hormones into female hormones (decreasing in those converting factors, meaning, more unconverted male factors), (Godwin, 2009) (Robert H. Devlin, 2002). A condition of external signaling and behavior receiving, which creates chain of endocrinal events which result in sex change, AVT\textsuperscript{14} over estrodiol\textsuperscript{15}, the investigation was taken on the paraoptic area\textsuperscript{16} of the wrass (Thalassoma bifasciatum) and was assumed that there is a connection between sex change and brain function and secretion in the fish’s brain, which means, that they could notice male expression gets bigger on the expense of female expression during the sex change. There are special areas in the brain, assisting in the sex change process by silencing some cell expression and increasing activation of others, most of them, are producing or involving with hormones production involved with sex change, another important area which involved with sex hormones production in the fish’s body are the sex organs and gland (Robert H. Devlin, 2002) (Godwin, 2009).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image10.png}
\caption{Functional gland responsible for homeostasis and covering mucous in the Wrasses brain, the AVT marked in green- for male phenotype emphasized over the estrodiol (converts to female hormones from male hormones) female depression in the brain and maleness establishments. An endocrinology direct connection (Robert H. Devlin, 2002).}
\end{figure}

14 - AVT- Arginine vasotocin, neurohypophysis hormone responsible male phenotype and it’s expression.
15 - Estrodiol converts male hormones to female hormones.
16 - The paraoptica area, responsible for the homeostasis and the mucosal glands covering the fish.
7. Hermaphrodites (Few Words).

Experiment, results and conclusions for a steroids treated female, which inverted its sex in the presence of the dominant male, which induces the sex inverting for the female in the pack, they wanted to investigate this phenomenon, therefore, several individuals from different species of coral reef with "Sequential hermaphroditism" were investigated and a fascinating finding was discovered which shown that all hermaphrodite individuals are infect females and only when the time is right (and that is the strategic) a sex invert will occur, which means, that the individual begins its life as a female whether if it is a Protandrous or protogynous (male first or female first respectively). Histological and physiological investigations were preformed to many hermaphrodites fish and they reached the conclusion that the juvenile stage is in a female form, even if the final stage as adult is in female form, they will change their sex at the beginning to a male and then to the alpha female (growing up stage as a male) for multi-male sequential hermaphroditism (e.g. Amphiprion percula or the Gobies fish), that can change sex more than once, from male to female and from female to male. (Godwin, 2009). The classical and most common pathway for hemaphrodites of the coral reef fish is strategic A: Juvenile female → Adult female → Alpha male.

Image 1. Labile sex determination in sex-changing fishes. (A and B) Monandric and Diandric protogyny seen in many wrasses and parrotfishes, (C) protandry as observed in anemonefishes, (D) Bi-directional sex change as exhibited in many gobies. Social cues can be important in directing both juvenile and adult sexual development.
8. GSD Molecular Genetics of Sex Determination (Genomic Data).

With the advent of modern biotechnology over the past decade, such as transgenicity, array-based gene profiling and proteomics, the field of mammalian sex determination has witnessed a remarkable boost in the understanding of the genetics and complex molecular mechanisms that regulate this fundamental biological event. Consequently, a number of excellent reviews have been devoted to this topic. The purpose of the present chapter is to provide an overview of selected aspects of mammalian sex determination and differentiation with emphasizing on studies that have marked this field of study (Robert S Viger, 2005), and abnormalities, (Appendix for further information about molecular techniques).

8.1. Molecular genetics background.

In mammals, sex development is a genetically and hormonally controlled process that begins with the establishment of chromosomal or genetic sex (XY or XX) at conception. At approximately 6 to 7 weeks of human gestation, expression of the Y chromosome-linked sex determining gene called SRY. Then initiates gonadal differentiation, which is the formation of either a testis (male) or an ovary (female). Male sex differentiation (development of internal and external reproductive organs and acquisition of male secondary sex characteristics) is then controlled by three principal hormones produced by the testis: (1) Mullerian inhibiting substance (MIS) or anti-Mullerian hormone (AMH), (2) Testosterone and (3) Insulin-like factor 3 (INSL3). In the absence of these crucial testicular hormones, a female sex differentiation ensues. This sequential, three-step process of mammalian sex development is also known as the “Jost paradigm” (A. Jost, 1970).

8.1.1. PAGE\textsuperscript{17} XX/XY.

True genotype sexing XY/XX and ZW/ZZ, small tissue samples (approx. 1 cubic cm) were collected from 235 dead Pacific walruses \textit{(Odobenus rosmarus)} (71 M and 164 F). The walruses harvested by Alaska Native hunters and also sampled skin from beach-cast carcasses. Also positively identified sex by either inspection of the whole animal or excised reproductive organ from which the tissue sample was taken, a genomic DNA was extracted, then a PCR\textsuperscript{18} amplifications (The PCR reactions began with denaturation at 94 C for 2 minutes, followed by 35 cycles consisting of 15 seconds at 94C, 15 seconds at 50 C, and 90 seconds at 728 C. They included a negative control (all PCR conditions identical to those described above but without added DNA) in each reaction), an electrophoresis was done for the PCR-amplified products and recorded results on Polaroid film. The Y-chromosome intron fragment was smaller than the X-chromosome fragment.

