Evolution of Mammalian Sex Chromosomes and Sex Determination Genes: Insights from Monotremes

A thesis submitted for the degree of Doctor of Philosophy

Deborah Fernanda Toledo-Flores

School of Molecular and Biomedical Science

Discipline of Genetics

December 2014
Table of Contents

Abstract .............................................................................................................................. 1
Thesis Declaration ........................................................................................................... 3
Acknowledgments ........................................................................................................... 4
CHAPTER 1: Introduction ................................................................................................ 5
  Chapter overview ......................................................................................................... 5
  Introduction .................................................................................................................. 6
    Sex chromosome evolution in vertebrates .................................................................. 8
    Sex chromosomes evolved from a pair of autosomes ................................................. 9
    The therian XY sex chromosome system ................................................................... 14
    The prototherian multiple sex chromosome system ................................................. 17
    Molecular aspects of mammalian sex determination ............................................... 20
    Sex determination genes in therian mammals ......................................................... 20
    Sex determination in monotremes ............................................................................ 26
    Sex ratios in captive animal populations .................................................................. 28
Aims of the Project ........................................................................................................ 30
Summary .......................................................................................................................... 31
References ...................................................................................................................... 34
CHAPTER 2: Prediction of Y-linked genes in the platypus reveals Amhy as the most likely sex-determining candidate gene ................................................................. 48
  Chapter overview ......................................................................................................... 48
  Statement of authorship ............................................................................................. 49
Publication: .................................................................................................................... 50
  Cortez, D., Marin, R., Toledo-Flores, D., Froidevaux, L., Liechti, A., Waters, P.D.,
  chromosomes across mammals. Nature 508, 488-493. .................................................. 50
CHAPTER 3: Non-random meiotic segregation of the therian proto sex-chromosome in platypus may provide insights into differentiation of sex
  chromosomes in mammals ......................................................................................... 51
  Chapter overview ......................................................................................................... 51
  Statement of Authorship ............................................................................................. 52
Manuscript in preparation: ............................................................................................. 53
  Deborah Toledo-Flores, R. Daniel Kortschak, Frank Grützner. Non-random
  segregation of the therian proto-sex chromosome at platypus male meiosis. .............. 53
CHAPTER 4: Identification and characterization of a male-specific change in
  Sox3 in the platypus ....................................................................................................... 89
  Chapter overview ......................................................................................................... 89
  Identification of a male-specific Sox3 allele in the platypus might suggest a role in
  male sex determination ............................................................................................... 90
CHAPTER 5: Investigating sex bias in captive bred echidnas ..................................... 117
  Chapter overview ......................................................................................................... 117
  Statement of authorship ............................................................................................. 118
Manuscript in preparation: ............................................................................................. 119
  Deborah Toledo-Flores, Wan Xian Kang, Arthur Ferguson, Belinda Turner, Enkhjargal
  Tsends-Ayush, Shu Ly Lim, Frank Grützner. Genetic Sexing Reveals Female Bias in
  Echidnas Born in Captivity ......................................................................................... 119
  Statement of authorship ............................................................................................. 144
Letter in preparation: ..................................................................................................... 145
Deborah Toledo-Flores, Frank Grützner. Identification of a Sox3 deletion in a captive-bred echidna. .................................................................145

CHAPTER 6: Significance and future directions ..................................152
Chapter overview ...........................................................................152
Significance and future directions ....................................................153

Amendments .....................................................................................156
Abstract

Genetic sex determination systems are generally based on the presence of differentiated sex chromosomes. Birds have a ZZ/ZW sex chromosome system in which males are ZZ and females ZW, whereas mammals have an XX/XY system with males being XY and females XX. Monotremes have an extraordinary sex chromosome system that consists of multiple sex chromosomes: 5X5Y in platypus and 5X4Y in echidna. Intriguingly, the monotreme sex chromosomes show extensive homology to the bird ZW and not to the therian XY. However, sex determination in monotremes is still a mystery; the Y-specific Sry gene that triggers male sex determination in therian mammals is absent and so far very few genes have been identified on Y chromosomes in monotremes. To gain more insights into the gene content of Y-chromosomes and to identify potential sex determination genes in the platypus a collaborative large scale transcriptomic approach led to the identification of new male specific genes including the anti-Muellerian hormone AMH that I mapped to Y5, this makes Amhy an exciting new candidate for sex determination in monotremes.

Platypus chromosome 6 is largely homologous to the therian X and therefore it represents the therian proto sex chromosome. In addition, this autosome features a large heteromorphic nucleolus organizer region (NOR) and associates with the sex chromosomes during male meiosis (Casey and Daish personal communication). I investigated chromosome 6 heteromorphism in both sexes and found a number of sex-specific characteristics related to the extent of the NOR heteromorphism, DNA methylation, silver staining patterns and interestingly, meiotic segregation bias. This
raises the possibility that chromosome 6 may have commenced differentiation prior to monotreme therian divergence.

These results led me to investigate the chromosome 6 borne gene *Sox3*, from which *Sry* evolved in therian mammals. This revealed a platypus male-specific *Sox3* allele, which differs from the alleles observed also in females on the length of one of the *Sox3* polyalanine tracts. This raises the possibility that *Sox3* may be working differently in males and females.

We have used our unique knowledge of monotreme sex chromosomes to determine the sex of captively bred echidnas. I used a PCR based genetic sexing technique that utilizes DNA from small hair samples and primers that amplify male-specific genes. Interestingly, I found that seven out of eight echidnas born in captivity were females. Furthermore, I found a *Sox3* deletion in the only male echidna born in captivity. This gives us the unique opportunity to investigate the sexual development of an animal in which this gene is naturally deleted providing an exceptional situation in which to study monotreme sex determination. Furthermore, this sexing technique has the potential of being applied in the wild to investigate sex ratio in natural populations of monotremes, including the critically endangered long-beaked echidna.
Thesis Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

The author acknowledges that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University’s digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Deborah Fernanda Toledo-Flores

20 – Dec – 2014
Acknowledgments

I would like to thank all the people that have supported me and guided me throughout my Ph.D. studies. Thanks to my supervisor Prof. Frank Grützner for his constant advice and support and to all the current and former members of the Grützner’s lab for being excellent colleagues and friends. Thanks to our collaborator Prof. Henrik Kaessmann and his group at the University of Lausanne, Switzerland, and to Prof. Vincent Harley and his group at the Prince Henry’s Institute, Melbourne. Thanks to Dr. Dan Kortschak, Prof. Jeremy Timmis and Prof. Jenny Graves for constructive discussion and feedback.

I would like to acknowledge the support of the Mexican National Council for Science and Technology (CONACYT) for sponsoring me during my Ph.D.

Thanks to all the people that made this journey so enriching and enjoyable; the School of Molecular and Biomedical Science students and staff, my friends and my boyfriend. I want to particularly thank Nicole and Reuben for their support, feedback and for so many good chats and lunch sessions. Also, thanks to Nicole for keeping my cookies in her drawer!

Finally, I would like to specially thank my family because without them this would not have been possible. Thanks to my mom, dad and my little brother for always being there to support me, advice me, encourage me and inspire me. Thank you. – Finalmente, quisiera agradecer muy especialmente a mi familia porque sin ellos, esto no hubiera sido posible. Gracias a mi mamá, papá y a mi hermanito por siempre estar ahí para apoyarme, aconsejarme, motivarme e inspirarme. Gracias. ¡Soy hija de mi apá y de mi amá! ¡Y hermanita de mi hermanito!
CHAPTER 1: Introduction

This chapter consists of a conventional thesis introduction.

Chapter overview

In this chapter I present an overview of the evolution and function of sex determination and sex chromosome systems in vertebrates, which is relevant for the work presented in this thesis. I will discuss the complexity of the monotreme sex chromosomes and their homology to the ZW-system in birds. In addition, I will discuss how the absence of Sry in monotremes together with the lack of information on Y-specific genes leads to a gap in the knowledge of sex determination in these animals. This will allow a better perspective of how this work on monotremes contributes to our understanding of the evolution of sex determination and sex chromosomes. In addition, I will briefly discuss the relevance of these studies in monotreme conservation.
Introduction

This research project is based on the study of monotremes in order to obtain insights onto the evolution of sex determination in the mammalian clade. This basal group of mammals have amazed and intrigued scientists for more than 200 years due to their fascinating appearance and extraordinary biology. Monotremes consists of the platypus (*Ornithorhynchus anatinus*) and four extant species of echidnas (*Tachyglossus aculeatus*, *Zaglossus bruijni*, *Zaglossus attenboroughi* and *Zaglossus bartoni*). They diverged from therian mammals about ~200 million years ago [1], whereas mammals diverged from birds and reptilians 315 million years ago (Fig. 1) [2]. Monotremes possess several traits that establish them as mammals such as fur and milk-producing mammary glands to feed their young [3], but they have also retained some features seen in birds and reptiles such as oviparous reproduction and a single opening for the intestinal, genital and urinary tracts [3].

Interestingly, this mixture of characteristics is also observed at the molecular level. Similar to therian mammals monotremes have a XY male heterogametic system, which is comprised by multiple sex chromosomes (male platypus have 5X5Y and male echidna 5X4Y) [4, 5] that share homology to the bird sex chromosomes [4-6] and no homology to the therian sex chromosomes. With such a complex system, it is unclear what are the genes involved in triggering male sex determination in monotremes representing an important gap in the knowledge of the evolution of mammalian sex determination.
Figure 1. Variation in sex determination genes and sex chromosome systems among amniotes. Divergence times between clades are represented in millions of years (Mya). Homology relationships are represented in blue, green and orange. TSD: Temperature sex determination. Croc: Crocodilians. Figure adapted from [7].
Sex chromosome evolution in vertebrates

Sexual reproduction is the predominant mode of reproduction in vertebrate species. It is achieved through the combination of the genetic content of two gametes. Usually, females are defined as the sex that produces the larger gamete (ovum) whereas males produce the smaller gamete (sperm). Whether an organism develops as a female or as a male is determined either by genetic or environmental variables. These mechanisms are known as genetic sex determination (GSD) and environmental sex determination (ESD), respectively [8]. GSD relies on the presence of one or more loci that drive the sex determination process and which, in most cases, are located on sex chromosomes. Heteromorphic sex chromosomes represent an amazing example of convergent evolution that evolved independently in different lineages but share several common features [9].

All mammals, birds and snakes rely on the presence of heteromorphic chromosomes to determine male or female fate during development. Similarly, some reptilians, amphibians and fish species also possess heteromorphic sex chromosomes, even though ESD is equally common among these clades (Fig. 1). Mammals have an XY sex chromosome system whereas birds and snakes have a ZW system. However, there is no gene homology between the mammalian XY and the avian ZW [10]. Although both birds and snakes have ZW systems, there is no gene homology between their sex chromosomes [11]. The human X-chromosome is largely homologous to the chicken chromosomes 1, 4 and 12 [12]; the chicken Z, to the human chromosomes 9 and parts of the 5, 8 and 18 [10]; and the snake Z, to the human 3, 7, 10 and 17 and to the chicken 2 and 27 [11]. Another example of sex chromosome variation is the dragon lizard *Pogona vitticeps*. This animal has a micro-ZW system that is not homologous to birds nor to snakes sex chromosomes [13]. Interestingly, the gecko *Gekko hokouensis* also has a
ZZ/ZW female heterogametic system, which shares homology to the bird ZW [14]. However, it has been suggested that sex chromosomes in this gecko species evolved only recently and independently from the bird sex chromosomes [15]. These different examples illustrate how sex chromosomes have evolved several times in different clades and even in different species.

**Sex chromosomes evolved from a pair of autosomes**

It has been proposed that these different sex chromosome systems evolved from different pairs of autosomes from the common ancestor independently in each lineage [16]. It has been suggested that the differentiation process between the proto-sex chromosomes started when one of them acquired a sex-determining allele (Fig. 2). This was subsequently followed by the accumulation of male-advantageous alleles surrounding the novel sex-determining locus. Furthermore, a chromosomal rearrangement (for example an inversion), which might have occurred before or during this process prevented recombination between the proto-X and proto-Y [17]. This process was likely accelerated and exacerbated by the addition of one or more sexually antagonistic genes to this area [9, 18]. A sexually antagonistic gene has contrasting effects on the fitness of the two sexes [19], therefore, the gain of a gene that is advantageous for males but disadvantageous for females in the rearranged fragment could have favored selection against recombination [9]. Lack of recombination consequently led to the degradation of the proto-Y, as it allowed mutations, deletions and insertions to accumulate in the non-recombinant region. Therefore, once a mutation arises in this area there is no possibility of reverting it due to the inability to repair a non-recombinant region [17, 20]. This resulted in the differentiation of the ancestral autosomal pair into heteromorphic sex chromosomes where the X largely maintained its gene content while the Y underwent massive gene loss and heterochromatization.
Figure 2. Sex chromosomes evolved from a pair of autosomes in the common ancestor. The differentiation process between the proto sex chromosomes (X and Y represented in this figure) involved the acquisition of a sex determining allele as well as accumulation of a number of male advantageous alleles on one of the chromosomes. Additionally, suppression of recombination triggered the process of Y-chromosome degradation. Figure adapted from [21].
Presumably, an analogous process led to the evolution of the ZW system in birds and snakes. However, different pairs of autosomes became sex chromosomes independently in each lineage (Fig. 3), which explains the lack of homology amongst the sex chromosomes of different species.

There is extensive evidence that supports this theory of sex-chromosome evolution. Sex chromosomes at different stages of differentiation have been identified. In birds, for example, the Z chromosome is similar in size across most species while the W-chromosome varies significantly in size and heterochromatinization level [22-27]. Flightless ratites have a larger W chromosome that is similar in size to the Z-chromosome, representing an early stage of sex chromosome evolution. In contrast, among carnites the Z and W chromosomes are highly differentiated, with the W being small and heterochromatic indicating a more advanced stage in evolution [28, 29]. A similar scenario is also observed in snakes, where again the Z chromosome is similar in size across species and the W varies greatly representing different stages of W-chromosome degradation [16].

Another piece of evidence supporting this theory is the existence of gametologous genes. It is known that the X and Y gene content overlaps, thus it is possible to find a X-borne homologue for most of the genes located on the Y. The candidate spermatogenesis gene \(Rbmy\) located on the Y chromosome of both humans and mice evolved from the X-borne gene \(Rbmx\) [30-32]. Similarly \(Tspy\) represents a Y-borne gene and \(Tspx\) is its X-borne counterpart [33]. Another example is the sex-determining region Y gene (\(Sry\)), which is on the Y chromosome of therian mammals and triggers male sex determination in these organisms. \(Sry\) evolved from its X-borne parent gene \(Sox3\) [34]. These pairs of genes are called gametologous; they were homologous in the ancestral autosomal pair and evolved independently due to suppression of recombination [35].
Figure 3. Sex chromosomes evolved from an ancestral pair of autosomes. This figure represents the autosomes in the common ancestor and how the ZW from snakes, the ZW from birds and the XY from therian mammals evolved from different autosomes, whereas in birds and monotremes sex chromosomes evolved from the same ancestral autosome.
Gametologous genes exist both in XY and in ZW systems. However, different pairs of gametologous exist in different species. This is because each species might have lost a different set of genes from the Y-chromosome, therefore, the content of sex chromosomes that share ancestry might overlap but is not necessarily the same [21]. An example of this is the X-borne gene Atrx, which is widely conserved among therian mammals but only marsupials have a Y-borne Atrx gametolog, Atry [36]. However, it is important to add that the degraded sex chromosomes, i.e. Y and W, carry other types of genes too. Not all the genes on the Y chromosome are necessarily gametologous to X-borne gene. Y-chromosomes also carry amplicons of other genes present on the Y as well as elements that arrived to this chromosome by retrotransposition from autosomes [37]. The Y-borne gene Daz is an example of this. Daz is more closely related to an autosomal gene and studies indicate that it was retrotransposed onto the Y-chromosomes in the lineage of great apes [38, 39].

**The therian XY sex chromosome system**

Therian mammals (marsupials and placental mammals) have an XY/XX male heterogametic system. The X chromosome is highly conserved in gene content and order amongst placental mammals [40-42], except in the case of rodents in which the gene order seems to be scrambled [43]. When comparing the gene content of the placental X and the marsupial X it was discovered that the placental X is composed of ancient and added regions [40]. These were called X-conserved region (XCR) and X-added region (XAR), respectively (Fig. 4). The XCR is present both in marsupials and placentals and it comprises the long arm of the therian X chromosome as well as the region above the centromere [44]. The XAR includes the short arm of the placental X and the pseudoautosomal regions. The XAR was found to be autosomal in marsupials.
Figure 4. Human X and Y chromosomes. The human X chromosome consists of the XCR region shared by all therian mammals and the XAR, which is absent in marsupials. The X and Y chromosome retain homology in the PAR1 and PAR2, of 2.6 Mb and 320 kb respectively. In these regions the two chromosomes are able to pair and recombine. Sry is located on the male specific Y region (MSY) and its X-borne parent gene, Sox3, forms part of the XCR. The gray region on the Y chromosome represents heterochromatin. Figure adapted from [40].
and completely represented in the chicken chromosome 1 [45]. Therefore, the XAR was added to the placental X after their divergence from marsupials, around 100-180 million years ago [46].

Similar to the X chromosome, the Y chromosome also possess a layered structure [47] that reflects its evolutionary history. The Y chromosome bears five layers that go from a high degree of divergence with respect to their X-borne gametologs (if any) to a lower degree of divergence. The first layer, with the higher degree of divergence includes the gene *Sry*, while the fifth layer is formed by the pseudoautosomal regions [47]. The pseudoautosomal regions (PAR) are located on the tips of the X and Y chromosome. These are areas of conserved homology between the X and the Y and consequently are still able to pair and recombine during meiosis behaving as autosomal regions [48]. PARs are also considered evidence of the autosomal origin of sex chromosomes. However, in therians they are part of the XAR, which means that they are absent in marsupials [44, 49]. In humans and other great apes, PAR1 is located on the tip of the short arm of the X and it covers 2.6 MB [50] whereas PAR2 is on the tip of the long arm and is only 320 kb [51]. Interestingly, mice and rats have lost PAR1. Comparisons of the gene content of the PARs amongst different mammalian species [52] have led to the conclusion that in each species the homologous regions between the X and Y chromosomes have reduced to a different extent due to independent Y-specific mutations and rearrangement events in each lineage.

Given the process of degradation Y chromosomes have gone through, they are generally much smaller than their X counterpart, which is very gene rich. For example, the human X chromosome comprises 5% of the total genome and it contains around 1,000 genes [53] with functions biased toward reproduction [54]. On the other hand, the human Y chromosome represents only 2% of the genome and contains a few active genes [37].
The Y-chromosome is rich in pseudogenes and amplified copies, mainly due to the suppressed recombination between X and Y [37]. From the 54 genes found on the Y chromosome, 24 map to PAR1, 5 to PAR2 and 25 to the non-recombinant region [55], called male specific Y region, i.e. MSY. This highly degenerated nature of Y chromosomes in mammals has made sequencing this chromosome a technically challenging task excluding them from most sequencing projects [56].

The prototherian multiple sex chromosome system

Similar to therian mammals, monotremes have a male heterogametic XY sex chromosome system. However, this system is unique among mammals because it is composed of multiple sex chromosomes that have no homology to the therian XY but share extensive homology to the avian ZW system [6]. Echidna and platypus females have ten X chromosomes that form five homologous pairs, whereas males have 9 and 10 sex chromosomes in echidna and platypus respectively [4, 5, 57]. During male meiosis, the sex chromosomes form an alternating chain: \(X_1Y_1X_2Y_2X_3Y_3X_4Y_4X_5Y_5\) in platypus and \(X_1Y_1X_2Y_2X_3Y_3X_4_Y_4X_5\) in echidna (Fig. 5d) [57], which segregates into X-bearing and Y-bearing sperm [4]. Sex chromosomes in the platypus chain pair through the pseudoautosomal regions in an ordered fashion starting from the \(X_5Y_5\) to the \(X_1Y_1\) end [58]. PARs have been identified for all the chromosome pairing regions except for the platypus \(X_5Y_5\) [6], for the echidna \(X_3Y_3\) and \(Y_3X_4\) [57], possibly because they are small chromosomes and the PARs would be very small as well.

Interestingly, echidna and platypus sex chromosomes are not completely homologous to each other. Platypus chromosomes \(X_1Y_1X_2Y_2X_3\) are homologous to the echidna \(X_1Y_1X_2Y_2X_3\), respectively. However, the platypus \(X_5\) is homologous to echidna \(X_4\), the platypus \(Y_5\) is represented in the echidna \(Y_3\) and surprisingly the platypus \(X_4\) and \(Y_3\) are
represented in the echidna autosome 27 whereas the echidna Y₄ and X₅ are also represented by autosomes in platypus (Fig. 5a,b) [57]. In addition to the changes on sex chromosomes various autosomal rearrangements have occurred after the divergence of platypus and echidna [57].

Monotremes share homology to the bird sex chromosomes and not to the therian XY [6, 57]. The XAR maps to the platypus chromosome 15 and 18 [6] and the XCR is homologous to the platypus chromosome 6 [6, 59-61] (Fig. 5c) and echidna chromosome 16. These chromosomes are interesting because they contain the gene from which Sry evolved, Sox3 [60]. Interestingly, recent research (Casey and Daish personal communication) shows that chromosome 6 in the platypus stays in close proximity to the sex chromosomes during prophase I in male meiosis. Additionally, chromosome 6 is a remarkably heteromorphic autosome [57, 62] (Fig. 5c) and it has been reported that length heteromorphism leads to segregation distortion [63]. Moreover, it has been suggested that non-random segregation during meiosis could facilitate the transmission of sexually antagonistic alleles [64]. This warrants further investigation of this proto-sex chromosome.
Figure 5. Monotreme sex chromosomes. (A) Echidna male sex chromosomes. The numbers on the right of each chromosome indicate their homologs in the platypus. (B) Platypus male sex chromosomes. The numbers on the right of each chromosome indicate their homologs in echidna. (C) Platypus chromosome 6 is remarkably heteromorphic as shown by G-banding. (D) Echidna male meiotic chain. The red signal indicates the positions of the telomeres. Figure adapted from [57, 62].
Molecular aspects of mammalian sex determination

At the histological level, sex determination is highly conserved: testes are distinguished by the presence of testis cords and ovaries are characterized by the presence of follicles surrounding the developing eggs. The genes involved in the male and female sex determination pathways are also highly conserved among vertebrates independently of differences in the sex chromosome system or even in the type of sex determination, i.e. GSD or ESD. These genes, however, show some variation in sequence, expression patterns and timing. Particularly, the factor that triggers the sex determination cascade varies greatly between species and it can be environmental or genetic. This section describes some of the most relevant genes involved in the therian sex determination pathway.

Sex determination genes in therian mammals

It has been shown that Sry (sex-determining region Y) acts as the testis determining factor (TDF) in therian mammals [65]. This gene is the founder of the SOX family, which is characterized by a high mobility group (HMG) domain. The HMG box is responsible for DNA-binding, DNA-bending, protein interactions and nuclear import and export among SOX genes [66]. Sry was discovered in mice and humans [65, 67] over two decades ago. It was shown that replacement of the HMG box in the mouse Sry for the HMG boxes of the SOX genes Sox3 or Sox9 was still able to induce female-to-male sex reversal when introduced into XX mice [68]. These findings show that the HMG box from these different SOX genes are functionally interchangeable.
Sry is an intronless gene (except in marsupials) that encodes 204 amino acids from which only the 80 that correspond to the HMG box are highly conserved among therians, whereas the rest are highly divergent. As previously mentioned, Sry originated from the intronless gene Sox3 [69-71]. Mutations in the human Sox3 cause X-linked hypopituitarism, recessive hypoparathyroidism, mental retardation and significant gonadal defects, including small testes [72, 73]. It has been shown that Sox3 expression occurs predominately in type A undifferentiated spermatogonia and that loss of this gene is associated with failure of the germ cell maturation. Thus Sox3 is directly related to spermatogenesis [74]. Based on amino acid homology it has been proposed that a portion of the Di-George syndrome critical region 8 (DGCR8) corresponding to its first exon, was inserted upstream of the ancestral Sox3 on the proto-Y chromosome [75]. Therefore, Sry is a hybrid of a portion of DGCR8 and Sox3.

Sry spatial expression is specific to the bipotential gonad, where the process of sex determination begins once the cells of the bipotential gonad commit to either male or female development [76]. The bipotential gonad primordium originates from the mesonephros, a primitive organ that functions as the embryonic kidney and forms part of the developing urogenital system. In mouse, the urogenital system forms at 9-9.5 days post coitum (d.p.c.) and the bipotential gonad primordium at 10.5 d.p.c. Sry expression starts in male-fated gonads and finishes at 12.5 d.p.c. [77] due to a negative feedback loop by its target gene Sox9 [78, 79]. At this stage it is possible to distinguish between a female and a male gonad because the male gonad is twice the size of the female gonad and it has highly structured testis cords. These are composed of the male supporting cells (Sertoli cells) and the primordial germ cells (PGC), and are surrounded by the interstitium. By this time, the gonad in female is smaller and less organized than in males as no structural tissue is observed [80]. Once the fate as male or female is chosen, sex differentiation starts and the set of genes that will act in each specific sex
will repress the opposite sex pathway in order to reinforce the chosen one [76]. Hormones secreted from the supporting cells, such as the anti-Müllerian hormone (AMH) in males, actively repress the opposite pathway.

Supporting cell precursors have their origin in the coelomic epithelium (CE) from cells that express the steroidogenic factor 1 (Sf1) and migrate to the bipotential gonad. Sf1 expression starts in the CE during 9.5 d.p.c in both sexes [81]. It has been demonstrated that Sf1 expression is essential for mice gonadal development in the two sexes [82], however, humans heterozygous for Sf1 missense mutations show XY female sex reversal highlighting its relevance for male sex determination [83]. The CE cells that arrive to the bipotential gonad undergo an asymmetrical division in which one of the daughter cells will turn into an interstitial cell and the other will become a supporting cell precursor and will retain Sf1 expression [78, 84]. Once this decision has been taken the supporting cell has to undergo a second decision, which is whether to become Sertoli or granulosa cells. Sry expression at 10.5 d.p.c. leads to the supporting cell precursors to become Sertoli cells.

Sry acts synergistically with Sf1 to activate Sox9 through the testis-specific enhancer of Sox9 (TES) (Fig. 6) [85]. TES is a 3.2 kb regulatory motif located 13 kb upstream of the Sox9 initiation site. TES contains a 1.9 kb region called TES core (TESCO) which is sufficient for Sox9 gonadal specific expression. From these 1.9 kb, 1.4 kb are highly conserved in human, dog and rat [85] and 180 bp are highly conserved in marsupials, monotremes, birds, reptiles and amphibians [86]. This region is called the evolutionarily conserved region (ECR) and it contains modules that predict regulatory roles for SOX, TCF/LEF, Forkhead, DMRT and GATA proteins [86]. Therefore, it may have an important role in the regulation of Sox9 across most vertebrates. After Sry expression is repressed, Sf1 and Sox9 expression are highly maintained in Sertoli cells [81], which
means that Sox9 is regulated by other factors, such as Fgf9/Fgfr2 [76, 87] (Fig. 6). SOX9 itself is very efficient binding TESCO in presence of SF1, both in vivo and in vitro [85]. This suggests that SOX9 and SF1 might interact physically to regulate TESCO, establishing a positive feedback loop. This idea has led to the proposition that Sry evolved by mimicking Sox9 autoregulation [86]. Thus Sox9 expression consists of three phases: initiation, promoted by Sf1 expression in the bipotential gonad; upregulation, dependent on the synergistic action of SF1 and SRY; and maintenance through autoregulation and positive feedback loops, such as the FGF9/FGFR2. 1,000 genes have been identified to act downstream of Sox9, from which many are specifically expressed in Sertoli cells [88]. Those genes that are activated by SOX9 must be related to repression of bipotential gonad expressed-genes and female-pathway genes [89].

It has been shown that Sox9 loss of function in male mice triggers male to female sex reversal whereas gain of function in female mice triggers sex reversal in the opposite direction [90, 91]. This shows that Sox9 is necessary for testis differentiation and that it is the only gene downstream of Sry that is relevant for male sex determination [89]. Sry might activate other genes beside Sox9, e.g. cerebelin4 (Cbln4), but their biological function in testis differentiation is still unclear [92]. Another function preformed by Sry is the inhibition of the β-catenin pathway, perhaps directly or through a downstream target such as Sox9 [93]. Sry expression seems to be triggered by the genes Gata4 and Wt1 as it has been demonstrated that they can act synergistically and activate human, mouse and pig Sry promoters [94].
Figure 6. Sex determination in therian mammals. The Sox9 pathway, promoted by Sry and Sf1, determines the formation of the male gonad and actively represses the female pathway. Ovary development involves the activity of genes that actively repress the male pathway, such as $\beta$-catenin. $\beta$-catenin expression is stabilized by Wnt4 and Rspo1 activity, and it represses Sox9 expression. Foxl2 is expressed after birth and it also represses Sox9 activity. Figure adapted from [89].
In mice, Sox3 is also expressed in the somatic cells of the embryonic gonad (overlapping with Sry expression) as well as in the Sertoli cells of adult testes [34, 95]. Interestingly, it was found that overexpression of Sox3 in the bipotential gonad of XX mice induces female-to-male sex reversal [96]. It has been shown that this Sox3-mediated sex reversal requires Sf1 expression and occurs only in a normal Sox9 context, as is the case of Sry induced sex determination [96]. This implies that Sry and Sox3 act through a similar mechanism and therefore are functionally interchangeable, suggesting that the modifications that gave rise to Sry should have conferred it a specific early gonad expression.

The time at which Sry is activated is crucial for its activity as testis determining factor. It has been proven that induced Sry expression in XX mice after 11.5 d.p.c. fails to induce complete female to male sex reversal [97, 98]. In the female pathway, formation of the Müllerian duct starts between 11.5 and 12.5 d.p.c. through an invagination of the surface epithelium of the mesonephros. The Müllerian duct gives rise to the oviduct, uterus and the upper part of the vagina. In males, SOX9 and SF1 interact physically to activate the anti-Müllerian hormone gene (Amh) [99]. This hormone is secreted by fetal Sertoli cells and it represses the formation of the Müllerian duct. Therefore, if Sry does not trigger Sox9 expression on time the Müllerian duct formation commences and it results in female development [98, 100]. Interestingly, some exceptions exist in other animals. For example, in chickens and alligators Amh expression precedes Sox9; hence it is not possible that Sox9 activates Amh in these organisms [101, 102]. Moreover, it has been shown that a male-specific duplicated copy of Amh is involved in male development in the teleost fish Patagonian pejerrey (Odontesthes hatchery) [103], which suggests some interesting alternative roles for Amh among vertebrates.
Despite the fact that most of the information presented in this section comes from studies done in mice, the results can be extrapolated to other therian mammals with some variations. For example, \textit{Sry} in humans and pigs is expressed both during the postnatal stage as well as during embryogenesis as in mice [104, 105]. Human \textit{Sry} expression is not restricted to Sertoli cells but it is also detected in germ cells [105]. However, the function of these genes is still conserved. For instance, human \textit{Sry} and goat \textit{Sry} can promote male sex determination in mice under mouse regulatory sequences [106, 107]. Another example is the previously discussed TES enhancer of \textit{Sox9} because it contains the ECR region that is present in most vertebrates [86], suggesting a highly conserved role.

\textbf{Sex determination in monotremes}

Monotremes have a unique reproductive biology that represents an amalgam of mammalian and reptilian characteristics. Their female tracts are similar to those of reptiles and marsupials; in the platypus the right ovary is inactive and the left is functional similar to birds, whereas in echidna both ovaries are functional [108]. Males have seminiferous tubules that resemble the structure of other amniote species, in which at least four generations of germ cells are supported by a group of Sertoli cells. Therefore, several stages of spermatogenesis can be visualized in a cross section of the monotreme seminiferous tubules [108, 109]. Given their position as basal mammals and their amazing mixture of features, monotremes are very important for comparative genomic analysis to understand the evolution of sex determination and differentiation in mammals.

There is nothing known about genes underlying sexual development in monotremes. These prototherian mammals have an XY sex chromosome system in which males are
heterogametic. However, their multiple sex chromosomes are homologous to the bird sex chromosomes. Several efforts have been made in order to identify orthologs to the therian mammal and bird sex determination triggers in the platypus, such as Sox9, Gata4, Wt1, Sf1, Atrx, Fgf9 and Wnt4; but their autosomal or pseudo-autosomal locations [60, 110-112] excluded them from functioning as primary testis determining factors. Therefore, the molecular bases underlying the initiation of sex determination in monotremes are still unknown.

\(Dmrt1\), the best candidate to be the bird sex determination trigger, has been mapped to the platypus chromosome X\(_3\) and to the echidna X\(_4\) [4, 5, 57]. The location of this gene in a X chromosome in monotremes might be the relic of a \(Dmrt1\)-controlled system [113]. In birds, \(Dmrt1\) is expressed in Sertoli and male germ cells [114] whereas in mouse it is expressed in the supporting cells of both sexes [115, 116]. \(Dmrt1\) expression in platypus was first detected only in adult testis through RT-PCR. Immunohistochemistry experiments showed that the DMRT1 protein is expressed in the Sertoli cells as well as in granulosa cells, however lower in the later; hence, \(Dmrt1\) expression is higher in males than in females but it is not sex-specific [111, 113]. These findings imply that the function of \(Dmrt1\) is highly conserved in the gonads of both sexes in mammals, however, the presence of two \(Dmrt1\) copies in male chicken versus one copy in male platypus do not agree with a role as sex determining switch in monotremes. However, we cannot exclude the possibility that a \(Dmrt1\) Y-gametolog might have diverged to an extent at which is no longer recognizable.

Several attempts have been made in order to identify Y-borne sex determining candidate genes in monotremes. However, orthologs of genes involved in the therian or bird sex determination pathways such as Sox9, Gata4, Wt1, Sf1, Atrx, Fgf9 and Wnt4 have been mapped to autosomal or pseudo-autosomal regions [60, 110-112] excluding
them from functioning as the primary testis determining factors. Sox9, which seems to act universally in the vertebrate male sex determination pathway has the TES enhancer that is highly conserved among marsupials, monotremes, birds, reptiles and amphibians and it contains modules that predict regulatory roles for DMRT and SOX proteins, among others [86]. The fact that the therian X-chromosome is represented by an autosome in these animals led to the conclusion that Sry is absent in monotremes. This was later confirmed by mapping Sox3 to chromosome 6 in the platypus [60]. However, a different SOX protein might be still responsible of Sox9 activation. Efforts to identify this putative monotreme sex determining candidate gene have been fruitless due to the lack of Y-specific sequence, since the sequenced platypus genome comes from a female [2]. Around 700 kb of platypus Y-specific sequence have been obtained [117]. Y-chromosomes are the obvious male-specific niches in which we could find male sex determination factors. Therefore, in order to facilitate the search for the monotreme sex-determining gene it is necessary to extract and analyze more Y-specific sequence in these animals. Moreover, investigating candidate genes belonging to the SOX family could shed light on potential sex-determining triggers.

**Sex ratios in captive animal populations**

Sex ratio bias in populations of captive-bred animals has been described in several mammalian species [118-120]. Even though the molecular mechanisms underlying this phenomenon are still unknown, different theories have been proposed in order to explain why this might happen [121-125]. In general, they all predict that skewing the sex ratio should lead to an increase in the fitness of the mother [126]. There are a number of examples where the factors influencing the sex ratio of captive animals are known. For instance, it has been shown that group size affects the offspring sex in lemurs; mothers housed in groups tend to produce more males whereas in isolation they
produce more females [118]. Another example is given by the captive pygmy hippopotamus; these animals also show a female bias that has been related to high levels of manipulation and feeding [119]. Accurately determining the sex ratio is a vital part of animal biology in captivity as well as in the wild and it plays an important role in conservation [127]. Early identification of sex ratio bias in captive-bred populations is important for zoos in order to make decisions about housing animals as well as to design their population management plans [128].