\textsuperscript{17} - Polyacrylamide gel electrophoresis, a separation technique by Mobility (function of length, conformation and charge of molecule).
\textsuperscript{18} - Polymerase chain reaction, used to amplify DNA sequence(s).
They assigned sex based on banding pattern of the products: 2 bands of different sizes (920 bp and 1,000 bp products) indicated male and 1 band (1,000 bp co-migrating products) indicated female. (Anthony S. Fischbach, 2008).

8.1.2. PAGE ZZ/ZW.
A simple PCR-based approaches for sex discrimination in three European Phalacrocoracidae species that tested, using 93 individuals of known sexes, for amplification of the Avian Sex-Specific chromo-helicase –DNA-binding protein gene. As expected, the PAGE for the male (homozygote for sex chromosomes) samples had 1 band (ZZ), while the female (heterozygote for sex chromosomes) PAGE result was 2 bands (Evanthia Thanou, 2013).

Image 12 Electrophoresis banding pattern from the product of Pacific walrus samples, collected from Alaskan waters, USA, 2002–2003, and one negative control amplified in a polymerase chain reaction with the Shaw et al. (2003) genetic sexing primers and electrophoresed on a 2.0% agarose gel. Samples in lanes with 2 bands are males and with 1 band are females. (Anthony S. Fischbach, 2008).

Image 13 (Evanthia Thanou, 2013).
8.2. X- and Y-Linked Specific Sequences.
Methods used nowadays in genetic labs and their reliability.

8.2.1. Y-Linked Specific Sequences.
The male-specific region of the Y chromosome (MSY) differentiates the sexes and comprises 95% of the chromosome's length. MSY is a mosaic of heterochromatic sequences and three classes of euchromatic sequences: (1) X-transposed, (2) X-degenerate and (3) ampliconic. These classes contain all 156 known transcription units, which include 78 protein-coding genes that totally encode 27 distinct proteins. The X-transposed sequences exhibit 99% identity to the X chromosome. The X-degenerate sequences are remnants of ancient autosomes from which the modern X and Y chromosomes evolved. The ampliconic class includes large regions (about 30% of the MSY euchromatin) where sequence pairs show greater than 99.9% identity, which is maintained by frequent gene conversion (non-reciprocal transfer). The most prominent features here are eight massive palindromes, at least six of which contain testis genes. Six of the eight palindromes carry recognized protein-coding genes, all of which seem to be expressed specifically in testes. MSY deletions have emerged as the most common of the known genetic causes of spermatogenic failure in human (Helen Skaletsky, 2003). The Sex-determining Region Y (Sry in humans) is a gene found on Y chromosomes that leads to the development of male phenotypes, such as testes, located on the short branch of the Y chromosome, initiates male embryonic development in the XY GSD-sex determination system. The SRY gene follows the central dogma of molecular biology; The DNA encoding the gene is transcribed into messenger RNA (mRNA), which then produces a single SRY protein. The SRY protein is also called the testis-determining factor (TDF). The SRY protein is a transcription factor that can bind to regions of testis-specific DNA, bending specific DNA and activating or enhancing its abilities to promote testis formation, marking the first step towards male rather than female development in the embryo (Troy, 2013), Y-chromosome has lost almost all traces of the ancestral autosome, including the genes that were once shared with the X chromosome.

X-chromosome inactivation (XCI) in mammals achieves dosage compensation between males and females for X-linked gene products. Inactivation of one X chromosome occurs early in female development and is initiated from the X-inactivation centre (XIC). The XIST-transcript (X (inactive)-specific transcript) is expressed initially on both X chromosomes, but later the transcript from the chromosome that is destined for inactivation becomes more stable than the other. Finally, the transcript is expressed only from the inactive X chromosome (Xi). Coating with the XIST transcript is the earliest of many chromatin modifications on The Xi, a progressive loss of XY recombination (Mark T Ross, 2005).

---

19- Any sequence of characters which reads the same backward or forward.
8.2.2. X-linked specific sequences.
The sex chromosome are shared by males and females, it has been determined that 99.3% of the euchromatic sequence of the X chromosome. Their analysis illustrates the autosomal origin of the mammalian sex chromosomes, process that led to the progressive loss of recombination between X and Y, and the extent of subsequent degradation of the Y chromosome.
LINE1 \(^{20}\) repeat elements cover one-third of the X chromosome, with a distribution that is consistent with their proposed role as way stations in the process of X-chromosome inactivation.
There are 1,098 genes in the sequence, of which 99 encode proteins expressed in testis and in various tumor types. Females inherit an X chromosome from each parent, but males inherit a single maternal X chromosome. Gene expression on one of the female X chromosomes is silenced early in development by the process of X-chromosome inactivation (XCI), and this chromosome remains inactive in somatic tissues however, in the female germ line, the inactive chromosome is reactivated and undergoes meiotic recombination with the second X chromosome. The male’s X chromosome fails to recombine along virtually its entire length during meiosis, instead, recombination is restricted to short regions at the tips of the X chromosome arms that recombine with equivalent segments on the Y chromosome. Genes inside these regions are shared between the sex chromosomes, and their expression can be described as ’pseudoautosomal’. Genes outside these regions of the X chromosome are strictly X-linked, and the vast majority, are present in a single copy in the male genome (Mark T Ross, 2005)
For sequencing, go to appendices (10.2.).