So far no data have been available about sex ratio bias of captive bred monotremes. In echidnas identification of the animals sex is challenging due to the lack of obvious sexual dimorphisms [129]. Therefore, developing our understanding of the genetics of sex determination in monotremes could help us to develop genetic sexing techniques to accurately and non-invasively identify the sex of captively bred echidnas and to investigate whether they are affected by sex ratio distortions.
Aims of the Project

Monotremes occupy a key position in the evolution of mammals. In contrast to birds and therian mammals, there is no information about the gene or group of genes that trigger sex determination in these organisms. The aim of this study was to gain more information about Y chromosomes and to identify potential sex-determining genes. The first approach followed was to look at candidate regions, such as the Y chromosomes and the autosome that shares homology to the therian X, chromosome 6 in the platypus. By studying this autosome the aim was also to characterize the therian proto-sex chromosome to obtain insights into the evolution of the XY system in mammals. The next aim was to study candidate genes, particularly, Sox3 given its relationship to the therian male sex-determining factor Sry. Finally, this study aimed to apply our knowledge in monotreme sex chromosomes to aid zoos in genetically determining the sex of captive bred echidnas and to investigate any potential sex ratio bias.
Summary

The thesis is divided in six chapters; the first chapter provides background information on sex chromosomes and sex determination. In chapter 2, I present the results of the first approach that was followed which consisted of identifying Y-chromosome genes. Chapter 3 talks about the outcomes of investigating platypus chromosome 6, which was chosen for analysis as this chromosome shares extensive homology to the eutherian mammal X. Chapter 4 focuses in the study of the candidate gene Sox3 – the Sry parent gene in therian mammals – while Chapter 5 provides a practical application of these sex determination studies onto ecology and conservation of monotremes. Finally, Chapter 6 summarises and discusses the major outcomes of work presented.

In chapter 2 I present the general approach, which was to gain more insight into gene content of monotreme Y-chromosomes. Y-chromosomes are the most obvious place to look for male-specific sex-determining genes given that they are male specific, however, the platypus genome was obtained from a female and, as it is the case with many other sequenced mammals, this meant that we were lacking information about Y-chromosome genes. In order to obtain more information about genes on the platypus Y-chromosomes I joined a collaboration with a research group in Lausanne, Switzerland; our collaborators predicted a number of male-specific genes based on male and female transcriptome data. I then experimentally validated this approach through PCR and fluorescent in situ hybridization (FISH) experiments. I found that Amhy, predicted to be in one of the platypus Y chromosomes, maps to the Y5. Since Y5 is proposed to be the oldest Y-chromosome in the platypus and a Y-copy of Amh acts as the primary sex determination trigger in the Patagonian pejerrey fish this renders Amhy as a likely sex determination candidate in the platypus.
Chapter 3 describes my results regarding the second candidate region I investigated, the platypus chromosome 6. This chromosome was of interest because it is homologous to the therian X-chromosome and it associates with the monotreme sex chromosomes complex at meiosis. I started by investigating the NOR heteromorphism of chromosome 6, showing that it is heteromorphic in males and females but it is more prominent in females. I found a number of other sex-specific differences. For example, chromosome 6 is hypermethylated in females but not in males and the male NOR has a different silver-staining pattern compared to the pattern observed in females. I next investigated chromosome 6 segregated in male meiosis, finding that this autosome is non-randomly segregated in X-bearing sperm; the short version of chromosome 6 segregates preferentially with the X chromosomes. Further experiments suggested that this bias is related to negative selection of X-bearing sperm carrying the long version of chromosome 6. This is of interest since it has been proposed that non-random segregation of autosomes could facilitate the transmission of sexually antagonistic alleles and even participate in the evolution of new complex sex determination systems, which raises the possibility that chromosome 6 may have commenced differentiation prior to monotreme therian divergence.

In chapter 4 I focus on the Sox3 gene. This gene is located on the platypus chromosome 6 and, in therian mammals, Sry evolved from Sox3. Interestingly, I found that there is a male specific variation of Sox3. This gene has four polyalanine tracts in humans, which have been related to transcriptional activity. Expansions of as little as one residue in polyalanine tracts have been associated with disease in humans. Platypus has only two poly-alanine tracts and I found that the second poly-alanine tract (which corresponds to the fourth polyalanine tract in humans) has a reduction of two residues exclusively in males. I speculate that this variation may lead to a male-specific Sox3 function. In order
to answer this, I progressed to modify the mouse Sox3 to resemble platypus Sox3 and to test its potential to trigger male sex-determination in vitro.

Chapter 5 describes work towards using our information of monotreme sex chromosomes to assist the conservation and breeding of monotremes, particularly echidnas. Early identification of the sex of these animals is important to zoos in order to make captive management plans. However, due to the lack of obvious sexual dimorphisms the only way to determine the sex of these animals is by palpation, which is quite invasive for the echidna puggles. I successfully established a genetic sexing technique, which allowed us to identify a striking bias in the sex ratio of these captively bred animals. Also, through this technique I identified a deletion of Sox3 in the only sexed male. This gives us the unique opportunity to investigate the sexual development of an animal in which this gene is naturally deleted helping us to overcome the limitations of genetically manipulating monotremes.
References


CHAPTER 2: Prediction of Y-linked genes in the platypus reveals *Amhy* as the most likely sex-determining candidate gene

This chapter consists of one published paper.

Chapter overview

This paper describes work where a bioinformatics and molecular cytogenetic approach was combined to identify and confirm novel Y-chromosome genes. This successfully identified a large number of novel Y-borne genes across different mammalian species. In the platypus, 25 Y-linked protein-coding genes were identified and I confirmed their male-specific nature after establishing a novel FISH technique specifically to map short PCR products. The establishment of this technique proved to be challenging and it required several rounds of optimization. However, it provided a crucial result in this study that was the physical mapping of *Amhy* onto platypus chromosome Y₅. AMH is known to repress the female developmental pathway in vertebrates and in some organisms it acts as the primary sex-determining gene, rendering *Amhy* as the most likely sex-determining gene in the platypus so far.
## Statement of Authorship

<table>
<thead>
<tr>
<th>Title of Paper</th>
<th>Origins and functional evolution of Y chromosomes across mammals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Publication Status</td>
<td>Published, O O O O</td>
</tr>
</tbody>
</table>

### Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate’s thesis.

<table>
<thead>
<tr>
<th>Name of Co-author (Candidate)</th>
<th>Deborah Toledo-Flores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contribution to the Paper</td>
<td>Established a novel technique to physically map PCR products predicted by the computational approach. A key result and contribution was the mapping of Amhy on a platypus Y-chromosome (Extended data Fig7e-f).</td>
</tr>
<tr>
<td>Signature</td>
<td></td>
</tr>
<tr>
<td>Date</td>
<td>19/12/14</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of Principal Author</th>
<th>Diego Cortez</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contribution to the Paper</td>
<td>Performed most data processing and biological analyses.</td>
</tr>
<tr>
<td>Signature</td>
<td></td>
</tr>
<tr>
<td>Date</td>
<td>15 December 2014</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of Co-Author</th>
<th>Ray Marin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contribution to the Paper</td>
<td>Processing and data analysis of platypus genomic data.</td>
</tr>
<tr>
<td>Signature</td>
<td></td>
</tr>
<tr>
<td>Date</td>
<td>18 December 2014</td>
</tr>
<tr>
<td>Name of Co-Author</td>
<td>Laure Frodevaux</td>
</tr>
<tr>
<td>-------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Contribution to the Paper</td>
<td>Prepared samples and generated RNA-seq and genomic sequencing libraries. Also, preformed the large-scale PCR/Sanger sequencing validation experiments. Unfortunately none of the authors have been able to contact Laure. We do not know her current address or contact details.</td>
</tr>
<tr>
<td>Signature</td>
<td>Date</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of Co-author (Candidate)</th>
<th>Angélica Liechti</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contribution to the Paper</td>
<td>Prepared samples and generated RNA-seq and genomic sequencing libraries.</td>
</tr>
<tr>
<td>Signature</td>
<td>Date</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of Principal Author</th>
<th>Paul D. Waters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contribution to the Paper</td>
<td>Provided elephant and tammar wallaby fibroblast samples and advised on these species' sex chromosome biology.</td>
</tr>
<tr>
<td>Signature</td>
<td>Date 19/12/2014</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of Co-Author</th>
<th>Frank Grützner</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contribution to the Paper</td>
<td>Provided platypus and echidna samples, supervised FISH experiments, and provided advice on the sex chromosome biology of these species.</td>
</tr>
<tr>
<td>Signature</td>
<td>Date 19/12/2014</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of Co-Author</th>
<th>Henrik Kaessmann</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contribution to the Paper</td>
<td>Originally designed and supervised this project. H.K. and D.C. wrote the paper with input from all the authors.</td>
</tr>
<tr>
<td>Signature</td>
<td>Date 18.12.2014</td>
</tr>
</tbody>
</table>
Publication:


NOTE:
This publication is included between pages 50-51 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

[http://dx.doi.org/10.1038/nature13151](http://dx.doi.org/10.1038/nature13151)
CHAPTER 3: Non-random meiotic segregation of the therian proto sex-chromosome in platypus may provide insights into differentiation of sex chromosomes in mammals

This chapter consists of one manuscript in preparation.

Chapter overview

Platypus chromosome 6 shares extensive homology to the therian X chromosome and hence represents the therian proto-sex chromosome. During meiosis the NOR, chromosome 6 and the sex chromosome chain are closely associated. Chapter 3 and 4 describes work to investigate if chromosome 6 is segregated non randomly or shows signs of sex chromosome differentiation.

Utilizing a number of molecular and cytogenetic techniques, I have identified sex-specific characteristics between chromosome 6 homologs. Moreover, I found that chromosome 6 is non-randomly segregated during male meiosis. To our knowledge, this is the first autosome shown to be non-randomly segregated during male meiosis in a mammal. This may suggests that non-random segregation of chromosome 6 may have facilitated its evolution as the therian sex chromosome.
# Statement of Authorship

<table>
<thead>
<tr>
<th>Title of Paper</th>
<th>Non-random segregation of the therian proto-sex chromosome at male meiosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Publication Status</td>
<td>Published, Accepted for Publication, Submitted for Publication, Publication style</td>
</tr>
<tr>
<td>Publication Details</td>
<td>Toledo-Flores, D., Kortschak, R.D., Grützner, F., Non-random segregation of the therian proto-sex chromosome at male meiosis</td>
</tr>
</tbody>
</table>

## Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

<table>
<thead>
<tr>
<th>Name of Principal Author (Candidate)</th>
<th>Deborah Toledo-Flores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contribution to the Paper</td>
<td>Performed experiments, processed all samples, interpreted and analysed data and wrote manuscript.</td>
</tr>
<tr>
<td>Signature</td>
<td></td>
</tr>
<tr>
<td>Date</td>
<td>15/12/2014</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of Co-Author</th>
<th>R.D. Kortschak</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contribution to the Paper</td>
<td>Helped in data interpretation and statistical analysis.</td>
</tr>
<tr>
<td>Signature</td>
<td></td>
</tr>
<tr>
<td>Date</td>
<td>15/12/2014</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of Co-Author</th>
<th>F. Grützner</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contribution to the Paper</td>
<td>Supervised development of work, helped in data interpretation and manuscript preparation and evaluation.</td>
</tr>
<tr>
<td>Signature</td>
<td></td>
</tr>
<tr>
<td>Date</td>
<td>15/12/2014</td>
</tr>
</tbody>
</table>
Manuscript in preparation:

Deborah Toledo-Flores, R. Daniel Kortchak, Frank Grützner. Non-random segregation of the therian proto-sex chromosome at platypus male meiosis.
Non-random segregation of the therian proto-sex chromosome at platypus male meiosis

Deborah Toledo-Flores¹, R. Daniel Kortschak¹, Frank Grützner¹

¹The Robinson Research Institute, School of Molecular and Biomedical Science, The University of Adelaide, Adelaide, Australia

Abstract

Random segregation of chromosomes during meiosis is a widely accepted key aspect of sexual reproduction. However there is increasing evidence that chromosomal heteromorphisms can lead to non-random segregation at meiosis [1]. The platypus features a large NOR heteromorphism on its singular NOR on chromosome 6. This autosome shares extensive homology to the X chromosome of other mammals. Here we investigated whether this NOR heteromorphism is associated with non-random meiotic segregation of this proto-sex chromosome. First we determined that the heteromorphism is found in both sexes although it is more prominent in females than in males. Our results suggest that this is in part due to hypermethylation of the NOR region in females but not in males. Importantly we found first evidence of non-random segregation of this autosome in platypus male meiosis. Our data suggests that negative selection might act against X-bearing sperm carrying the chromosome 6 with the large NOR. This work provides a possible mechanism that may have influenced the evolution of chromosome 6 as a sex chromosome in therian mammals.
Introduction

The platypus is one of three extant monotremes. Its karyotype is arguably the most unusual amongst mammals; with 21 pairs of autosomes [2] and an unusually large sex chromosome complement that consists of $X_1X_2X_3X_4Y_1Y_2Y_3Y_4Y_5$ in males and $X_1X_2X_3X_4X_5X_1X_2X_3X_4X_5$ in females [3]. This sex chromosome complex shares extensive homology with the birds Z chromosome [3-6] but not with the therian X [6, 7]. Instead, the therian X is largely homologous to platypus chromosome 6 [8-10]. Chromosome 6 is a very distinctive autosome carrying a large heteromorphic nucleolus organizer region (NOR) located on its short arm [5, 10-12].

The NOR is the chromosomal locus around which nucleoli are formed [13, 14]. It consists of clusters of tandemly repeated rRNA genes typically organized in a head-to-tail orientation [15-20]. Each rRNA gene codes for the 45S pre-rRNA precursor that is subsequently cleaved forming the mature 18S, 5.8S and 25-28S (the exact size is species-dependent), which will later assemble with the 5S into ribosome subunits. rRNA gene expression is tightly regulated to ensure that the cell maintains a sufficient amount of ribosome subunits to support protein synthesis levels during cell growth and division [21-24].

In mammals, rRNA gene expression is controlled mainly through DNA methylation, epigenetic modifications, number of rRNA genes and gene silencing due to positional effects [25-30]; rRNA genes that have been hypermethylated and that are associated with unacetylated histones are often inactive, whereas those that show DNA hypomethylation coupled with histone acetylation are often active [28, 31]. Active NORs form secondary constrictions in metaphase chromosomes. This is due to the accumulation of RNA Pol I transcription factors on rRNA genes that were actively
transcribed during the previous interphase, which keeps them relatively decondensed [13, 14, 32, 33]. These transcription factors contain acidic/argyrophilic domains, hence active NORs can be easily identified through silver staining [14, 34-39]. Differences in NOR expression lead to both polymorphisms in the number of silver-stained NORs (active NORs) [30, 40-46] as well as to NOR size heteromorphisms [47-51] between individuals of the same species. Another factor influencing NOR size between homologous chromosomes is unequal crossing over, since it can induce loss or tandem duplications of rDNA regions [30, 52-54].

Interestingly, structural differences between homologous chromosomes, such as size heteromorphism, have been shown to bias meiotic segregation [1]. In the nematode Caenorhabditis elegans, for example, indels ranging from 1 kb to 7.3 Mb introduced in any of its five autosomes produced a significant segregation bias; the shorter chromosome in a homologous pair segregated preferentially with the X chromosome whereas the longer segregated away from the X. They also showed a positive correlation between indel size and transmission bias ratio [1].

In monotremes, both platypus and echidna, there are a number of autosomes that present variation in size between homologous and between individuals [11, 12]. However, the platypus chromosome 6 bears the most remarkable heteromorphism given its large NOR. In mammals NORs are commonly found in autosomes [55] with the exception of marsupials [56] and some bats where NORs are on the sex chromosomes [55]. Interestingly, in insects NORs are commonly found on sex chromosomes [57-61].

In this study we investigated whether this heteromorphism is sex-specific and whether it is a result of differential DNA methylation or transcriptional activity. Importantly we
used this heteromorphism to trace chromosome 6 segregation during male meiosis and found evidence of non-random segregation of chromosome 6 in sperm.

**Results/Discussion**

*Chromosome 6 heteromorphism is observed in both sexes in platypus but it is more prominent in females*

Platypus chromosome 6 has been reported as a remarkably heteromorphic autosome [11] however this observation has not previously been quantified or investigated systematically in males or females. We first investigated whether this heteromorphism was consistently found in both sexes. For accurate quantification we performed a two-color FISH (fluorescence *in situ* hybridization) experiment utilizing chromosome 6-specific BACs (bacterial artificial chromosomes) that flank the heteromorphic NOR (Figure 1) to determine the length of this region on each homologous chromosome. The measurements were normalized to different condensation levels by dividing each NOR length by the average length of chromosome 1 in the corresponding metaphase.

This analysis was performed in fibroblast-derived metaphase spreads of three male and female individuals each. 45-109 metaphases were scored per individual (Table 1), the average length of each homologue was obtained and then a paired t-test was performed to determine whether the population of long chromosome 6 had the same NOR mean length as the population of short chromosome 6 in each individual. We found that the lengths of the short and long chromosome 6 are significantly different in the 6 analyzed individuals (Table 1). This shows that chromosome 6 is heteromorphic in both sexes.
We also analyzed the chromosome 6 length distribution. For this, we plotted the kernel density distribution for the pooled short and long chromosome 6 of each individual. We found that there is variation in the shape of the density distribution of different individuals (Figure 2). Interestingly, in two out of three females we observed a bimodal distribution (Figure 2A) whereas in males a shoulder-type distribution was observed in two out of three cases (Figure 2B) but no bimodality was observed. The density graphs thus suggest that the heteromorphism is more prominent in females than males. Therefore, even though the difference in length between the short and long chromosome 6 is consistently significant in all the analyzed individuals (Table 1), the variation between individuals and sexes results in different shapes of density distributions (Figure 2). This result raises the question of whether the variation can be attributed to a difference in rDNA copy number or explained by a difference in condensation and activity.

**DNA hypermethylation contributes to the observed heteromorphism in females, but not in males**

In mammalian cells, extensive DNA methylation is a hallmark of silent rRNA gene repeats [24, 26, 27] and it has been shown that inhibition of DNA methylation activates rDNA transcription from previously silent cistrons [26]. Based on this, we decided to investigate whether inhibition of DNA methylation had an impact on the observed chromosome 6 heteromorphism. We treated male and female platypus fibroblasts with 5-aza-2’-deoxycytidine (5-aza-dC), a drug that inhibits cytosine methylation when incorporated to DNA [26, 62]. Metaphase spreads were prepared from 5-aza-dC treated cells and also from control untreated cells from the same individuals. The length of the
whole chromosome 6 was measured, as it was not possible to retrieve sharp and reliable FISH signals in the metaphase spreads prepared from 5-aza-dC treated cells.

Consistent with our previous results, both male and female untreated cells showed a statistically significant chromosome 6 heteromorphism (Figure 3). Interestingly, the treated cells also showed a statistically significant heteromorphism in both sexes. We then investigated whether there was any difference between the untreated short or long chromosome 6 and their treated counterparts. Indeed the female long chromosome 6 remained unchanged whereas the short chromosome 6 was significantly elongated after 5-aza-dC treatment (Figure 3). In contrast, the 5-aza-dC treatment had no significant effect in either chromosome 6 homologue in males (Figure 3). Therefore, only the shorter female NOR is hypomethylated presumably resulting in reduction of its activity, which may consequently increase condensation and contribute to the heteromorphism observed in this sex.

It has been reported before that amongst homologous or non-homologous NORs, those with the fewest ribosomal cistrons are preferentially inactivated [47, 50, 63, 64]. An example of this is given by the preferential inactivation of the shorter NOR in inter-specific hybrids [63]. Something similar may be occurring in the female NOR, where a difference in the number of ribosomal cistrons on each homologous NOR might cause the initial heteromorphism [52], and this could be exacerbated by preferential inactivation of cistrons in the shorter NOR.

*Silver staining shows different NOR activity between sexes*

In order to investigate whether there are any sex-specific differences between the activities of homologous NORs we performed silver staining of the proteins associated
with NOR activity on male and female platypus metaphase spreads. We found that in females, both NORs are entirely covered by silver staining (Figure 4A). In males, we observed that one of the homologous NORs bears a gap of unstained chromatin surrounded by silver deposits over the rest of the NOR (Figure 4B). This is inconsistent with the observation that demethylation affected the short female NOR and not the male NOR. Therefore, the female short NOR must represent a mosaic of active and inactive ribosomal cistrons [31, 63].

To investigate whether the gap in the male NOR is the result of localized rDNA silencing or if the gap represents an area containing non-rDNA we performed a FISH experiment using a 28S specific PCR probe (Figure S1A) on the same male. We observed binding of the 28S probe to the entire NOR region with no evident gaps (Figure S2 B,C). However, when we measured the intensity of the signal of the silver staining and of the FISH we observed correspondence between the intensity plots for both experiments (Figure S1 B,C). This is consistent with the observations of other researchers that found that the size of the rDNA FISH signal is associated with the size of the silver staining signal [65, 66]. Our FISH results suggest that the silver staining gap observed in the male NOR might contain a lower number of rDNA genes as indicated by the drop in FISH signal intensity. Alternatively, a mechanism other than DNA methylation might be involved in the inactivation of the rDNA contained in this region, as shown by silver staining. In horse, for example, it has been observed that NOR hypomethylation can occur together with lack of silver staining deposits [30], showing that DNA methylation does not always correlate with NOR activity.
Chromosome 6 heteromorphism is present in uncultured meiotic cells in platypus

In order to investigate segregation bias of the heteromorphism at meiosis we first had to determine whether the different chromosome 6 homologs could be identified in sperm nuclei. We measured the distance between NOR flanking BAC clone hybridization signals in platypus sperm cells. The density distribution showed a clear bimodality (Figure 5A). In addition, we analyzed this set with Bayesian Information Criterion (BIC), which estimates the number of clusters that best represent the data. This analysis indicated that the chromosome 6 measurements obtained from sperm are best represented by two clusters (Figure 5B). This shows that the heteromorphism is not a cell culture artifact and can be observed in uncultured cells. Importantly this allows us to investigate segregation bias of chromosome 6 at male meiosis.

The short chromosome 6 segregates preferentially with the X chromosomes at platypus male meiosis.

Heteromorphisms have been shown to bias meiotic segregation of autosomes with sex chromosomes. In monotremes the large NOR associates closely with the sex chromosomes complex (Casey and Daish, personal communication) raising the possibility of non-random segregation. To identify X and Y bearing sperm we performed a three-color FISH experiment; we used two chromosome 6 specific BACs (Figure 6) and a third X- or Y-specific BAC. We then counted the number of X- or Y-bearing sperm carrying the long and short versions of chromosome 6. We found that the long and short chromosome 6 were randomly segregated in Y-bearing sperm (Table 2, Figure 7). Interestingly, we observed a significant bias in X-bearing sperm, which in most cases carried the short version of chromosome 6. In the three individuals we observed between 66 and 78% of the X-bearing sperm carrying the short version of
chromosome 6 and only between 21 and 33% carrying the longer version (Table 2, Figure 7).

As previously discussed, a similar scenario was described before in *C. elegans*, where length variations of autosomes affected their segregation. Consistent with our results in the platypus, this study showed that the shorter version in a homologous pair segregated preferentially with the X chromosome whereas the longer version segregated against it [1]. Importantly, this study also showed that indels of as little as 1 kb were able to induce the distortion, therefore, it is possible that even a subtle difference in the number of ribosomal cistrons may result in a segregation bias in the platypus.

*Depletion of X-bearing sperm with the long chromosome 6*

The finding that there is a greater frequency of Y-bearing sperm compared to X-bearing sperm (Table 3, Figure 8) might suggest that this is a case of gamete competition, where a significant proportion of those male gametes carrying the X and the long version of chromosome 6 must be somehow eliminated. If this were the case we would expect to see a bias in X/Y sperm frequency. In order to investigate this, we counted the frequency of X/Y sperm on the same three individuals in which we observed the segregation bias. For this we performed sequential one-color FISH experiments. We firstly performed FISH utilizing a red-labeled Y<sub>5</sub> BAC (Figure 8A); we counted the number of observed Y<sub>5</sub> positive and negative sperm and recorded the location of the scored cells. In the second FISH we used a X<sub>1</sub> BAC also labeled in red (Figure 8B) and we counted the number of X<sub>1</sub> positive cells. As expected, most of the cells that were negative for Y<sub>5</sub> in the first FISH were positive for X<sub>1</sub> in the second FISH. In the first experiment we observed an overrepresentation of Y-bearing sperm (Figure 8C). Similarly, we observed the same bias while comparing the percentage of X-bearing
sperm from the second experiment to the percentage of Y-bearing sperm from the first FISH (Figure 8D). In the three studied males around 60% of the scored sperm were Y positive, 30% were X positive and 10% of the sperm were uninformative with no visible signal (Table 3). This supports the idea that there is a bias towards Y-bearing sperm and this is consistent with our hypothesis that a proportion of X-bearing sperm may be reduced by the removal of cells that bear the X and the long chromosome 6. We evaluated our results under three different assumptions; (1) the total of X-sperm is represented by the sum of X-positive and uninformative sperm, (2) Y sperm is represented by the sum of Y-positive sperm and uninformative sperm and (3) we assume that uninformative sperm represent cases in which either the X1 or Y5 were lost in the scored sperm (Figure S2). In all these possible scenarios it is evident that the X/Y bias exists in platypus sperm.

To explain the length distributions observed in males and females (Fig. 2) it is necessary to consider the mode of inheritance of chromosome 6 from females (Fig. 9). It is likely that females transmit both types of chromosome 6 (Fig. 9C). However, given that no males were identified with a bimodal distribution of the chromosome 6 types, there should be a mechanism eliminating or preventing presence of the two variants in males. This might be through pre-zygotic mechanisms such as incompatibility between oocytes carrying the short chromosome 6 and Y-bearing sperm carrying the long chromosome 6 as well as between oocytes carrying the long chromosome 6 and Y-bearing sperm carrying the short chromosome 6. Alternatively, XY zygotes carrying the long and short chromosome 6 might be eliminated post-fertilization or these males may have increased mortality (post-zygotic).

If this were the case, these mechanisms could also help to balance the ratio of males and females. Given the fact that there is a bias towards Y-bearing sperm in the platypus (Fig. 8), if no compensatory mechanisms exist there would be more males than females.
in the population, which could put at risk this species and eventually lead to extinction [67]. However, if there were increased mortality (or gamete incompatibility) of XY males carrying the short and long chromosome 6, this could help to balance the ratio of males and females. Future work may reveal more information about males and females ratios in the wild to obtain and provide insights on whether the observed segregation distortion and X/Y sperm bias has an impact on the platypus population.

**Conclusion**

Our findings show that the platypus chromosome 6, which is the proto-sex chromosome in therian mammals, presents a number of sex-specific characteristics; its NOR is heteromorphic predominantly in females, demethylation has an effect in females but not males and there is a differential silver staining pattern in both sexes that suggests sex specific NOR activity patterns. Importantly chromosome 6 is non-randomly segregated in X-bearing sperm. Such sex-biased segregation of autosomes could facilitate the transmission of sexually antagonistic alleles and even participate in the evolution of new complex sex determination systems [68]. At this stage, we do not know whether there is any relationship between our observations and the possible evolution of chromosome 6 as a sex chromosome. However, to the best of our knowledge, this is the first time non-random segregation of an autosome that has evolved as a sex chromosome has been reported.
Materials and methods

BAC clones

BAC clones were obtained from the CUGI BAC/EST resource centre (Clemson, SC, USA) from the library Oa_Ab or from the Children’s Hospital Oakland Research Institute (CHORI, CA, USA) from the library CH236.

Preparation of chromosomes and sperm cells

Mitotic metaphase spreads were produced from established platypus fibroblast cell-lines [3, 69-71]. Established sperm and meiotic cell suspensions were used [69]. For new preparations, cells were obtained from extracted testes; the cells were then directly fixed in methanol/acetic acid (3:1). Slides were prepared according to standard procedures.

Demethylation assay

Established cell lines were cultured in 50% Dulbecco’s Modified Eagle Medium High Glucose 1x (Life technologies) supplemented with glutamine, heat-inactivated FCS and Penicillin/Streptomycin; and 50% AmnioMAX-C100 (1X) (Life Technologies) at 32°C. Demethylation was performed for 72 hrs at a concentration of 15 µM 5-aza-dC. For chromosome preparation information refer to Preparation of chromosomes and sperm cells section.

Fluorescence in situ hybridization

BAC DNA (1 µg) was labeled with spectrum orange or green (Abbot Molecular) using random primers and Klenow polymerase and hybridized by fluorescence in situ hybridization (FISH) on female and male metaphase cells according to standard procedures previously described in [69]. For FISH on sperm cells, one step was introduced into the standard procedure; after pepsin treatment (10 min incubation in
0.01 % pepsin in 10 mM HCl at 37°C), sperm slides were incubated in 8 mM DTT/PBS at 37°C for 8 min. Followed by the standard 10 min re-fixation in 1 x PBS, 50 mM MgCl₂, 1 % formaldehyde. Slides were then dehydrated in ethanol series followed by a 5 min denaturation at 75°C in 70% formamide, 2x SSC and again dehydrated. Slides were hybridized overnight in a moist chamber at 37°C with a mix of DNA probe, 10-20 µg of sonicated genomic DNA and 50 µg of salmon sperm redissolved in 50% formamide, 10% dextran sulfate, 2x SSC. Prior to hybridization the probe mix was denatured for 10 min at 80°C and preannealing of repetitive DNA sequences was performed for 30 min at 37°C. Post-hybridization, the slides were washed three times for 5 min in 50% formamide, 2x SSC at room temperature followed by one wash for 5 min in 2x SSX at 42°C, once for 5 min in 0.1x SSC at 60°C and finally once for 5 min in 2x SSC at 42°C. Cells were counterstained with 1 µg/µl DAPI in 2x SSC for 1 min and then mounted with one drop of Vectashield. Images were taken using a Zeiss AxioImagerZ.1 epifluorescence microscope and the Zeiss Axiovision software.

**Chromosome measurement and data analysis**

In metaphase and sperm cells, FISH was performed with two chromosome 6 specific BACs and the distance between the signals was measured. In 5-aza-dC treated metaphase cells, the length of the whole chromosome was measured instead. In both cases, measurements were taken with Olympus AnalySIS software by manually drawing a line from one BAC signal to the other or from one end of the chromosome to the other (5-aza-dC treated-cells). As described in [69], a macro was written to incorporate information about the magnification to calibrate the measurements. In metaphase spreads, an average length of the two chromatids was contained for each chromosome followed by normalization for different levels of condensation. Normalization was carried by dividing the chromosome length by the average length of chromosome 1, which was used because it can be easily identified by eye.
In sperm cells, normalization was carried by dividing the distance between the chromosome 6 signals by the total length of the sperm since it was previously shown that chromatin extension depends on sperm elongation [69]. Measurements were processed using perl (v5.10.0) and statistical analyses were carried in R (version 2.11.0). Clustering and BIC analyses were performed with the R package mclust (version 3.4.8).

Silver staining

Silver staining on metaphase spreads was carried following standard procedures. Briefly, two drops of gelatin solution (2% w/v gelatin / 1% v/v formic acid) plus two drops of silver nitrate solution (50% w/v silver nitrate) were placed onto metaphase-spread slides. Solutions were mixed by gently tilting the slide. Slides were then covered with cover slips and placed on a 70°C hotplate for approximately 2 min, or until golden brown. Slides were mounted with a drop of Vectashield.

Acknowledgements

Thanks to Dr. Jonathan Tuke for advice in statistical analysis and to Aaron Casey for very useful discussions and for providing a chromosome 6 BAC. Thanks to Prof. Jeremy Timmis for very constructive feedback and for providing the used rDNA primers. DT-F is funded by the National Council of Science and Technology from Mexico (CONACYT). FG is an ARC research fellow.

Author contributions: DT-F performed sample processing, laboratory work and wrote the manuscript. RDK helped in data interpretation and statistical analysis. FG contributed to the design and supervision of the project as well as to the manuscript preparation and data interpretation.
Figure 1. Two colour FISH with chromosome-6 specific BACs on platypus metaphase spread.

(A) FISH on platypus metaphase cell with chromosome 6 specific BACs in red (849O23) and green (171L02) hallmarking the heteromorphic NOR region. White brackets indicate the length of the NOR. (B) Platypus chromosome 6 and chromosome 1 homologues. Brackets indicate how the measurements were performed.
Figure 2. Density distribution of chromosome 6 length measurements in metaphase spreads of 3 males and 3 females.

The length of both chromosome 6 homologues was measured in a number of cells (Table 1) and the density distribution for each individual was plotted. (A) Density plots for three different females show variation but clear bimodality in individuals 1 and 3. (B) Density plots for three different males show variation and a “shoulder” type of distribution instead of the clear bimodality observed in females.
Figure 3. 5-aza-dC treatment of platypus cultured fibroblasts elongates the female short chromosome 6.

Methylation was inhibited in platypus fibroblasts in culture. 5-aza-dC treatment significantly elongates the short chromosome 6 in females (n = 127), whereas it had no effect in the female long chromosome nor in male cells (n = 92). Treatment failed to erase the observed heteromorphism since the long and short chromosome 6 are significantly different in both sexes in treated and untreated cells.
Figure 4. Silver staining on female and male platypus metaphase spreads.

Panel (A) shows silver staining performed on a female metaphase spread, (B) shows the same experiment on a male metaphase spreads. NORs are indicated by black arrows in both cases. In the female we observed an uniform staining of the NOR as indicated both in panel A and in C, where we show different examples of female chromosome 6 pairs with a uniform silver staining on the NOR. In males, however, we can see both in panel B and in the different examples depicted in panel D that while one of the NORs is uniformly stained with silver, the second one presents a gap in the middle of the NOR.
Figure 5. Chromosome 6 heteromorphism is observed in platypus sperm.

(A) Density plot of chromosome 6 length measured in 127 platypus sperm cells of one individual. The bimodality of this graph represents the existence of two populations of chromosome 6 in platypus sperm. (B) Bayesian Information Criterion (BIC) estimates the number of clusters that best represent the data. In this case, 2 clusters represent chromosome 6 data better than 1 cluster. This is true assuming both equal (E) and different (V) variance.
Figure 6. Three-color FISH was used to study chromosome 6 segregation in male meiosis.

FISH was performed on platypus sperm using a green-labeled BAC (171L02) specific to the long arm of chromosome 6 and a red-labeled BAC (849O23) specific to the short arm. Either X (A) or Y (B) specific BACs (456I13 or 456I13, respectively) were labeled in yellow (mix of green and red). The distance between the two chromosome 6 BACs was measured, if it was above the individual’s chromosome 6 median value it was classified as long and below, as short.
Figure 7. Percentage of short and long chromosome 6 on Y- and X-bearing sperm.

This figure shows the three different individuals in which chromosome 6 was measured. In the case of Y-bearing sperm, in all the cases there was an equal proportion of short and long chromosome 6 observed. However, in X bearing sperm we observed a bias with an overrepresentation of short X chromosome 6 in all the cases. Male #1 X-sperm n = 66, Y-sperm n=105; male #2 X-sperm n = 71, Y-sperm n = 56; male #3 X-sperm n = 59, Y-sperm n = 60.
Figure 8. XY sperm counting through two sequential 1-colour FISH experiments.