8.2.2.1. Coding and Features of the X chromosome sequence:
Analysis of the sequence reveals a gene-poor chromosome that is highly enriched in interspersed repeats and has a low (G + C) content (39%) compared with the genome average (41%). The Genes contain: known genes (699), novel coding sequences (132), novel transcripts (166), and putative transcripts (101). Also identified 700 pseudogenes in the sequence (4.6 pseudogenes per Mb), of which 644 are classified as processed and 56 as non-processed. The gene density (excluding pseudogenes) on the X chromosome is among the lowest for the chromosomes that have been annotated to date, also, evolutionarily conserved regions (ECRs) by comparing the X chromosome sequence to the genomes of mouse, rat, zebrafish and the pufferfishes Tetraodon nigroviridis and Fugu rubripes. (Mark T Ross, 2005)

---

20 Long interspersed elements (LINEs) are a group of non-LTR (long terminal repeat) retrotransposons.
8.2.2.2. Non-Coding RNA Genes (ncRNA).
Using a complementary approach, it has been analyzed the X chromosome sequence using the Rfam\textsuperscript{21} (Sam Griffiths-Jones, 2003), database of structural RNA alignments, and predicted 173 ncRNA genes and/or pseudogenes. The most prominent of the ncRNA genes on the X chromosome is XIST (X (inactive)-specific transcript) which is crucial for XCI. The XIST locus spans 32,103 bp in Xq13, and its untranslated transcript coats and transcriptionally silences one X chromosome. The RefSeq\textsuperscript{11} transcript of XIST is an RNA (19,275 bases), which includes the largest exon on the chromosome.

Repetitive sequences. Interspersed repeats account for 56% of the euchromatic X chromosome sequence, the long terminal repeat (LTR) Retroposon (repetitive DNA fragments which are inserted into chromosomes after they had been reverse transcribed from RNA molecule) coverage is above average, but the most remarkable enrichment is for long interspersed nuclear elements (LINEs) of the L1 family, which account for 29% of the X chromosome sequence compared to a genome average of only 17%.

8.2.2.3. X Chromosome Centromere.
The most proximal 494 kb and 360 kb of the Xp and Xq sequences, respectively, consist of extensive regions of satellite DNA\textsuperscript{22}, adjacent to euchromatin of the chromosome arms that is exceptionally high in L1 content (a group of LTR) (Mark T Ross, 2005).

\textsuperscript{21} Database containing information about non-coding RNA (ncRNA).
\textsuperscript{22} Main component of functional centromeres.
8.2.2.4. **Single-nucleotide polymorphisms (SNP).**
The heterozygosity level on the X chromosome is known to be well below that of the autosomes, and this difference can be explained partly or entirely by genetic of population (Mark T Ross, 2005).

8.2.2.5. **Comparison of the human X and Y chromosomes.**
The evolutionary process has eradicated most traces of the ancestral relationship between the human X and Y chromosomes. At the cytogenetic level, the Y chromosome has a large and variably sized heterochromatic block and is considerably smaller than the X chromosome.
The euchromatic part of the X chromosome is six times longer than that of Y’s, only few genes on human chromosome X have an active counterpart on the Y chromosome, and the majority of these are contained in regions where XY homology is of relatively recent origin. Has been discovered an extent of Y chromosome decay in non-recombining regions. The tip of the short arm of the X and Y chromosomes comprises the 2.7 Mb pseudoautosomal region (PAR1), homology between the X and Y chromosomes in PAR1 is maintained by an obligatory recombination in male meiosis (Mark T Ross, 2005).
There was a progressive loss of XY recombination (Mark T Ross, 2005).

8.3. Sexual Characteristics and Ranking, Levels of Sexuality.

“Sex” is a biological quality or classification of sexually-reproducing organisms. Sex generally female, male, and/or intersex, according to functions that derive from the chromosomal complement, reproductive organs, or specific hormones or environmental factors that affect the expression of phenotypic traits that are strongly associated with females or males within a given species. A range of traits are expressed within each sex, with considerable overlap of “female” and “male” phenotypic traits. Those factors that determined sex may be defined or classified (especially for “secondary sex characteristics”) (Schiebinger, 2011-2015) According to:

8.3.1. Primary Sex Characteristics and Genetics.

In humans and many other animals include Chromosomal make-up (female/male), such as ZW/ZZ (birds) and XX/XY (most mammals). In mammals the sex-determining region of the Y chromosome, SRY, plays the greatest role in sex differentiation. (Females and males may have karyotypes other than 46, XX and 46, XY, respectively) Regardless of karyotype, the presence of sex-determining genes means that every nucleated human cell has “sex.” (Schiebinger, 2011-2015)