(A) A Y-specific BAC (285J12) was labeled in red and hybridized onto platypus sperm spreads. The number of Y-positive and Y-negative sperm was counted. (B) The same platypus sperm spreads were hybridized against a X-specific BAC (456I13) labeled in red in a second FISH experiments. The same sperm cells recorded in experiment (A) were observed and counted in this second FISH. (C) This graph reports the percentage of Y positive sperm (Y) and Y negative sperm (no Y) observed in the first FISH experiment (A), showing a clear bias towards Y positive sperm. (D) This graph reports the percentage of X positive sperm (X) observed in the second 1-colour FISH experiment (B), which was recorded as no Y in graph (C). The percentage no X represents all the X-negative cells that were reported Y-positive in (C), and the reported Non-informative percentage corresponds to sperm cells that did not yield a signal in either experiment.
Figure 9. Potential modes of inheritance of platypus chromosome 6 variants in females. (A) Assuming that females transmit only eggs that carry the short chromosome 6, the possible offspring will include males showing bimodal distribution that have not been identified yet and only females showing bimodal distribution, however, we found one case of non-bimodality in females. Therefore, this assumption does not fit our data. (B) If females transmitted only the long version of chromosome 6, we would expect to see bimodal males, but again no bimodality has been identified in males so far. Also, this model does not allow the occurrence of non-bimodal females, therefore, it does not explain our data either. (C) If females were to transmit both alleles we would be able to observe all the distributions that we have observed in our studies. However, it would also imply to have bimodal males. Therefore, a mechanism should exist to prevent the development of bimodal males.
Figure S1. The signal intensity on the NOR after rDNA FISH resembles the signal intensity obtained after silver staining. The chromosomes we present in this figure correspond to the same male platypus. The silver staining chromosomes are homologous as well as the FISH chromosomes, but they belong to different cells. (A) A probe based on the human 28S rDNA was designed and used for DNA FISH. (B) On the left we find the chromosome 6 homologue that presented a gap after silver staining with its respective signal intensity graph, to the left we found the signal intensity for a chromosome on which FISH rDNA was performed and we notice the resemblance between the two intensity graphs. (C) On the left we observe the chromosome in which FISH was performed with its respective signal intensity graph to the left, showing similarity to the intensity graph of the silver staining chromosome on the right.
Figure S2. Expected XY sperm counting differences assuming negative selection against X-sperm carrying the long chromosome 6 versus observed XY sperm counting differences under three different assumptions. Based on the data in Figure 3 and Table 1, the black column represents the difference Y-X sperm that we expect to observe in each individual. The white, dark gray and light gray columns represent the observed Y-X sperm differences based on the data presented in figure 4, under three different assumptions. (1) We assume that X sperm is represented by the sum of X positive sperm and non-informative sperm; (2) we assume X sperm is exclusively represented by X positive sperm whereas Y sperm is Y positive sperm plus non-informative sperm; (3) we assume uninformative sperm represent cases in which either the $X_1$ or $Y_5$ were lost in the scored sperm, therefore, we exclude them from the calculation.
Tables

Table 1. Chromosome 6 NOR heteromorphism in platypus males and females.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>P-value</th>
<th></th>
<th>N</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male #4</td>
<td>56</td>
<td>&lt; 2.2e-16</td>
<td>Female #1</td>
<td>103</td>
<td>&lt; 2.2e-16</td>
</tr>
<tr>
<td>Male #5</td>
<td>45</td>
<td>1.3e-12</td>
<td>Female #2</td>
<td>62</td>
<td>&lt; 2.2e-16</td>
</tr>
<tr>
<td>Male #6</td>
<td>104</td>
<td>&lt; 2.2e-16</td>
<td>Female #3</td>
<td>109</td>
<td>&lt; 2.2e-16</td>
</tr>
</tbody>
</table>

Table 2. Percentage value of long and short chromosome 6 in X/Y sperm.

<table>
<thead>
<tr>
<th></th>
<th>X-Sperm</th>
<th></th>
<th>Y-Sperm</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Short %</td>
<td>Long %</td>
<td>N</td>
</tr>
<tr>
<td>Male #1</td>
<td>66</td>
<td>66.67</td>
<td>33.33</td>
<td>105</td>
</tr>
<tr>
<td>Male #2</td>
<td>71</td>
<td>78.87</td>
<td>21.13</td>
<td>56</td>
</tr>
<tr>
<td>Male #3</td>
<td>59</td>
<td>76.27</td>
<td>23.73</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 3. XY sperm counting in two sequential 1-color FISH experiments showing a bias towards Y sperm in all three analyzed individuals.

<table>
<thead>
<tr>
<th></th>
<th>1st FISH</th>
<th>2nd FISH</th>
<th>Non-Informative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Y %</td>
<td>no Y %</td>
</tr>
<tr>
<td>Male #1</td>
<td>151</td>
<td>57</td>
<td>43</td>
</tr>
<tr>
<td>Male #2</td>
<td>109</td>
<td>59.6</td>
<td>40.4</td>
</tr>
<tr>
<td>Male #3</td>
<td>100</td>
<td>59</td>
<td>41</td>
</tr>
</tbody>
</table>
References


CHAPTER 4: Identification and characterization of a male-specific change in Sox3 in the platypus

This is a conventional thesis chapter.

Chapter overview

In this chapter I aimed to investigate the genes located on chromosome 6, focusing on Sox3. The interest in this gene stems from the fact that Sry evolved from it and that it has been shown to function in a similar way to Sry when expressed in the gonad. Although circumstantial data suggested that Sox3 is on platypus chromosome 6, actual information was lacking for this important gene. Furthermore, in light of the results presented in the previous chapter Sox3 appears an interesting gene to look for any signs of sex chromosome differentiation.

Platypus Sox3 location was confirmed to be on chromosome 6. Analysis of a fragment of this gene in ten different individuals showed a male-specific variation on one of the polyalanine tracts. Expansion and reduction of polyalanine tracts have been related to disease in humans, therefore, a male-specific change in one of these regions could have an impact on its transcriptional activity in a sex-specific fashion. Furthermore, given that Sry evolved from Sox3 it is possible that this male-specific variant could be involved in male sex determination or development. In the future, obtaining the full-length Sox3 transcript in both sexes would be crucial to spot further sex-specific variations. Moreover, testing the capability of the male-specific and female-occurring Sox3 variants to activate Sox9 in vitro would be key to further confirm Sox9 possible implication on male sex-determination.
Identification of a male-specific $Sox3$ allele in the platypus might suggest a role in male sex determination

Abstract

$Sox3$ is the gene from which the therian male sex-determining gene ($Sry$) evolved. It is normally expressed in the developing central nervous system and in the developing urogenital ridge, as well as in adult gonads and neurogenic regions. Interestingly, it has been shown that $Sox3$ can mimic $Sry$ when ectopically expressed in the XX bipotential gonad in mice. In platypus $Sox3$ is located on chromosome 6, which is the proto-sex chromosome in therian mammals. The autosomal location of this gene confirmed the lack of a $Sry$ orthologue in the platypus. However, $Sox3$ has not been characterized in this animal yet. Given the lack of information of $Sox3$ in the platypus, we decided to characterize this gene. In this study we confirmed $Sox3$ location on chromosome 6 and interestingly, we discovered a male-specific variant. The observed male-specific $Sox3$ has a reduced second polyalanine tract coding for seven residues instead of nine. We analyzed five males and five females; none of the females carried the seven alanine $Sox3$ whereas it was observed in four out of five males. Polyalanine tract reductions and expansions have been related to disease in humans, with expansions of as little as one residue producing a phenotype. This raises the interesting possibility that $Sox3$ might have adopted a male-specific role in the platypus.

Introduction

$Sox3$ is the gene from which the therian male sex-determining gene ($Sry$) evolved [1, 2]. This is a very interesting gene that has been investigated in therian mammals and in other vertebrates, but mostly overlooked in the platypus. $Sox3$ is a one-exon gene and it contains a high mobility group (HMG) box, which is responsible for DNA-binding,
DNA-bending, protein interactions and nuclear import and export [3, 4]. This domain is highly conserved across vertebrates, with around 86% identity among Sox3 orthologues [5]. Interestingly, it has been shown that the HMG box from Sox3 and Sox9 can replace Sry’s HMG box in sex determination [6]. SOX3 additionally contains a variable N-terminal region that bears homopolymeric runs of alanine (A), glycine (G) and proline (P), and it has been suggested that this region is important for the modulation of SOX3 in different species [5].

Polyalanine tracts are of particular interest since expansions of these regions in SOX3 and several other transcription factors have been linked to disease in humans [7, 8]. Interestingly, expansions of as little as one amino acid have been associated to a phenotype. For example, expansions from 16 to 17 residues in the first polyalanine tract in ARX results in non-syndromic mental disability in humans [9]. Polyalanine tracts have shown to be mammalian specific in SOX3 as well as in the HOX and GATA protein families [5, 10]. SOX3 has four polyalanine tracts, the first tract is therian-specific and expansions of this tract have been associated with X-linked mental retardation with hormone deficiency and hypopituitarism in humans [7, 11]. The second and fourth tracts have been observed in all mammals including monotremes, which is the only group missing the third tract [5]. Thereafter, monotremes have only two polyalanine tracts that correspond to the second and fourth polyalanine tracts from the eutherian SOX3 [12].

It is known that Sox3 is normally expressed in the developing central nervous system in mouse [4, 13-15] and together with Sox1 and Sox2 participates in the initiation and progression of neuronal differentiation [16]. Sox3 expression is also found within adult neurogenic regions in the subventricular and subgranular zones as well as in a subset of differentiated hypothalamic cells [17, 18]. On the other hand, Sox3 is also expressed in
the developing urogenital ridge [4] and later in the adult gonad in both males and females [19, 20]. Unlike Sry, Sox3 is not required for sex determination but it is important for normal oocyte development as well as for testis differentiation and gametogenesis [20].

However, it has been shown that Sry and Sox3 are functionally interchangeable [21]. In mice, SRY acts synergistically with SF1 to trigger male sex determination by up-regulating Sox9 through its testis-specific enhancer (TES) [22]. SOX9 in turn activates AMH, the anti-Müllerian hormone [23], which suppresses the formation of the Müllerian duct repressing the female developmental pathway and allows the progression of male development [24]. It has been shown that ectopical expression of Sox3 in the bipotential gonad of XX mice leads to female-to-male sex reversal and that this occurs only if there is also Sf1 expression as well as a normal Sox9 context [21]. This implies that Sry and Sox3 are able to induce male sex-determination through a similar mechanism making them functionally interchangeable.

Sox3 has not been characterized yet in the platypus, however, mapping of this gene to chromosome 6 [25] helped to exclude the possibility of a Sry orthologue in this animal. It has been recently shown that chromosome 6 in the platypus shows non-random segregation during male meiosis (Toledo Flores et al, in preparation) and this could potentially facilitate the transmission of sexually antagonistic alleles [26]. Since chromosome 6 is homologous to the therian X [25, 27, 28] we asked whether this autosome could bear a male sex determination or sex development factor. Given that Sry evolved from Sox3 we decided to investigate this gene in the platypus.

In this study we physically mapped and characterized platypus and echidna Sox3. Importantly, we identified a variation in the platypus second polyalanine tract that
showed to be male-specific. This raises the interesting possibility that the Sox3 male-specific variant might have evolved a male-specific role in the platypus.

Materials and Methods

Phylogenetic analysis

Sequences of the following genes were included in the analysis: Sox1, Sox2, Sox3, Sox14 and Sox21. The following species were used: Gallus gallus (GG), Ornithorhynchus anatinus (OA), Monodelphis domestica (MD), Homo sapiens (HS) and Caenorhabditis elegans (CE) as outgroup. The reference numbers of the used genes are the following: Sox1 HS (NM_005986.2), Sox1 MD (XM_007501279), Sox2 GG (D50603.1), Sox2 HS (NM_003106.3), Sox2 MD (XM_001368783.3), Sox2 OA (XM_007669055.1), Sox3 GG (AB753847.1), Sox3 HS (NG_009387.1), Sox3 MD (XM_007507371.1), Sox14 GG (NM_204761.1), Sox14 HS (NM_004189.3), Sox14 MD (XM_007493921.1), Sox14 OA (AY112710.1), Sox21 GG (AB026623.1), Sox21 HS (NM_007084.2), Sox21 MD (XM_007501393.1). The utilized Sox3 OA sequence was obtained from Ultracontig 15 (release Oana-5.0 Ensembl gene build May 2006). Sequences were aligned using MUSCLE [29] implemented in Geneious v5.3.4 using default parameters. MrBayes [30] was used to reconstruct the tree using $10^6$ iterations and GTR + gamma.

BAC clone isolation

We used the partial platypus Sox3 sequence identified in Ultracontig 15 (release Oana-5.0 Ensembl gene build May 2006) to identify the Sox3 positive BAC clones KAAH-752L02, KAAH-171118 and KAAH-643C06 (http://genome.wustl.edu/tools/blast).
**RNA extraction**

Adult male echidna and platypus tissues (testis, brain, liver) were used to isolate RNA using standard protocols [31]. Briefly, total RNA was extracted from -80°C frozen tissues using TRIzol reagent (Invitrogen), according to the manufacturer’s instructions. RNA was resuspended in nuclease free water and stored at -80°C. RNA was later treated with DNase I (Invitrogen) to remove genomic DNA, precipitated and resuspended in RNAase-free water.

**cDNA synthesis**

Samples of 1 µg of RNA were used to obtain cDNA from each tissue using a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen), as previously described [31]. In summary, RNA was incubated for 5 min at 65°C with 1 µl of 50 µM oligo(dT) and 1 µl of 10 mM dNTP. This was followed by further incubation for 50 min at 50°C with 2 µl of 10x RT buffer, 4 µl of 25 mM MgCl₂, 2 µl of dithiothreitol (DTT, 0.1 M), 1 µl RNaseOUT™ (40 U/µl), and 1 µl SuperScript III RT (200 U/µl). Termination was performed at 85°C for 5 min. Reverse transcription was followed by RNase H treatment; 1 µl of RNase H was added to each reaction and incubated for 20 min at 37°C. The cDNA samples were stored at -20°C.

**PCR and gene expression analysis**

Each PCR had a final volume of 25 µl, which consisted of 0.5-1 µl (50 ng) of genomic DNA, BAC DNA or cDNA, 5 µl of 5x PCR buffer with MgCl₂ (Promega), 5 U/µl of *Taq* DNA polymerase, 0.4 µM of each forward and reverse primers and 0.1mM dNTPs.
Initial denaturation was carried at 96ºC for 3 min followed by 35 cycles of: denaturation at 96ºC for 30 s, annealing at 55-59ºC for 1 min and extension at 72ºC for 2 min. Final extension was performed at 72ºC for 7 min. The primers used were \textit{SoxF-GCACAACTCGGAGATCAGCAAG}, \textit{SoxR-GGCAGGTACATGCTGATCATATC} at 59ºC (expected size \(~600\text{bp}\)) or the gene-specific \textit{Sox3F-CTACTCGCTGTGAGCCCGACC} and \textit{Sox3R-GAGCTGGGCTCCGACTTTGAC} at 59ºC (expected size \(~300\text{bp}\)), designed based on ENSOANG00000002448; \textit{β–actin-F-GCCCATCTACGAGGTTACGC} and \textit{β–actin-AAGGTCGTTTCGTGGATACCAC} at 55ºC. A 1.5% agarose gel was used to visualize the products.

\textit{Preparation of mitotic chromosomes}

Mitotic metaphase chromosomes and interphase preparations were made from previously established platypus fibroblast cell lines [32]. Fibroblast cells were cultured in DMEM Gibco BRL plus Hepes, 10% FCS, 2 mM L-glutamine (Invitrogen), penicillin/streptomycin at 32ºC until they reached 75-85% confluence, changing media two or three times per week. To harvest metaphase chromosomes, cells were treated with 0.5 µg/ml colcemid for 2-3 hrs. Cells were then trypsinized and spun at 1,200 rpm for 5 min to recover pellet. Supernatants were removed and the cell pellet was slowly resuspended in 0.075 M KCl hypotonic and incubated at 37ºC for 10 min. Cells were recovered after spinning at 1,200 rpm for 5 min and resuspended in fresh-made ice-cold fixative, containing 75% methanol and 25% acetic acid. After washing the metaphase preparation four to six times with fixative, cells were dropped in methanol-washed glass slides and allowed to age overnight at -20ºC.
Fluorescence in situ hybridization

BAC DNA (1 µg) was labeled with spectrum orange or green (Abbott Molecular) using random primers and Klenow polymerase and hybridized by fluorescence in situ hybridization (FISH) on metaphase cells according to standard procedures previously described in [33]. Briefly, slides were treated with 100 µg/ml RNase A/2x SSC for 30 min at 37ºC followed by pepsin treatment (10 min incubation in 0.01 % pepsin in 10 mM HCl at 37ºC). Next, slides were re-fixed for 10 min in 1 x PBS, 50 mM MgCl₂, 1 % formaldehyde and then dehydrated in ethanol series, denatured for 5 min at 75ºC in 70% formamide, 2x SSC and again dehydrated. Slides were hybridized overnight in a moist chamber at 37ºC with a mix of DNA probe, 10-20 µg of sonicated genomic DNA and 50 µg of salmon sperm redissolved in 50% formamide, 10% dextran sulfate, 2x SSC. Prior to hybridization the probe mix was denatured for 10 min at 80ºC and preannealed for 30 min at 37ºC. Post-hybridization, the slides were washed three times for 3 min in 50% formamide, 2x SSC at room temperature followed by one wash for 5 min in 2x SSX at 42ºC, once for 5 min in 0.1x SSC at 60ºC and finally once for 5 min in 2x SSC at 42ºC. Cells were counterstained with 1 µg/µl DAPI in 2x SSC for 1 min and then mounted with one drop of Vectashield. Images were taken using a Zeiss AxioImagerZ.1 epifluorescence microscope and Zeiss Axiovision software.

Construct modification

Expression plasmid pcDNA3.1(+) containing mouse Sox3 was used as a template for inverse PCR. Primers flanking the first polyalanine tract were used for standard PCR reaction (see above) with the next modifications: TM of 65ºC, elongation for 8 min and final elongation for 10 min; the used primers were MM-1st_polyA_F-
AGCAGCCCGGTGGGCGTGG and MM-1st_polyA_R-
GCCCCGGGGGCAGGAGGCCGC. PCR products were purified using QIAquick PCR
purification kit (QIAGEN) according to the manufacturer’s instructions. 20 ng of the
obtained inverse PCR product were self-ligated in a 20 µl ligation reaction with 2 µl
10x Buffer (NEB), 1 µl ligase T4 (NEB) and PEG4000 50% w/v. The ligation reaction
was performed overnight at 16ºC. 10 ng of ligated vector were used to transform 50 µl
of competent recA, endA E. coli cells by heatshock at 42ºC for 45-50 secs. Vector was
propagated and collected following standard procedures. Deletion was confirmed by
standard PCR and Sanger sequencing.