8.3.1.2. Gametes and Gonads.

Germ cells, in species that produce two morphologically distinct types of gametes with each individual producing only one type, the egg-sperm distinction is the basis for distinguishing between females and males, respectively, (Schiebinger, 2011-2015).
8.3.2.1. Secondary Sex Characteristics.
Are phenotypic traits that strongly associated with females or males and become prominent at puberty when the ovaries and testes produce higher levels of hormones (estrogens and androgens respectively), often referred as “gonadal hormones” (however, also produced by the adrenal gland and metabolized in many body tissues), or “sex hormones” (also other hormones and genetic factors influence female and male phenotypic traits and “sex hormones” have roles unrelated to sex differentiation), both classes of hormones have important biologic effects in both sexes. For example, estrogens are critical to skeletal development in both sexes, and androgens are responsible for pubic and axillary hair growth at puberty in both sexes. Examples of secondary sex characteristics in humans include shorter stature and wider pelvis, breast development, and more fat in the thighs and buttocks in women and broader shoulders, greater muscle mass, more facial and other body hair, and “male pattern” baldness in men. These traits vary within each sex and ranges overlap. For instance, many women are taller than many men and some women are stronger than many men. These traits can also be promoted for both sexes, by exogenous hormones, (Schiebinger, 2011-2015) (Gilbert, 2010). Morphology and physiological traits that differentiate female and male phenotypes.

8.3.2.2. Internal reproductive organs and genitalia.
That derived from “bipotential” organs (e.g. “indifferent gonads” that become ovaries or testes) and dual structures. Usually, one structure is maintained and the other regresses. For example, human embryos have both 'mesonephric' ducts, which become Müllerian ducts (and form the fallopian tubes, uterus and proximal vagina) in females and regress in males and 'paramesonephric' ducts that become Wolffian ducts (and form the seminal vesicles, epididymis, and ductus deferens) in males, but regresses in females (Schiebinger, 2011-2015).

8.3.2.3. External genitalia.
Which generally differentiate toward one of two basic forms: distal vagina, labia and clitoris in females; and scrotum and penis in males; nevertheless, external genitalia may not reflect karyotypical or internal genital sex (Schiebinger, 2011-2015).

8.3.2.4. Sexually Dimorphic Prenatal Neural Structures.
Many morphological and functional brain dimorphisms arise during late gestational and neonatal periods. They may be due to differentiating effects of fetal hormones and other sex-biased regulatory mechanisms, including genetic and environmental factors (Schiebinger, 2011-2015).
8.3.3. Tertiary Sex Characteristics- Behavior and Gender Orientation.
This stage is mostly can be observed in human and rarely for animals. Sexual orientation refers to an enduring pattern of emotional, romantic, and/or sexual attractions to men, women, or both sexes. Sexual orientation also refers to a person’s sense of identity based on those attractions, related behaviors, and membership in a community of others who share those attractions. Research over several decades has demonstrated that sexual orientation ranges along a continuum, from exclusive attraction to the other sex to exclusive attraction to the same sex. However, sexual orientation is usually discussed in terms of three categories: heterosexual, gay/lesbian and bisexual (American Psychological Association , 2008). There is a distinct difference between Gender and Sex (Gender refers to the attitudes, feelings, and behaviors that a given culture associates with a person’s biological sex) (American Psychological Association , 2008). This is a multi-factorial complicated topic, involving sociology, psychology and law studies. Animals however, show a same sex pairing as well (Nathan W. Bailey, 2009).
Intersex may be defined as variations or combinations of what are considered XY male-typical and XY female-typical chromosomal, gonadal, and genital characteristics (WHO, 2015).

8.4. Genomic Abnormalities and Infertility.
Because of other transcription factors, such as DAX1 and FOXL2 in females and SOX9 in males, or translocation of the SRY to the X chromosome or an autosome, females and males may have karyotypes other than 46, XX and 46, XY, respectively. (Schiebinger, 2011-2015). A research suggests, however, that in a few births per thousand some individuals will be born with a single sex chromosome (45X or 45Y), sex monosomies and some with three or more sex chromosomes (47XXX, 47XYY or 47XXY, etc.), sex polysomies. In addition, some males are born 46XX due to the translocation of a tiny section of the sex determining region of the Y chromosome. Similarly some females are also born 46XY due to mutations in the Y chromosome. Clearly, there are not only females who are XX and males who are XY, but rather, there is a range of chromosome complements, hormone balances, and phenotypic variations that determine sex. More than 95% of the Y chromosome is a "male-specific" and a single copy of the Y chromosome is able to induce testicular differentiation of the embryonic gonad. The Y chromosome acts as a dominant inducer of male phenotype and individuals having four X chromosomes and one Y chromosome (49XXXXY) are phenotypically male. When a Y chromosome is present, early embryonic testes develop around the 10th week of pregnancy. In the absence of both a Y chromosome and the influence of a testis-determining factor (TDF), ovaries develop (WHO, 2015) (Gilbert, 2010).

23- Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1
24 contains a fork-head DNA-binding domain and may play a role in ovarian development and function
Aneuploidy is the condition of having less than (monosomy) or more than (polysomy) the normal diploid number of chromosomes. Aneuploidy occurs in at least 5% of all pregnancies and is the most commonly recognized chromosome abnormality in humans (WHO, 2015).

### 8.4.1. Few Examples Sex Chromosome, Abnormality.

#### 8.4.1.1. "Turner's Syndrome".
Monosomy X (45X) occurs in individuals that have one X chromosome, no Y chromosome, and are phenotypically female (WHO, 2015). People who have Turner syndrome develop as females (Genetic Science Learning Center, 2015). Generally lack prominent female secondary sexual characteristics and are sterile in some instances of "Turner syndrome" (WHO, 2015). Some species have "Turner Syndrome" (*Ellobius lutescens*, mole voles as mentioned earlier) for both, male and female (Ferguson-Smith, 2006).

#### 8.4.1.2. "Klinefelter's syndrome".
Klinefelter syndrome (47XXY or XY/XXY mosaic) with male phenotype is the most pervasive sex chromosomal anomaly affecting approximately 1:600 males. Males with Klinefelter syndrome carry two or more X chromosomes which results in abnormal development of the testis, leading to hypogonadism and infertility. Affected individuals are often tall and produce relatively small amounts of testosterone. As a result of this hormone imbalance, affected males have incompletely developed secondary male sex characteristics. (WHO, 2015) Similar conditions are caused by additional X chromosomes (48, XXXY; 49, XXXXY), (Genetic Science Learning Center, 2014). Klinefelter was documented in several animal cases, "tricolor" male cats, (Rieck, 1984).

#### 8.4.1.3. Congenital Adrenal Hyperplasia.
Called adrenogenital syndrome (AGS), results from a genetically caused deficiency of cortisol, a steroid hormone produced by the adrenal cortex. The disorder occurs with a frequency of 1:5000 and results in incomplete female sex differentiation and increased androgenic effects due to a compensatory increase in adrenocortical hormone (ACTH) (WHO, 2015).
9. Discussion and Conclusion.

There are two mechanisms and strategies for sex determination in vertebrates GSD and ESD and one mechanism which combines them both, the MSD. In the GSD system, the sex determination is known and determined already in the early stages of the embryonic development, the fertilized egg is sexually bi-potential but the fate is known and detectable, the GSD has advantage of resistance from external environmental pressure and factors and does not let the environment to influence the sex determination, resulting in sex ratio of 1:1 male to female. The disadvantage is the need to have a supportive genome (maintenance enzymes and intracellular factors) for the sex determination, (Gilbert, 2010) also at the maintenance and protection of the offspring, almost in all cases of GSD (the will to keep sex ratio, egg nursing incubation or pregnancy). The ESD sex determination is not instantly made and is undetermined immediately but throughout the embryonic developmental life. The fertilized egg is a sexually bi-potential egg for longer time than the mammalian embryo, the advantage of the ESD is that this mechanism is taking the environmental pressure factors and using them for the sex determination (temperature or pH), the offspring nursing is minimal to not existing and there is no energetic effort and investment in the cellular level for sex chromosomes (maintenance, transcription etc.). The disadvantage of the ESD is that it is environmental (external) influenced, which means, that any change in the embryo’s external environment might lead to a stress for the whole species. For all strategies, in the cellular level, there is a constant involvement of genes, for maintenance and expression of the reproductive organs or the phenotype of the vertebrate, even for the ESD. My estimation is that there was evolutionary progress from the ESD toward GSD, however, due to environmental pressure, convenience and adjustment to changing condition, we could notice reinstating of the vertebrates to different sex determination regardless of their location on the phylogenetic tree, different models for different vertebrates (e.g. fish with ESD and a relative fish species with GSD). As we progress in the evolution, along the phylogenetic tree, the GSD mechanism is more frequent, entire systems have kept on a certain mechanism, combined in between the mechanisms and even "return" back in evolution in order to increase their survival chances and the ability to reproduce. I believe that here, the MSD came into expression with genetic and environmental combination, in fact, any ESD with genetic influence, known and will be classified as MSD, who knows, maybe some of the GSDs are actually MSDs and we haven’t discovered the environmental-external factor that is influencing the sex determination. The MSD was discovered accidently when heterogametes were discovered in vertebrates with ESD and also, with the sex ratio shifting in extreme environments for GSDs. For mammals, female needs two X chromosomes in order to achieve a complete female development. There is a "default program" for sexual fate in mammals, which is "female". The evidence is the fact that for a female result, it is enough to have one X chromosome while for male formation, we need both X and Y chromosomes, the body can handle even with poly-X, by silencing the excess X, we also can observe a poly-gene state but the cause is still GSD or a
set of sex chromosomes and genes (e.g. *platypus*). In sex changing or invert for coral reef fish- hermaphrodites, endocrinology is the factor that drives this process, even if it is only behavioral and can be changed by external factors, the fact that the hermaphrodite individuals are starting their lives as female (some species, even silencing this expression and live as males) and there is constant endocrinological involvement for the coral reef fish (Robert H. Devlin, 2002), which means, that the environment is stimulating the individuals in the pack and causing, not only physiological change but also changing in the hormonal level, changes in glands in the brain and neurological changes as well (Godwin, 2009), that are triggering for chain of events inside the brain, which initiates the sex change. More ever, hormonal sex change is being used in aquaculture for both directions and purposes, to increase growth rate as male and for breeding packs, poly-female with very few males to breed them. This topic is interesting and unclear completely, yet. My conclusion from Image 7, "Histology during sex change", the blue head wrass fish as a model, the left side of the picture, we can see a fertile female with functioning ovary, in the middle picture we can see the sex change process with cells in the gonads have atretic follicle formation and their role is to prevent and end further more activity of the ovary (it happened to women in stress and bad conditions like in the concentration camp during the 2nd world war, they didn’t ovulate because of nutritional and/or mental stress). In coral reef fish, it means either stress or sex change, we can also notice cells that will change into spermatogenesis cells and hormonal producing cells when the sex change will be completed, at the right image of the picture, we can see a fully functioning and forming spermatogenesis cell with arrows pointing at the hormones producing cells in the testicle. Apparently, the precursor for this mechanism for the coral reef fish (hermaphrodites) is bi-directional, on one hand, the male, which induces factors, physicals e.g. size, color, shape and chemicals e.g. scent (pheromones) and on the other hand, the female, which neurologically, endocrinologically and physiologically, inhibits the sex change by social-behavioral dominance. Many researches are needed to understand those reproduction mechanisms and understanding the control and regulation for them, which maybe, some day, could help resolving infertility for some species or even mankind or species in extinction risk. The contribution could be significantly big. We can find evidences for almost any phenomenon (e.g. XX-male and XY-woman due to chromosomes translocations) and unnecessarily understood completely but the will to reproduce was pushing different organisms to different mechanisms of the sex determination throughout, exploiting the environmental conditions. There is no such thing as “the right” or “wrong” sex determination mechanism, nor primitive or advanced mechanism. Each mechanism fits their environmental niche of the organism living in it. The knowledge of sex change has significant contribution, for science and for the environment as alternative for medication and food source, if we understand better those mechanisms or rather "sex-reactivation" we might even resolve the infertility among human being, extinct organisms, coral reef fish-protection (global warming and acidification of the sea water, increasing CO₂ in the water and decreasing of the total coral reef cover, diminishing their
Sex Determination in Vertebrates