Results/Discussion

Sox3 is located on platypus chromosome 6

The first attempts to map Sox3 in the platypus using a Sox3 positive BACs did not
succeed due to unspecific signals in the FISH experiments [25]. To overcome this
problem, a BAC containing Sox3 contiguous sequence was used instead. This anchor
sequence was obtained from the Ultracontig 15 from the platypus genome (release
Oana-5.0 Ensembl gene build May 2006) and it comprised the region expanding
positions 1622 kb to 1799 kb. In this same ultracontig, a region matching Sox3 is
located around position 337.5 kb [25], however, it is surrounded by unresolved
sequence (Fig. 2). Besides this region, there are no direct matches to Sox3 in Ultracontig
15.

Chromosome 6 contains many other genes that have been mapped to the therian X
chromosome [34-36]; therefore, it is very likely that it contains Sox3 as well. However,
the existence of unresolved sequence surrounding Sox3 leads to the possibility that the
mapped BAC and Sox3 are not physically contiguous. Therefore, we first wanted to confirm the physical location of Sox3. In order to do this, we first determined the identity of the publicly available Sox3 sequence through a Bayesian phylogenetic reconstruction, which show that the platypus Sox3 clusters together with Sox3 from other species and not with other SOX genes, further confirming its identity (Fig. 3). Next, we searched the platypus BAC sequence library of the Genome Institute at the University of Washington (http://genome.wustl.edu/tools/blast) with the confirmed platypus Sox3 sequence. Through this, three different BACs containing fragments of Sox3 were retrieved KAAH-752L02, KAAH-171I18 and KAAH-643C06 (Fig. 4A). We physically mapped these three BACs, which colocalized on the long arm of platypus chromosome 6 (Fig. 4B-D), in a region similar to that where the previous BAC containing adjacent sequence has been localized, confirming previously reported work [25].

Absence of detectable Sox3 mRNA expression in adult monotreme gonads

Next we wanted to investigate whether the expression is conserved in the monotreme male gonad. As previously discussed, Sox3 is normally expressed in the developing urogenital ridge [4] as well as in the adult gonad in both sexes [19, 20] and in specific regions of the developing [4, 13-15] and adult brain [17, 18]. We prepared cDNA from adult male platypus and echidna brain, testis and liver. Expression analysis was performed using RT-PCR. As expected, no Sox3 expression was detected in liver of either species. Surprisingly, we were able to observe mRNA expression in echidna brain but no platypus brain expression and no mRNA was detected in gonads either (Fig. 5B). This is surprising since Sox3 was previously detected in the adult gonad of both sexes in mice and chickens [19, 20] and it has been proposed that it has an important function in gametogenesis [20]. It is possible that Sox3 has a function earlier in development but to
investigate this we would require access to monotreme embryonic samples. The lack of this type of material represents a limitation to this project.

The absence of Sox3 expression in the platypus brain is also surprising particularly after observing expression in the echidna brain (Fig 5B). PCR amplification of the platypus Sox3 gene has been a challenging task showing inconsistent PCR results. PCR triplicates in the same individual showed that under the same conditions and using the same reagents the reactions successfully amplified a product only sometimes, whereas others it failed (Fig. 6). Interestingly, this has been observed only in the platypus but not in the echidna. The lack of Sox3 expression in platypus brain, in contrast to the clear Sox3 expression in echidna brain, could be the result of a failed RT-PCR or to the inexistence of Sox3 expression in this tissue.

It is important to note, however, that these brain samples were taken more likely from the brain cortex. It has been previously reported that in mice Sox3 expression in the adult brain is limited to neuroprogenitor cells in the subventricular and subgranular zones and in some differentiated hypothalamic cells [17]. Therefore, it is possible that our platypus sample comes from a non-Sox3 expressing region.

*A male-specific Sox3 variant shows a reduction of the second polyalanine tract*

The published Sox3 sequence is incomplete; it comprises 660 bp that cover part of the HMG box and the region coding for the polyalanine tracts. Several attempts using genome-walking, TAIL-PCR and RACE-PCR were followed in order to amplify the whole length of the platypus Sox3. However, these different attempts failed due to the inconsistency of the Sox3 PCR, which is likely a result of the high GC content of this gene (Fig. 6). Importantly, β-actin was used as a control to ensure that the genomic
DNA was suitable for PCR amplification. However a number of Sox3 fragments were amplified from different individuals. Sequencing of the products revealed variation in the polyalanine tract of Sox3. Specifically this variant was defined by a reduced second polyalanine tract (fourth tract in conserved terms). Previously in the literature it was reported that the platypus second polyalanine tract contains nine residues [12], however, we identify two common variants: a nine-alanine and a seven-alanine Sox3.

Next we identified and sequenced this region in ten animals. Interestingly, we found that the seven-alanine Sox3 was found exclusively in males and not in females; five females were sequenced and all of them carried a nine-alanine Sox3 variant whereas four out of five males carried the seven-alanine Sox3 and one of them carried a five-alanine tract (Fig. 7). The Sox3 PCR product was cloned for four individuals, two males and two females. Through cloning, we observed that the two males were heterozygous and carried a seven- and nine-alanine Sox3. Whereas females carried both a nine- and ten-alanine variants, confirming that females do not carry a seven-alanine Sox3.

This is very interesting because it has been shown that transcription factors are five times more abundant in polyalanine tracts than other proteins [10], which suggests that these tracts could be involved in transcriptional activity. Furthermore, the fact that expansions of as little as one residue produce a phenotype [9] raises the possibility that the male-specific Sox3 observed in platypus might function slightly differently in males compared to females. Additionally, clinical and in vitro studies have shown that deletions in the first polyalanine tract of the human Sox3 lead to increased transcriptional activity [37].

Therefore, future work will aim to investigate the effects that the reduction in the second polyalanine tract could have on the platypus Sox3 transcriptional activity in vitro. We are particularly interested in investigating whether this variant could
participate in platypus sex determination by adopting a similar activity as Sry in therian mammals in upreguating Sox9 via its TES enhancer. Therefore, we aim to test our hypothesis that the Sox3 seven-alanine variant could efficiently activate Sox9 TES core element (TESCO) using a reporter gene assay. This will consist in cloning the platypus Sox3 variants into an expression vector that would be later transfected into cells containing the TESCO enhancer linked to a luciferase reporter. Next, the relative luciferase activity will be measured as an indication of the potential of Sox3 to activate Sox9 through its TESCO enhancer. Other genes may be included in this assay, such as Sf1 since it is known that it acts synergistically with Sry to activate Sox9 [22]. Similar approaches have been followed in the past to test the potential of other male sex-determining candidate genes to trigger Sox9 activity in the platypus [31].

Given the difficulty to amplify the entire Sox3 transcript, we have designed an experiment that aims to mimic in a mouse Sox3 construct what we have observed in platypus. Since platypuses have only two polyalanine tracts (Fig. 8A), which correspond to the second and fourth polyalanine tracts in mouse (Fig. 8B), we have decided to start by reducing the length of the mouse first polyalanine tract from 14 to only six residues (Fig. 8C). Our aim is to later assess the impact this might have on Sox3 transcriptional activity through the previously described reporter gene assay. The next step would be to completely eliminate the first polyalanine tract and reduce the length of the fourth polyalanine tract by two residues, from ten to eight (Fig. 8D) to reproduce the change we observed in the platypus male-specific Sox3. We hypothesize that there should be an increase in the transcriptional activity of the modified mouse Sox3 construct (Fig. 8D). This will be tested using the described reporter gene assay.

Importantly, these samples were obtained from a platypus population in New South Wales. Future work will aim to investigate whether the observed variant is specific to
this region or if it is widespread across different populations in Australia. Furthermore, it would be important to find out what mechanisms are responsible of maintaining this difference. Our previous work (chapter 3) has shown that chromosome 6 present distinct characteristics between males and females as well as non-random segregation during male meiosis. It is then possible that this chromosome commenced to differentiate before monotremes and therians divergence. Suppression of recombination between chromosome 6 homologues could then help to maintain the seven-alanine Sox3 variant in males, however, further work is necessary to investigate this.

Conclusion

Sox3 has shown to be able to trigger male sex determination through a similar mechanism as Sry [21] and its location on the non-randomly segregated chromosome 6 makes it a worthy gene to study in terms of sex determination in the platypus. Our finding of a male-specific variant of Sox3 raises interesting questions about whether it might have potentially acquired a male-specific function that could be involved in male sex determination or differentiation. Investigating the transcriptional activity of this variant in vitro could provide us with insights about potential novel functions. Additionally, it might help us to understand the function of the fourth polyalanine tract in Sox3, which may have clinical relevance. Moreover, this could shed light on the evolution of Sox3 as a sex-determining factor in therian mammals by looking at its function in monotremes.

Acknowledgements

We would like to thank Arthur Ferguson and Belinda Turner from the Perth Zoo for providing essential material for this study.
Figure 1. Sox3 HMG box and polyalanine tracts in mammals. This diagram shows the structure of Sox3 in mouse, opossum, platypus and echidna. Mouse Sox3 has an HMG box followed by 4 spaced polyalanine tracts. Opossum lacks the 1st polyalanine tract, whereas platypus and echidna lack both the 1st and 3rd polyalanine tracts. In echidna, the 4th tract seems to be split in two by three serines.
Figure 2. Platypus Ultracontig 15 (release Oana-5.0 Ensembl gene build May 2006). The identified Sox3 sequence is indicated at 337.5 kb and it is surrounded by unresolved sequence indicated by “NNN”. The anchor sequence that was previously used to map Sox3 [25] is between positions 1,622 kb and 1,799 kb.
Figure 3. Bayesian phylogenetic reconstruction of Sox genes. Species used for Bayesian reconstruction: CE, *Caenorhabditis elegans*; MD, *Monodelphis domestica*; GG, *Gallus gallus*; HS, *Homo sapiens*; OA, *Ornithorhynchus anatinus*. The OA Sox3 sequence used for this analysis was taken from the Ultracontig 15 in the platypus genome (release Oana-5.0 Ensembl gene build May 2006). This phylogeny shows that the platypus Sox3 clusters with the Sox3 of other species and not with any other Sox gene, supporting that the sequence contained in the Ultracontig 15 is effectively Sox3.
Figure 4. *Sox3* location on chromosome 6. (A) PCR on BACs 171I18, 752L02 and 643C06 using primers *Sox_F* and *Sox_R* that amplify platypus *Sox3* confirmed these BACs contain *Sox3*. (B) FISH onto female platypus metaphase spreads with 171I18 labeled in green and a known chromosome 6 BAC 849O23 labeled in red, confirms location of 171I18 on chromosome 6. In panel (C) we observe the same FISH but with BAC 752L02 labeled in green, and panel (D) shows colocalization of 752L02 and 643C06. This confirms that *Sox3* is on platypus chromosome 6.
Figure 5. Sox3 expression in platypus and echidna adult tissues. (A) β-actin amplification in echidna and platypus adult tissues was used as positive control. First three samples belong to an adult male echidna, next three samples belong to an adult male platypus. (B) RT-PCR with Sox3 specific primers (SoxF and SoxR) reveals expression in echidna adult brain. Samples are presented in the same order as in (A). Expected size: ~600 bp. Tes: testis; Li: liver; Br: brain; g: echidna genomic DNA; 1: negative control. White arrow indicates Sox3 expected size.
Figure 6. *Sox3* amplification in the platypus is inconsistent. (A) PCR amplification of *Sox3* from male and female platypus genomic DNA showed to be inconsistent. Whenever the amplification was successful we retrieved a band of the expected size, which after sequencing showed to be positive to *Sox3*. However, as indicated by the triplicates for these four individual, PCR amplification was not successful in all the cases. Used primers: *Sox3F* and *Sox3R*, expected size: ~300bp. (B) PCR amplification of β-actin as a positive control.
Figure 7. A Sox3 male-specific allele was identified in the platypus. A fragment of Sox3 was amplified in five different females and five different males. A Sox3 coding for seven alanines on its 2nd polyalanine tract (4th in conserved terms) was detected only in males and not in females.
**Figure 8.** Modified mouse Sox3 constructs to mimic platypus Sox3 structure. (A) The platypus Sox3 polyalanine region consists of two polyalanine tracts whereas the mouse Sox3 (B) features four polyalanine tracts. (C) To assess the impact that the lack of the first polyalanine tract could have in the platypus, we reduced the length of the first polyalanine tract in the mouse construct. (D) To fully mimic the structure of the platypus Sox3 in mouse, we aim to delete the first polyalanine tract followed by reduction of the fourth polyalanine tract.
Table 1. Cloning of PCR products reveals that four out of the ten analysed platypuses are heterozygous for Sox3. Although three forms of Sox3 were identified, the 7-Ala variant was observed exclusively in males.

<table>
<thead>
<tr>
<th></th>
<th>7-Ala</th>
<th>9-Ala</th>
<th>10-Ala</th>
</tr>
</thead>
<tbody>
<tr>
<td>♂1</td>
<td>✓</td>
<td>✓</td>
<td>?</td>
</tr>
<tr>
<td>♂2</td>
<td>✓</td>
<td>✓</td>
<td>?</td>
</tr>
<tr>
<td>♀1</td>
<td>✗</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>♀2</td>
<td>✗</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>
References


CHAPTER 5: Investigating sex bias in captive bred echidnas

This chapter consists of one manuscript and a brief letter, both in preparation.

Chapter overview

Determining the sex of captive born animals is of great relevance to zoos in order to implement this information to their captive management plans. The echidna, is a particularly difficult animal to sex given the lack of any obvious sexual dimorphisms and due to the fact that sexual organs are internal. Adult echidnas can be sexed by palpation, however, this is impossible on echidna puggles.

In this chapter, I describe how I utilized our knowledge about male specific genes to develop a PCR based technique to genetically determine the sex of echidna puggles.

This revealed a strong female bias on captive born echidnas providing important hints that suggest that factors during the captive breeding process might influence sex ratio. Importantly, this noninvasive sexing technique can be potentially applied to wild echidna and platypus populations, including the critically endangered long-beaked echidna.

Moreover, I have identified a naturally occurring deletion of *Sox3* in a male echidna. This provides a unique opportunity to study the impact this deletion might have on echidna sex development.
# Statement of Authorship

<table>
<thead>
<tr>
<th>Title of Paper</th>
<th>Sexing of juvenile echidnas using small hair and blood samples reveals sex bias in echidnas born in captivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Publication Status</td>
<td>○ Published, ○ Accepted for Publication, ○ Submitted for Publication, ● Publication style</td>
</tr>
</tbody>
</table>

**Author Contributions**

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate’s thesis.

<table>
<thead>
<tr>
<th>Name of Principal Author (Candidate)</th>
<th>Deborah Toledo-Flores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contribution to the Paper</td>
<td>Performed genomic DNA extractions from hair and blood samples, sexing through PCR, established lymphocyte cultures, performed FISH experiments, wrote manuscript.</td>
</tr>
<tr>
<td>Signature</td>
<td>Date</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of Co-author</th>
<th>W.X. Kang</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contribution to the Paper</td>
<td>Performed genomic DNA extractions from hair samples, sexing through PCR.</td>
</tr>
<tr>
<td>Signature</td>
<td>Date</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of Co-Author</th>
<th>A. Ferguson</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contribution to the Paper</td>
<td>Originally designed the project together with F. Gützner.</td>
</tr>
<tr>
<td>Signature</td>
<td>Date</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of Co-Author</th>
<th>B. Turner</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contribution to the Paper</td>
<td>Collected and provided echidna hair samples and advised on echidna biology.</td>
</tr>
<tr>
<td>Signature</td>
<td>Date</td>
</tr>
<tr>
<td>Name of Principal Author</td>
<td>E. Tseng-Ayush</td>
</tr>
<tr>
<td>--------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Contribution to the Paper</td>
<td>Designed probes for BAC library screening.</td>
</tr>
<tr>
<td>Signature</td>
<td>Date 18/12/14</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of Co-Author</th>
<th>S.L. Lim</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contribution to the Paper</td>
<td>Performed BAC library screening. Performed genomic DNA extractions from hair samples.</td>
</tr>
<tr>
<td>Signature</td>
<td>Date 15/12/14</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of Co-Author</th>
<th>F. Grützner</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contribution to the Paper</td>
<td>Originally designed this project together with A. Ferguson, supervised development of work and manuscript preparation and evaluation.</td>
</tr>
<tr>
<td>Signature</td>
<td>Date 18/12/14</td>
</tr>
</tbody>
</table>
Manuscript in preparation:

**Deborah Toledo-Flores**, Wan Xian Kang, Arthur Ferguson, Belinda Turner, Enkhjargal Tsend-Ayush, Shu Ly Lim, Frank Grützner. Genetic Sexing Reveals Female Bias in Echidnas Born in Captivity
Genetic Sexing Reveals Female Bias in Echidnas Born in Captivity

Deborah Toledo-Florea, Wan Xian Kangb, Arthur Fergusonb, Belinda Turnerb, Enkhjargal Tsend-Ayusha, Shu Ly Limb, Frank Grütznerb

aThe Environment Institute, School of Molecular and Biomedical Science, The University of Adelaide, Adelaide, SA 5005, Australia
bPerth Zoo, 20 Labouchere Road, South Perth, WA 6151, Australia

Abstract

Captive breeding of echidnas remains difficult and determining the sex of echidnas born in captivity as soon as possible is important for husbandry and continued breeding success. Accurately sexing juvenile echidnas is a challenging task due to the lack of visible external genitalia. Here we have established a non-invasive PCR based technique to determine the sex of echidnas using DNA from plugged hair. Genomic DNA was extracted from as few as 10 echidna hair follicles and subsequently PCR amplification of a male-specific gene was used for determining male or female sex. We tested eight echidnas born in captivity at Perth Zoo and found a striking sex bias, with seven females out of eight animals. In two individuals we confirmed the sex by culturing peripheral blood lymphocytes for chromosomal analysis and Fluorescence in situ hybridization (FISH) to identify an XX or XY sex chromosome constitution further confirming the results obtained by PCR. In conclusion we have established a straightforward and non-invasive molecular technique to reliably determine the sex of young echidnas and discovered a striking bias towards female offspring in captive bred echidnas.
Introduction

Echidnas and platypuses are the only living monotremes and amongst Australia’s most iconic animals. Echidnas are wide-spread in Australia and also found in New Guinea[1]. There are four species of echidnas; the short-beaked echidna (*Tachyglossus aculeatus*) and three species of the critically endangered long-beaked echidna (*Zaglossus bruijnii, Z. bartoni* and *Z. attenboroughi*) [2]. Echidnas are comparably easy to keep but difficult to breed in captivity [3-5]. A considerable amount of planning and effort is required to successfully breed Echidnas [8].