When a global-large scale environmental changes will happen and how will they deal with it or react to it?

ESD-fish: It is clearly shown that sex ratios and differentiation of both, fresh and marine fish are influenced by environmental factors. Mostly, temperature seems to be the main Environmental determinant of sex. The characteristics of thermosensitivity in fish is different from those observed in reptiles, in particular, because very few true monosex populations are induced by the modification of an environmental factor even at extreme values. This seems to be mainly associated with strong temperature genotype interactions. Therefore, at least in sensitive Fish species, sex is probably determined by genetic factors (both major and minor factors.) by environmental factors and their interactions. In temperature sex differentiation of fish there is (up and down) gene regulation.

Sea-turtles TSD: All species of marine turtles studied to date appear to exhibit male-female (MF) pattern in which incubation in cooler temperatures produce males whereas warmer temperatures during incubation lead to the production of females. The transitional range of temperatures (TRT) describes the range of temperatures in which the sex ratios shift from all female hatchlings (temperatures above the TRT) to an all male ratio (temperatures below the TRT). Within this range there is what is referred to as the pivotal temperature which yields a 1:1 MF ratio, (Yaron Tikochinski, 2010). This data may lay the basis for the potential manipulation of sex ratio of hatchlings, as an effort of enhancing reproductive outputs of populations and they enable researchers to assess aspects regarding TSD and how it may affect the reproductive ecology in a population. Hatchlings do not exhibit external characteristics that can assist in distinguishing sex. There are indeed methods of doing so but they involve dissections and histological studies. (Yaron Tikochinski, 2010). This hot topic is broad, and for many questions, we still do not know the answer, many of the mechanisms still unclear till these days, researches are taking place constantly, the knowledge is accumulating and the questions are piling up as well. I find this topic very interesting and I had the privilege to investigate it, I think that the solution for our global needs will come from the aquatic world, (food drinking water, medicine, habitat etc.) More research is needed. It is also estimated that one of three miscarriages is due to aneuploidy affecting the foetus, (WHO, 2015). Therefore, reproductive solutions can be assisted by further knowledge.
10. Appendices.

10.1. Methods of Investigation.
Molecular genetic methods for sex detection in different monomorphic species. In many bird species, males and females cannot be discriminated on the basis of phenotype (sexual monomorphism) and we could overcome the problem by molecular techniques in the 1990, based on DNA hybridization or polymerase chain reaction (PCR) (Anna Dubeic, 2006) and the amplification of primers related to the CHD gene is the most rapid, cheap and reliable technique of almost universal application. By the time of their common application, sex had been identified on the basis of: (1) behavioral observations, (2) presence of brooding patch, (3) differences in morphometric traits, (4) examination of the gonads by laparotomy or laparoscopy, and (5) examination of sex chromosomes. The first two methods can be generally applied only during the breeding season, and the analyses of morphometric traits may be ambiguous. The examination of gonads may be difficult in the adult birds outside the breeding season (when the gonads regress) and in nestlings because of their small body size, The W chromosome is rich in heterochromatic, repetitive DNA of the satellite type and both chromosomes contain a small pseudoautosomal region. The examination of the morphology of sex chromosomes requires establishing the cell cultures and stopping the mitotic division at the metaphase stage, when chromosomes are well visible and separate. Chromosome spreads are most commonly based on the cells from the pulp of growing feathers; the sex chromosomes are usually easy to distinguish from one another, because they differ in size. The Z chromosome falls into the group of large chromosomes, called macrochromosomes, usually being comparable in size with chromosomes 4 and 5, while the W chromosome represents smaller chromosomes, called microchromosomes. However, chromosome spreads of good quality are very laborious rarely used. The techniques are divided into two groups: 1 is DNA hybridization based, detection of sex-specific sequences in the genomic DNA by complement DNA-prob. And 2, PCR based- location by primer and amplifying the specific part. In avian, DNA sampling is taken from the blood, -small amount of non-heparinized sample, or feathers collection. DNA can be kept and keep stability in ethanol. (Anna Dubeic, 2006), DNA isolation can be done with several methods (e.g phenol-chlorophorm or guanidium thiocyanate) or even a commercial kit can be applied.

10.1.1. DNA HYBRIDIZATION. A large proportion of repetitive sequences are featured by the W chromosome, Those repetitive sequences contain tandems of repeated DNA: micro- and minisatellites, Microsatellites form clusters of usually less than 150 bp in length, with repeat units up to 13 bp, while minisatellites are longer, with clusters up to 20 kb in length and repeat units up to 25 bp, (Anna Dubeic, 2006) .detection of micro- and minisatellite W-sex linked markers, the DNA has to be digested in a specific areas by restrictin enzymes, then

---

25 - Chromo Helicase DNA-bi.
DNA-fragments separation according to size by electrophoresis and transferred to a nylon membrane (southern blot), the solution contain a complementing sequence probes and can be detecting depending on the labeling, usually p32 isotope, autoradiogram, fluorescence, or enzymatic reaction, the DNA profiles of reference males and females should be compared e.g trinucleotide repeat (TCC)and was shown to be sex-linked in pigeons and chickens.

**10.1.2. PCR Technique. Random Amplification of Polymorphic DNA (RAPD).** A single and randomly chosen 10-nucleotide primer that amplifies genomic fragments. If one of the bands on a gel is specific only for females, it most probably reflects a W-linked sequence, a single primer amplifies about 15 DNA fragments. Therefore, RAPD analyses start with testing many of primers to detect those that amplify the products specific for females. RAPD was developed for genetic polymorphism studies, differences between 2 individuals may be generated by - autosomal or Z chromosomal polymorphism, therefore, in order to locate the marker that most likely linked to the W chromosome they created a pooled DNA (a DNA sample from different subjects of the same sex) enabling to amplify products from only within each female pool (bulked segregant analysis) and should be compared with the PCR product from a male pooled sample. DNA fragment are resolved either on an agarose or polyacrylamide gel and fragments selected as a sex-specific marker should be among the 4 most intensive bands on a gel.

**10.1.3. Amplification fragment length polymorphism (AFLP):** Technique combines PCR with digesting DNA with restriction enzymes, a single reaction results in about 100 DNA fragments, steps are: digestion with 2 different restriction endonucleases (a 4 bases cut and a 6 bases cut), then ligation by oligonucleotide adaptors attach to "cohesive ends" (sticky ends that made by the endonucleases during splicing), then a preselective complementary primer for selective amplification (promoted by a "start cod primer") The last step of AFLP includes a selective amplification with the same primers, but usually with a 3-nucleotide extension and a detection on polyacrylamide gels. Unlike the RAPD, use of female-pooled DNA samples for detection is not recommended because PCR products may not reflect each of the individual samples in the pooled DNA and a sex-specific marker is needed to detect the probes. A positive control is highly recommended for both AFLP and RAPD, for exclusion of incorrect sexing result of non-optimal PCR reaction and will result in no visible W-linked marker that will result in identification of female as male (Anna Dubeic, 2006).

**10.1.4. CHD-based sex identification.** Only a few genes have been located on the W chromosome. The most universal tag for sex typing is provided by the CHD gene. It encodes the Chromodomain Helicase DNA binding protein and is located in both chromosomes in almost all bird species except ratites, which have undifferentiated sex chromosomes, there is no recombination between the Z and W copies of this gene, which hints that it is located elsewhere than the pseudoautosomal region, it has at least 2 introns, different in length
between the Z and the W chromosomes, which allow the differentiation between the Z and the W products on a gel. Consequently, males are identified by 1 band and females by 2 bands, with some exceptions however in those species, it can be detected by sizes of fragments and not by the number of bands. (Anna Dubeic, 2006).