The London Zoo holds the first record of housing an echidna back in 1845 [3], after which it became common practice to have echidnas as part of zoo collections. In 1908 the first captive-born echidna was reported in the Berlin Zoo, this came as a surprise since the breeding was not planned. Unfortunately, the puggle died three months after hatching [6]. A similar trend has been observed in a significant proportion of echidnas born in captivity, by 2007 there were 20 echidnas born in international zoos and 7 born in Australian zoos, with more than half dying before reaching 1 year of age [7]. Only recently, the Perth Zoo reported that they were able to breed echidnas in captivity during three consecutive years producing a total of five animals [8]. This breeding success led to the question of how to accurately determine the sex of juvenile animals to enable early population management succession planning.

In addition to the practical necessity of determining the sex of the animals bred in captivity, there is the question of whether there is any bias in the sex ratio of the offspring. Early identification of sex ratio bias is necessary so that population managers
at zoos can take compensatory measures into action [9], such as transferring known-sex animals to other institutions for future breeding. Different cases of sex ratio bias in captive animals have been described. For instance, ring-tailed and lesser mouse lemurs show housing-dependent offspring sex ratio bias [10]. Also, captive populations of pygmy hippopotamus present female-biased sex ratios associated with high feeding intensity and “hands-on” husbandry, however, the mechanisms underlying the observed segregation distortion remain unknown [11]. Similarly, this phenomenon has been observed in a number of other primate, rodent, marsupial, cervid, bovid, carnivore, chiropteran and cetacean species [12] but so far no data have been available about sex ratio bias of captive bred echidnas.

Determination of the sex of echidnas, particularly juveniles, is not a straightforward task since they do not have any externally obvious sexual dimorphism in body mass, dimensions or color [1]. In addition, some of the known sex-specific traits such as the female pouch or the male developed spur (without the sheath) are only established once the echidna reaches sexual maturity, which means that the young cannot be sexed using these traits. Even in adults, these characteristics cannot be unequivocally identified. The female pouch, for example, is not well developed outside of the breeding season [3], and it can be mistaken with the contraction of the longitudinal muscles of the abdomen even in males [13]. In the case of the male spur, at least 25% of the adult male population lose one of their spurs while 25% of mature females have one well developed spur; this stems from the fact that both sexes have spurs while juveniles, which are meant to regress in females during adulthood [13]. Furthermore, the reproductive organs are internal [1]. During the breeding season it is possible to identify reproductively active males because they show a degree of cloacal swelling, which is thought to be due to enlarged testicles [5, 14]. However, this technique requires an experienced eye and outside of the breeding season it is more difficult to accurately
determine the sex of individuals. Therefore, given the lack of obvious sexual
dimorphisms the only reliable way to determine the sex of an echidna is by palpation [1, 13, 15], which is quite invasive and impossible in echidna puggles.

Previously, a couple of fecal steroid analyses were established in echidna; however, they had a maximum accuracy of 80% and one of them was reliable only during the breeding season [16]. A number of non-invasive genetic sexing techniques available in and outside breeding season have been established in other species. These techniques rely on extraction of genomic DNA from unobtrusively collected samples such as hair and feces [17-24]. Importantly, it has been shown that it is possible to obtain consistent results from genomic DNA (gDNA) derived from hair, blood and other tissues [25-27]. To the best of our knowledge, no genetic techniques have been previously established to non-invasively determine the sex of echidnas or platypuses.

Here we report the establishment of a non-invasive PCR-based method to genetically determine the sex of echidna juveniles from genomic DNA extracted from small hair samples. Also, we validated our results by performing the same method on genomic DNA extracted from blood as well as through in situ hybridization with sex chromosome-specific probes on metaphase spreads derived from peripheral blood lymphocytes.

Materials and methods

Sample preparation and ethics

Hair samples with follicle attached were collected from the abdominal area of the echidna under manual restraint using sterile tweezers and placed immediately in a
sterile 15mL Falcon® tube. Echidna hair samples were stored at room temperature post-collection and shipped in 70% ethanol.

Blood samples were collected from the venous beak sinus under general anesthetic, into green-capped sodium heparin tubes. Blood samples were stored at room temperature and shipped within one day of collection.

Since this was a non-invasive procedure which was done opportunistically during routine examination no ethics permit had to be acquired.

*Genomic DNA extraction from hair and blood samples*

Hair follicles were cut under light microscope and each sample was placed in a tube containing 0.5-1 ml of 70% ethanol. The tubes were briefly spun to collect follicles in the bottom and ethanol was then removed. Follicles were allowed to dry at room temperature. Hair follicles were incubated at 55°C overnight while shaking at 700 rpm in 300 µl of lysis buffer (50mM TrisChloride, pH 8; 100mM EDTA, pH 8; 100mM NaCl; 1% SDS), 0.17µg/µl of proteinase K and 0.1M DTT. Once lysis was finalized, 0.6 µl of RnaseA (10 mg/ml) were added and the cell lysate was incubated for further 30 min at 37°C. Samples were allowed to cool down at room temperature, after this 100µl of 7.5M ammonium acetate were added to the samples and they were vigorously vortexed, followed by a 5 min incubation on ice. Samples were centrifuged for 5 min at 12,000 rpm, supernatants were then transferred to a new tube. DNA was then precipitated at -20°C overnight with 300 µl of 100% isopropanol in the presence of 0.5 µl of Glycogen solution (20 mg/ml). Centrifugation was carried at 12,000 rpm for 20 min, supernatants removed and the DNA pellet was resuspended in 10µl of 55°C pre-warmed Milli-Q water.
Genomic DNA from blood samples was extracted using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer’s instructions. Concentrations were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

**PCR and gel electrophoresis**

Each PCR had a final volume of 25 µl, which consisted of 0.5-1 µl of the extracted genomic DNA (depending on the obtained concentration), 5 µl of 5x PCR buffer with MgCl₂ (Promega), 5U/µl of Taq DNA polymerase, 0.4 µM of each forward and reverse primers and 0.1 mM dNTPs. In the case of PCRs using hair-extracted gDNA, 2.5 µl of BSA were used per reaction. Initial denaturation was carried at 96°C for 3 min followed by 40 cycles of: denaturation at 96°C for 30 s, annealing at 50-55°C for 1 min and extension at 72°C for 2 min. Final extension was performed at 72°C for 7 min. The primers used were **Crspy** for-ACCAGTAAATGCTGTGAAACCTC, **Crspy** rev-TTCTTTTTATTGGCTGGTTCTGA [28] at 50°C and **β-actin** for-GCCCATCTACGAAGGTTACGC and **β-actin** rev-AAGGTCGTTCGTGGATACCAC at 55°C. A 1.5% agarose gel was used to visualize the products.

**Culture of peripheral blood cells for chromosome analysis**

500 µl of echidna blood were cultured with 10 ml of PB-MAX medium (Life Technologies) for 72 hrs at 32°C (due to monotremes lower body temperature), according to the manufacturers suggested protocol. The 72 hr incubation was followed by a further 30 min incubation with 0.5 µg/ml of colcemid. A cell pellet was then obtained after a 5 min centrifugation at 1,200 rpm, supernatants were removed and cells were slowly resuspended in 10 ml of 0.075M KCl hypotonic and incubated at 37°C for
10 min. A cell pellet was recovered by spinning 5 min at 1,200 rpm, supernatants were removed and the cells were slowly resuspended in 10ml of fresh, ice-cold fixative made of 1 part of acetic acid and 3 parts of ethanol. Cells were then incubated for 10 min at 4ºC, washed 5 times with fixative and resuspended in a final volume of 0.5ml of fixative. Cells were dropped in methanol-washed glass slides and allowed to age overnight at -4ºC.

Fluorescence in situ hybridization of BAC clones

Y₃-specific BAC EAmhy2 and Y₃X₄-PAR BAC 48g5 [29] were used to perform FISH on echidna metaphase spreads under standard conditions, as previously described in [30]. Images were taken with a Zeis AxioImager Z.1 epifluorescence microscope equipped with a CCD camera and Zeiss Axiovision software.

Results

Echidna sexing through PCR on hair genomic DNA

We obtained small hair samples from eight echidnas (Fig. 1) born in captivity at the Perth Zoo during a period of five years 2009 – 2014. Each of the samples consisted of 10 – 50 hairs from which only the follicles were used for the DNA extraction protocol in order to minimize contaminants. The follicles have a characteristic conical shape (Fig. 2) compared to the typical bulb shape found in humans. We observed some variation in the efficiency of the DNA extraction protocol, however generally we obtained 190.5 ng/µl from an extraction of only 10 hairs.
The sex of the individuals was then determined through PCR using primers that amplify a platypus male-specific gene, \textit{Crspy}. \textit{Crspy}, the male specific copy of the \textit{Crsp70} gene that is located on platypus chromosome Y\textsubscript{5} [28], which has been shown to be homologous to the echidna male specific chromosome Y\textsubscript{3} [31]. These primers were tested in echidnas whose sex was known, confirming that the platypus \textit{Crspy} primers amplify a male specific product and no products in females (Fig. 3). Running this PCR on the DNA of eight echidna puggles showed that seven out of the eight captive-born echidnas were females (Table 1). Importantly, $\beta$--\textit{actin} primers were used as a positive control.

\textit{Echidna sexing validation through PCR on blood genomic DNA and FISH}

In order to validate our method we decided to back the PCR result with another method to genetically determine the sex of the animals. Monotremes have a unique heteromorphic sex chromosome system, which we have studied in detail [31-33]. We accessed blood samples for two of the eight echidnas (ID 7 and 8, Table 1) to prepare genomic DNA and metaphase chromosomes. PCR on the blood extracted DNA confirmed the results obtained with the hair samples (Fig. 4).

To chromosomally sex the two animals we prepared metaphase spreads derived from peripheral blood lymphocytes; we then performed DNA FISH using a Y\textsubscript{3} specific BAC together with a Y\textsubscript{3}X\textsubscript{4}-PAR BAC. In echidna number seven we observed two signals for the red-labeled Y\textsubscript{3}X\textsubscript{4}-PAR BAC and no signals for the green-labeled Y\textsubscript{3} BAC (Fig. 5A). In the echidna number eight we observed two signals for the now green-labeled Y\textsubscript{3}X\textsubscript{4}-PAR BAC and one signal for the red-labeled Y\textsubscript{3} BAC, which colocalized with one of the Y\textsubscript{3}X\textsubscript{4}-positive chromosomes (Fig. 5B). These results confirm the PCR based results.
Discussion

In this study we have established a reliable technique to genetically sex echidnas from small hair samples using PCR-based amplification of the male-specific gene Crspy [28]. Our results, which have been confirmed by chromosomal sexing, are consistent with previous studies that indicate that hair genomic DNA and DNA extracted from other tissues perform reliably [18, 25-27]. Furthermore, we obtained biological confirmations of the sex of three echidnas (number 1, 2 and 3) since they later had puggles or laid eggs (Table 1). There is scope to use this technique also in other contexts including study of sex ratios in wild populations where hair samples can be collected with hair traps, as it was successfully established for the American pika (Ochotona princeps) [25]. Adopting this approach may be particularly valuable in the study of the critically endangered long-beaked echidnas.

Interestingly, this study also revealed a striking bias in the sex ratio of these echidnas born in captivity. We observed that seven out of eight puggles were females (Table 1). Distortions in the sex ratios of captive mammals are not unusual and several examples have been described. Species such as the cotton-top tamarin (Saguinus Oedipus), the Persian onager (Equus hemionus onager), the ring-tailed lemur (Lemur catta), the sloth bear (Melursus ursinus) and the scimitar oryx (Oryx dammah) have shown male bias in captivity [9] whereas others like the pygmy hippopotamus (Hexaprotodon liberiensis) [11], the blue duiker (Cephalophus monticola) and the mangabey (Cercocebus albigena) [9] have presented female bias. Several factors have shown to affect sex ratio, for example, in the gray mouse lemur (Microcebus murinus) it was demonstrated that group size affected the sex ratio, if the mothers were kept in isolation their offspring was biased towards females, whereas if they were kept in groups they showed a male
bias instead [10]. Other studies have reported that in macaques with a matrilineal social structure the social status of the mothers influences the sex ratio of their offspring; high ranking females produce more daughters and lower ranks, produce more sons [34-36]. Also, in the pygmy hippopotamus, *Hexaprotodon liberiensis*, it was determined that high feeding intensity coupled with “hands-on” husbandry favored production of females [11].

A number of different hypotheses have been proposed in order to explain the observed sex ratio biases. In general, they all predict that manipulation of the sex ratio by an individual should lead to a greater offspring production (hence, increased fitness) through the favored sex [37]. This was recently shown to be true, demonstrating that sex ratio manipulation is quite common amongst species and that it represents a highly adaptive evolutionary strategy in mammals [37]. One of the first hypothesis was the sex allocation theory [38], which postulates that in species where the condition of the mother influences the sex of the offspring there should be a higher production of the evolutionarily less expensive sex (females) in poor quality mothers, which are females with suboptimal nutrition and stressed. Other hypotheses such as the Trivers-Willards hypothesis [39, 40] and the “cost of reproduction hypothesis” [41-44] also predict that mothers in good conditions will produce more sons and poor mothers will produce more females. The later suggests this is because mothers in poor conditions simply cannot afford to produce offspring of the energetically costly sex (males).

In the case of the captive-born echidnas studied here we observed a bias towards females that in the wild may be a sign of poor nutrition [12]. Stress is another factor that has been associated with female bias [40]. However, stress often results in inhibition of reproduction in several species [45-47] and the fact that these echidnas reproduced successfully suggests that stress levels were low. Therefore, it could be possible that
females are costlier than males amongst echidnas, hence the favorable conditions in the zoos may lead to a female bias [11]. More individuals over a longer period of time will reveal if this trend is indeed a sex bias or a result of the relatively small sample size [9, 48]. It would be necessary to monitor the sex ratio in this population in the future, since there have been cases in which populations showing strong sex ratio biases over a short period of time have lost it or decreased it over time [49-51]. In addition, looking at different variables such as diet composition (intake of fats versus carbohydrates) [52], could also shed light on the potential factors influencing sex ratios in monotremes.

Conclusions and future directions

In this study we successfully established several methods to determine the sex of eight echidnas born in captivity providing, to our knowledge, the first non-invasive genetic sex determination technique applied to echidna juveniles, which has the potential of being also applied to the study of wild echidnas. Future refinement will test other genes on sex chromosomes to provide a positive result in both males and females. Importantly we discovered a marked sex ratio bias in the studied group of echidnas born in captivity. Future work on more animals for extended periods of time will test our hypothesis that echidnas are vulnerable to a sex ratio bias during captive breeding.

Acknowledgements

Thanks to Prof. Steve Donnellan and Dr. Terry Bertozzi from the South Australian Museum for advice on the hair genomic DNA extraction protocol and for providing hair samples to test the protocol. DT-F is funded by the National Council of Science and Technology from Mexico (CONACYT). FG is an ARC research fellow. Thanks to Perth Zoo’s Veterinary staff for collection and shipment of samples.
Author contributions: DT-F performed sample processing, laboratory work and wrote the manuscript. WXK performed sample processing and laboratory work. AF contributed to the design of the project. BT collected samples and advised on echidna biology. ET-A and SLL performed laboratory work. FG contributed to the design and supervision of the project as well as to the manuscript preparation and data interpretation.
Fig. 1. Echidna juvenile 7-months old, individual B20282 (Photo: Perth Zoo).
Fig. 2. Echidna hair follicle. Samples of 10-50 follicles were used from eight different individuals.
Fig. 3. Sexing results for the eight analyzed echidnas. (A) Example of the PCR. On the left, the gel for the amplification of the positive control $\beta$-actin. A band of 600bp was obtained from male (♂) and female (♀) control gDNA as well as from the hair extracted gDNA of echidna number 6. On the right, the result of the sexing-PCR with Crspy primers. A product is obtained from the male control (♂) and no products are retrieved from the female control (♀) or from echidna 6, showing that echidna 6 is female. (B) PCR results for the sexing of the eight analyzed echidnas. On the left column, the results of $\beta$-actin amplification and on the right, the sexing-PCR results. Amplification of $\beta$–actin in all the individuals indicates that the lack of a Crspy band in females was due to the inexistence of this gene in those individuals and not to a failure in our PCR reactions. All the analyzed echidnas are females, except for echidna number 8. m: 100 bp marker; -: negative control.
**Fig. 4.** Sexing results through PCR using sex specific primers on genomic DNA from a male control (♂), a female control (♀), individuals 7 and 8 (7,8) and a negative control (-). *B-actin* primers were the positive control and *Crspy* primers were our sexing primers. (A) The gDNA of individuals 7 and 8 were extracted from blood samples. (B) The gDNA of individuals 7 and 8 were extracted from hair samples. m: 100 bp marker; -: negative control.
Fig. 5. FISH on metaphase spreads prepared from short-term culture of echidna peripheral blood lymphocytes. (A) FISH on metaphase spread from individual 7 with a red-labeled Y3X4 BAC which yielded 2 signals and a green-labeled Y3 specific BAC that yielded 0 signals. (B) FISH on metaphase spread from individual 8 with a green labeled Y3X4 BAC that produced 2 signals and a red-labeled Y3 specific BAC which produced 1 signal which co localized with one of the Y3X4 BAC positive chromosomes.
### Table 1 Information about the animals used in this study

<table>
<thead>
<tr>
<th>ID</th>
<th>Individual</th>
<th>Sex</th>
<th>Age when sampled</th>
<th>DOB</th>
<th>Sexing method</th>
<th>Fertile</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A80281</td>
<td>♀</td>
<td>6.5 months</td>
<td>6/8/08</td>
<td>PCR</td>
<td>Yes (Mother of B20282)</td>
</tr>
<tr>
<td>2</td>
<td>A80284</td>
<td>♀</td>
<td>6 months</td>
<td>20/8/08</td>
<td>PCR</td>
<td>Yes (Mother of B20300)</td>
</tr>
<tr>
<td>3</td>
<td>A70246</td>
<td>♀</td>
<td>18 months</td>
<td>25/7/07</td>
<td>PCR</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>A90273</td>
<td>♀</td>
<td>5 months</td>
<td>27/8/09</td>
<td>PCR</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>A90272</td>
<td>♀</td>
<td>5 months</td>
<td>24/8/09</td>
<td>PCR</td>
<td>NA</td>
</tr>
<tr>
<td>6</td>
<td>B10297</td>
<td>♀</td>
<td>10 months</td>
<td>12/9/11</td>
<td>PCR</td>
<td>NA</td>
</tr>
<tr>
<td>7</td>
<td>B20282</td>
<td>♀</td>
<td>19 months</td>
<td>17/8/12</td>
<td>PCR / FISH</td>
<td>NA</td>
</tr>
<tr>
<td>8</td>
<td>B20300</td>
<td>♂</td>
<td>19 months</td>
<td>27/8/12</td>
<td>PCR / FISH</td>
<td>NA</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not available.
References


# Statement of Authorship

**Title of Paper**
Identification of a Sox3 deletion in a captive-bred echidna

**Publication Status**
- Published
- Accepted for Publication
- Submitted for Publication
- Publication style

**Publication Details**

## Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate’s thesis.

<table>
<thead>
<tr>
<th>Name of Principal Author (Candidate)</th>
<th>Deboss Toledo-Flores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contribution to the Paper</td>
<td>Performed experiments, interpreted data and wrote manuscript.</td>
</tr>
<tr>
<td>Signature</td>
<td>Date 20-Dec-14</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of Co-Author</th>
<th>Frank Grützner</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contribution to the Paper</td>
<td>Supervised development of work, helped in data interpretation and manuscript preparation and evaluation.</td>
</tr>
<tr>
<td>Signature</td>
<td>Date 20-Dec-14</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of Co-Author</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Contribution to the Paper</td>
<td></td>
</tr>
<tr>
<td>Signature</td>
<td>Date</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of Co-Author</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Contribution to the Paper</td>
<td></td>
</tr>
<tr>
<td>Signature</td>
<td>Date</td>
</tr>
</tbody>
</table>
Letter in preparation:

**Deborah Toledo-Flores, Frank Grützner.** Identification of a Sox3 deletion in a captive-bred echidna.
Identification of a Sox3 deletion in a captive-bred echidna

Authors:

Deborah Fernanda Toledo-Flores and Frank Grützner

Affiliations:

The Robinson Research Institute, School of Molecular and Biomedical Science, The University of Adelaide, Adelaide, Australia

Abstract:

In mice and humans, Sox3 deletions have been associated with X-linked hypopituitarism. A deletion of the therian proto-Sry gene, Sox3, was identified in a captive born echidna at the Perth Zoo following genetic sexing from hair genomic DNA (Toledo-Flores et al, in preparation, chapter 5). Previous work indicates that in platypus, a male-specific Sox3 allele could potentially be involved in male sex-determination (Toledo-Flores and Grützner, in preparation, chapter 4). Work described in the first part of this chapter revealed a marked female bias in captive bred echidnas. In this section we describe the extraordinary finding that the only male identified in this population lacked the Sox3 gene. The discovery of this deletion in a male echidna provides a unique opportunity to investigate the role, if any, of Sox3 in male sexual development and fertility.

Key words: Sex determination, echidna, Sox3, male deletion, captivity, female bias.
Over many decades, researchers have been interested in the mechanisms of sex determination in monotremes. These unusual mammals have a rather peculiar sex chromosome system composed of multiple sex chromosomes that have been found to be homologous to sex chromosomes in birds and not in therian mammals [1-4]. So far the best male sex-determining candidate in monotremes is *Amhy* located on one of the Y chromosomes [5]. *Sox3* is also a very interesting gene, since the therian male sex determination gene *Sry* evolved from it [6-8]. *Sox3* is on the platypus chromosome 6, which represents the therian proto-sex chromosome [9-11]. The autosomal location of this gene excluded the existence of a *Sry* orthologue in the platypus and therefore, the relevance of *Sox3* in platypus sex determination has been overlooked.

It has been recently described that there is a male specific allele of *Sox3* in the platypus (Toledo-Flores and Grützner, in preparation, chapter 4). Furthermore, it was found that chromosome 6 in the platypus shows signs of divergence; it has a number of sex specific characteristics and it is non-randomly segregated during male meiosis (Toledo Flores *et al.*, in preparation, chapter 3). Therefore, it has been hypothesized that chromosome 6 might have commenced differentiation prior to monotreme therian divergence. This, together with the evidence of a male-specific *Sox3* in the platypus, might suggest that *Sox3* is involved in monotreme sex determination. However, due to the lack of the full *Sox3* sequence in both platypus and echidna, no male-specific alleles have yet been characterized in the later.

A recent study in captive born echidnas established a protocol to genetically sex echidna juveniles from hair-extracted genomic DNA samples (Toledo-Flores *et al.*, in preparation, chapter 5). This study found a striking sex ratio bias in these captive born animals, they observed that seven out of eight individuals were born genetically females and only one was genetically male. A PCR utilizing *Sox3* nested primers was performed
in two of the eight sexed echidna juveniles (echidna number seven and echidna number eight). Fig. 1A shows firstly the control PCR using β-actin primers for a male and a female echidna control genomic DNA as well as for echidna number seven and eight; secondly, it shows the sexing PCR using the male specific primers amplifying CrsPy (both control and sexing PCRs were taken from Toledo Flores et al, in preparation, chapter 5), which demonstrates that echidna seven is female and echidna eight is male; thirdly, it shows two sets of results for amplification with primers Sox3 – 1 and Sox3 – 2. The later demonstrates that this Sox3 region is depleted in echidna number eight, whereas it is present in the rest of the samples. Interestingly, this Sox3 region contains part of the HMG domain as well as the only two poly-alanine tracts present in the platypus Sox3 (Fig. 1B). Therefore, even though the rest of the Sox3 sequence was present in this individual, deletion of these functional domains could dramatically affect the activity of Sox3, if not completely abolish it.

The identification of this naturally occurring Sox3 deletion can thus provide a unique opportunity to study the impact that such a modification/deletion could have in the sex development and fertility of a male echidna. Identifying the parents of this animal and their genotype, in addition to following the development of this individual as well as measuring hormonal levels and investigating whether adjacent regions are also deleted could make a significant contribution to our understanding of monotreme sex determination and the role that Sox3 might play on it.
Figures

**Figure 1.** *Sox3* deletion in a male echidna. (A) PCR results for an autosomal positive control, β-actin; a male-specific gene, *CrspY*; and two sets of *Sox3*-specific primers, *Sox3* – 1 and *Sox3* – 2. ♂: control male genomic DNA; ♀: control female genomic DNA; 7: echidna number seven; 8: echidna number eight; -: negative control. (B) Structure of echidna/platypus *Sox3*. The gray area represents the extend of unknown sequence; the orange region, the sequence amplified by primers *Sox3* – 1 and the blue region, the sequence amplified by primers *Sox3* – 2. The HMG domain and the two polyalanine tracts are indicated by black lines labeled as HMG, 1st PolyA and 2nd PolyA, respectively.
References


CHAPTER 6: Significance and future directions

This chapter consists of a conventional significance and future directions section.

Chapter overview

In this chapter, I briefly summarize the main results of my projects and the significance these results have on the field.
Significance and future directions

Monotremes represent one of the three major mammalian lineages. With genomic data sets increasingly being accessible they have also become a valued species in comparative genomics. Work on their sex chromosome system has revolutionized our understanding of mammalian sex chromosome evolution. In addition they are the only mammalian species in which the therian sex chromosome is autosomal. Sex determination in monotremes has been a mystery and the work presented here provides novel insights into possible genes involved in sex determination as well as a new perspective into sex chromosome evolution in mammals. The mapping of Amhy onto a Y chromosome provides a key result that makes this gene a worthy candidate as a gene important for male sex determination in monotremes. This gives a new direction to the field of monotreme sex determination making of Amhy the most likely sex determining candidate gene in monotremes, potentially mimicking the function of male-specific copy of amh in the teleost fish Patagonian pejerrey. Future work should focus on finding functional evidence to support the involvement of Amhy in male sex determination. The biggest challenge in these studies is the lack of platypus embryonic material as well as the fact that genetic and hormonal manipulations in the platypus are not possible. Therefore, it would be necessary to find a suitable organism or in vitro system to model the effects of the platypus anti-Müllerian hormone.

Furthermore, by studying the therian proto-sex chromosome, chromosome 6, I discovered several lines of evidence that this is not an ordinary autosome. Firstly I found a unique case of non-random segregation of this autosome in platypus male meiosis. Secondly the extent in NOR heteromorphism is different in males and females and thirdly DNA demethylation has an effect on the heteromorphism only females. This might suggest that chromosome 6 is undergoing a process of differentiation and also

153
might show differential epigenetic changes in males and females. Future work may investigate the mechanisms underlying this segregation bias and identify further evidence of differentiation. Particularly interesting would be to see if recombination occurs along the whole length of chromosome 6.

One would predict that lack of recombination is responsible for my finding of a male-specific allele of Sox3 in the platypus. Since the observed variation consists of a two amino acid reduction in one of the two platypus Sox3 polyalanine tracts, this raises the possibility that such a change might confer a male-specific function that in turn could be involved in male sex determination or differentiation. Given the GC-rich nature of this gene and the fact that successful Sox3 amplification from platypus gDNA or cDNA is highly inconsistent, manipulating this gene and further investigating its potential to upregulate Sox9 activity in vitro has been technically challenging and the planned cloning and testing of the Sox3 deletions in a TESCO reporter gene assay where beyond the timeframe imposed by my candidature. In the future, it is necessary to obtain the full length of Sox3 in the platypus to test through reporter gene assay whether it can upregulate Sox9 in comparison to the female-occurring alleles. Alternatively, to mimic this change in the human or mouse Sox3 and to assess how this alters its transcriptional activity would also shed-light on the impact of this variation in the platypus.

Finally, I applied my research to assist zoos in determining the sex of young echidna puggles. Importantly, this technique has the potential to be used to study echidnas in the wild, including the critically endangered long-beaked echidna. This work revealed a strong female sex bias of echidnas born in captivity. This highlights the need to monitor sex rations in captively bred species as a possible indicator of the effects of husbandry on the breeding population. I found the first reported naturally occurring Sox3 deletion
in monotremes. This provides a unique opportunity to study the effects of such a deletion in a male echidna in vivo.

In conclusion, this work provided novel insights into sex chromosomes and sex determination in monotremes. It also highlights the need to further research into chromosome 6 as therian proto-sex chromosome. In addition to this basic research a useful approach to sex echidnas has been developed and applied revealing unmarked sex bias in a captive bred echidna population. This research provides more evidence for the extraordinary genetics found in monotremes, which nonetheless provides vital information about mammalian evolution. It also highlights that much is to be done to understand the biology of these fascinating creatures at the molecular level.
Amendments

Page 1, paragraph 2

Read:

In addition, this autosome features a large heteromorphic NOR and associates with the sex chromosomes during male meiosis (Casey and Daish personal communication).

Changed to:

In addition, this autosome features a large heteromorphic nucleolus organizer region (NOR) and associates with the sex chromosomes during male meiosis (Casey and Daish personal communication).

Page 71, figure 3

Modification:

Error bars and sample sizes (n) were added to figure 3.

Page 60, paragraph 1

Read:

We found that in females, both NORs are entirely covered by silver staining (Figure 3A). In males, we observed that one of the homologous NORs bears a gap of unstained chromatin surrounded by silver deposits over the rest of the NOR (Figure 3B).

Changed to:
We found that in females, both NORs are entirely covered by silver staining (Figure 4A). In males, we observed that one of the homologous NORs bears a gap of unstained chromatin surrounded by silver deposits over the rest of the NOR (Figure 4B).

**Page 75, figure 7**

Modification:
Average columns, error bars and sample sizes (n) were added to figure 7.

**Page 91, third paragraph**

Read:

*It is known that Sox3 is normally expressed in the developing central nervous system [4, 13-15] and together with Sox1 and Sox2 participates in the initiation and progression of neuronal differentiation [16].*

Changed to:

It is known that *Sox3* is normally expressed in the developing central nervous system in mouse [4, 13-15] and together with *Sox1* and *Sox2* participates in the initiation and progression of neuronal differentiation [16].

**Page 95, first paragraph**

Read:

*The primers used were SoxF-GCACAAACTCGGAGATCAGCAAG, SoxR-GGCAGGTACATGCTGATCATATC at 59ºC (expected size ~600bp) or Sox3F-CTACTCGCTGATGCCCGACC and Sox3R-GAGCTGGGCTCGACTTGAC at 59ºC*
(expected size ~300bp); β–actin-F-GCCCATCTACGAAGGTTACGC and β–actin-
AAGGTCGTTCGTGGATACCAC at 55ºC. A 1.5% agarose gel was used to visualize
the products.

Changed to:
The primers used were SoxF-GCACAACTCGGAGATCAGCAAG, SoxR-
GGCAGGTACATGCTGATCATATC at 59ºC (expected size ~600bp) or the gene-
specific Sox3F-CTACTCGCTGATGCCCAGACC and Sox3R-
GAGCTGGGCTCCGACTTGAC at 59ºC (expected size ~300bp), designed based on
ENSOANG00000002448; β–actin-F-GCCCATCTACGAAGGTTACGC and β–actin-
AAGGTCGTTCGTGGATACCAC at 55ºC. A 1.5% agarose gel was used to visualize
the products.

Page 99, last paragraph

Read:

However, these different attempts failed due to the inconsistency of the Sox3 PCR,
which is likely a result of the high GC content of this gene (Fig. 6). However a number
of Sox3 fragments were amplified from different individuals.

Changed to:

However, these different attempts failed due to the inconsistency of the Sox3 PCR,
which is likely a result of the high GC content of this gene (Fig. 6). Importantly, β–actin
was used as a control to ensure that the genomic DNA was suitable for PCR
amplification. However a number of Sox3 fragments were amplified from different
individuals.
Specifically this variant was defined by a reduced second polyalanine tract.

Specifically this variant was defined by a reduced second polyalanine tract (fourth tract in conserved terms).

Interestingly, we found that the seven-alanine Sox3 was found exclusively in males and not in females; five females were sequenced and all of them carried a nine-alanine Sox3 variant whereas four out of five males carried the seven-alanine Sox3 (Fig. 7).

Interestingly, we found that the seven-alanine Sox3 was found exclusively in males and not in females; five females were sequenced and all of them carried a nine-alanine Sox3 variant whereas four out of five males carried the seven-alanine Sox3 and one of them carried a five-alanine tract (Fig. 7).

The following sentence was added to the end of Fig. 5 legend: “White arrow indicates Sox3 expected size.”
Legend read:

**Figure 7.** A Sox3 male-specific allele was identified in the platypus. A fragment of Sox3 was amplified in five different females and five different males. A Sox3 coding for seven alanines on its 2\textsuperscript{nd} polyalanine tract was detected only in males and not in females.

Legend changed to:

**Figure 7.** A Sox3 male-specific allele was identified in the platypus. A fragment of Sox3 was amplified in five different females and five different males. A Sox3 coding for seven alanines on its 2\textsuperscript{nd} polyalanine tract (4\textsuperscript{th} in conserved terms) was detected only in males and not in females.

Page 127, first paragraph

Read:

The sex of the individuals was then determined through PCR using primers that amplify a platypus male-specific gene, Crspy. Crspy, the male specific copy of the Crsp70 gene that is located on platypus chromosome Y\textsubscript{5} [28], which has been shown to be homologous to the echidna male specific chromosome Y\textsubscript{3} [31]. These primers were tested in echidnas whose sex was known, confirming that the platypus Crspy primers amplify a male specific product and no products in females (Fig. 3). Running this PCR on the DNA of eight echidna puggles showed that seven out of the eight captive-born echidnas were females (Table 1).

Changed to:

The sex of the individuals was then determined through PCR using primers that amplify a platypus male-specific gene, Crspy. Crspy, the male specific copy of the Crsp70 gene
that is located on platypus chromosome Y₅ [28], which has been shown to be homologous to the echidna male specific chromosome Y₃ [31]. These primers were tested in echidnas whose sex was known, confirming that the platypus Crspy primers amplify a male specific product and no products in females (Fig. 3). Running this PCR on the DNA of eight echidna puggles showed that seven out of the eight captive-born echidnas were females (Table 1). Importantly, β–actin primers were used as a positive control.

Page 129, second paragraph

Read:

*In general, they all predict that manipulation of the sex ratio by an individual should lead to a greater offspring production through the favored sex, increasing in this way its fitness* [37].

Changed to:

In general, they all predict that manipulation of the sex ratio by an individual should lead to a greater offspring production (hence, increased fitness) through the favored sex [37].

Page 153, first paragraph

Read:

*This gives a new direction to the field of monotreme sex determination. Future work should focus on finding functional evidence to support the involvement of Amhy in male sex determination.*
Changed to:

This gives a new direction to the field of monotreme sex determination making of *Amhy* the most likely sex determining candidate gene in monotremes, potentially mimicking the function of male-specific copy of amh in the teleost fish Patagonian pejerrey. Future work should focus on finding functional evidence to support the involvement of *Amhy* in male sex determination.

Page 154, second paragraph

Read:

*Given the GC-rich nature of this gene and the fact that successful Sox3 amplification from platypus gDNA or cDNA is highly inconsistent, manipulating this gene and further investigating its potential to upregulate Sox9 activity in vitro has been technically challenging and the planned cloning and testing of the Sox3 deletions in a TESCO reporter gene assay where beyond the timeframe imposed by my candidature.*

Changed to:

Given the GC-rich nature of this gene and the fact that successful *Sox3* amplification from platypus gDNA or cDNA is highly inconsistent, manipulating this gene and further investigating its potential to upregulate *Sox9* activity *in vitro* has been technically challenging and the planned cloning and testing of the *Sox3* deletions in a TESCO reporter gene assay where beyond the timeframe imposed by my candidature.