10.1.5. SELECTION OF AVIAN SEXING TECHNIQUE: The unquestionable first method of choice will be the CHD based technique, high accuracy, easy to perform, cheap and fast. AFLP is more repeatable than RAPD and allows producing a higher number of bands per assay. (Anna Dubeic, 2006)

10.1.6. Platypus (Ornithorhynchus anatinus). Exhibits a fascinating combination of reptilian and mammalian characters. A fur coat adapted to aquatic lifestyle, females are lactating, yet lay eggs and males are equipped with venom similar to that of reptiles. (Wesley C. Warren, 2008). The platypus has 26 pairs of chromosomes in total while researchers confused about which ones are autosomal and which ones are sex chromosomes. A researcher (Australian National University in Canberra) used fluorescent tags to study the animal's chromosomes, discovered five separate pairs, which join together in a chain during cell division, that determine an individual's sex. (Roxanne Khamsi, 2004).

10.1.7. FISH: fluorescence in situ hybridization: Used for the duck-billed platypus, confirmed how the ten elements segregate. Remarkably, the five X chromosomes go into one cell, and the five Y into another, This results in two kinds of sperm—half with XXXX that determine female young and half with YYYYY that determine male young. A certain pattern has to take place - X1Y1X2Y2X3Y3X4Y4X5Y5—during meiosis (Atkinson, 2004).
10.2. X chromosome Sequencing:

http://www.nature.com/nature/journal/v434/n7031/fig_tab/nature03440_F1.html

10.3. Picture: was taken from an internet presentation
http://www.slideshare.net/GigaScience/graves-smbe-2014

10.4. Hermaphrodites. Protogynous species, no indication of testicular tissue may be apparent in young individuals of *Dascyllus reticulatus*. In the *protogynous* Serranid *Plectropomus maculatus*, sperm ducts do not form initially, but rather sperm is collected intra-gonadally (Robert H. Devlin, 2002).
**Bibliography**


Sex Determination in Vertebrates


http://www.nature.com/articles/nature03021.epdf?referrer_access_token=e2FLfC_iMBpAofIIuQ7PWrGn0jAjWeI9jnR3zoTv0NkmrzFX607BRVg-CqxmRRqEHJKowRF3iyzaOs1SwOkel8Kcmh3iMzoEuzuAmn6Kc-skXaqYLnUJoE.

Sex Determination in Vertebrates


Yaron Tikochinski Jeremy S. Edwards Complete genome sequencing of the green sea turtle (Chelonia mydas) [Conference]. - Michmoret : Ruppin Academic Center, 2010.

Acknowledgements.

I would like to thank Prof. Zöldág, László, much appreciation for giving me the opportunity to investigate this broad topic.
Copyright Declaration.
HuVetA - SZIA
AGREEMENT OF ELECTRONIC PUBLISHING AND DECLARATION REGARDING COPYRIGHT *
Name: Aaron I (Ron) Korabelnik
Contact information (e-mail): akorabelnik@gmail.com
Title of document (to upload): "SEX DETERMINATION AND DETECTION IN VERTABRATES"

Publication data of document:
Number of files submitted:
With the acceptance of the present agreement the author or the holder of copyright offers non-
exclusive rights for HuVetA and SZIA to archive the above document (including its abstract) converted into PDF format protected against copying without changing its content in order to preserve it and ensure access to it.
The author accepts that HuVetA and SZIA store more than one copy (accessible only for the administrators of HuVetA and SZIA) of the document provided by you exclusively for the purposes of ensuring safe storage and recovery if necessary.
You also declare that the provided document is your own work and/or you are entitled to give the permissions included in the present agreement. You also declare that the document is an original one and you do not know about it violating the copyright of anyone else. If the document has a part which you are not the copyright owner of, you have to remark that you got unlimited permission from the copyright owner to give permission for the use of the document according to the present agreement, and the name of the original author is clearly indicated by the parts the copyright of which belong to a third person.
The copyright owner defines the conditions of access as follows: (indicate your choice by putting an X in the proper box):
X - I give permission to make the document stored in HuVetA/SZIA accessible on the internet without any limitation for everyone,
I give permission to make the document accessible only within the intranet (IP range) of the Szent Istvan University,
I give permission to make the document accessible only on one single dedicated computer at the Veterinary Science Library,
I give permission only to upload the bibliographic data and abstract of the document (with unlimited access on the internet to these data only),
I do not give permission to make bibliographic data public or access to the document uploaded to HuVetA/SZIA.

* The present declaration is based on the rector’s order number 5/2011. regarding the database of scientific publications written at the Szent Istvan University.
Please, also make declaration regarding the in-house use of the document by writing an X into the box:
X - I give permission for the in-house reading of the printed version of my document in the library.
If the preparation of the document to be uploaded was supported or sponsored by a firm or an organization, I declare that I am entitled to sign the present agreement regarding the document.
The operators of HuVetA/SZIA do not assume any legal responsibility towards the author or copyright holders or organizations for the case if a user would use the material uploaded with permission to HuVetA or SZIA in an unlawful way.
Budapest, .day .month 2015.
Signature: Author/Copyright owner.

© Many thanks to Judy and Family.