Splitting the sexes

The birth and senescence of sex chromosomes

Cirulis, Aivars

2022

Document Version:
Publisher's PDF, also known as Version of record

Splitting the sexes

The birth and senescence of sex chromosomes

AIVARS CĪRULIS
DEPARTMENT OF BIOLOGY | FACULTY OF SCIENCE | LUND UNIVERSITY

List of papers

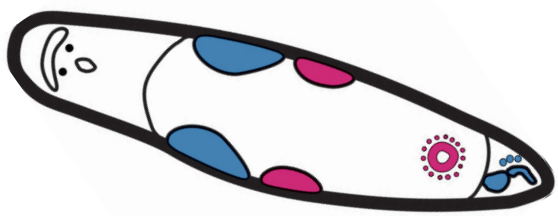
I. **Cirulis, A.,** Nordén, A. K., Churcher, A., Ramm, S., Zadesenets, K., Abbott, J. K. Effects of sex-limited experimental evolution on a hermaphrodite transcriptome. *Manuscript*

II. **Cirulis, A.,** Ramnath, V., Abbott, J. K. Effects of sex-limited experimental evolution on a hermaphrodite genome. *Manuscript*

III. **Cirulis, A.,** Qinyang, L., Pranter, R., Abbott, J. K. Effects of sex-limited experimental evolution on hermaphrodite sexual anatomy. *Manuscript*

IV. **Cirulis, A.,** Majvall, M., Abbott, J. K. Effects of sex-limited experimental evolution on mating behaviour in a hermaphrodite. *Submitted*

V. **Cirulis, A.,** Hansson, B., Abbott, J. K. Effects of degenerated sex-limited chromosomes on non-reproductive traits. *Submitted*



Printed by Media-Tryck, Lund 2022 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

Faculty of Science
Department of Biology

ISBN 978-91-8039-201-3

Splitting the sexes

The birth and senescence of sex chromosomes

Aivars Cīrulis

LUND
UNIVERSITY

DOCTORAL DISSERTATION

by due permission of the Faculty of Science, Lund University, Sweden.
To be defended in the Blue Hall, Ecology Building, Sölvegatan 37, Lund, Sweden
on the 29th of April, 2022 at 13.

Faculty opponent
Dr. Alison Wright

University of Sheffield

Organization	Document name
LUND UNIVERSITY	**DOCTORAL DISSERTATION**
Department of Biology	**Date of issue:** 2022-03-21
Author: Aivars Cīrulis	Sponsoring organization

Title and subtitle

Splitting the sexes: The birth and senescence of sex chromosomes

Abstract

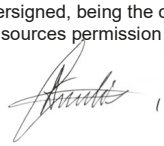
The evolution of gonochorism from hermaphroditism can be gradual by increasing investment in one sex role while decreasing in the other, or rapid through the fixation of sex-role sterility mutations, eventually leading to the evolution of sex chromosomes. It is expected that the transition will involve a temporary state of gynodioecy or androdioecy as the mutations are not expected to take place at the same time. If the first mutation is a dominant female-sterility mutation, later accompanied by a recessive male sterility mutation, then an XY sex chromosome system evolves, while the opposite combination of mutations will result in a ZW system. Later on sexually antagonistic (SA) genes can be linked to the newly established sex-determining regions on the sex chromosomes. This is followed by recombination arrest in the region, so that the inheritance pattern is sex-limited for all these sex-specific genes. However, the lack of recombination leads to degeneration of the genetic content on the sex-limited chromosomes, since recombination is important for repairing mutations. Nevertheless, recombination arrest does not necessarily mean a dead-end for the sex-limited chromosomes. As our understanding of the very early stages of sex chromosome evolution is mainly based on theory and comparative evidence, we developed a system which we hoped would make it possible to observe in real time what happens after the acquisition of a new sex-determining gene. We used a previously established green fluorescent protein (GFP) line of the simultaneous hermaphrodite *Macrostomum lignano*. We used the GFP locus as a dominant sterility mutation, which is inherited in a Mendelian fashion. By allowing the GFP allele to be inherited only through sperm, we created male-limited selection lines (resembling the early stages in XY chromosome evolution), and by allowing the GFP allele to be inherited only through egg cells, we created female-limited selection lines (resembling the early stages in ZW chromosome evolution). We also created control lines, where the inheritance pattern was equally mixed. After tens of generations, we investigated how these lines have responded on the level of the genome, the transcriptome, and the phenotype. We sequenced genomes and analysed changes in SNP frequency and structural variant (SV) distribution in pairwise comparisons to see changes across the genome, but particularly on the scaffold where the GFP is located. We also sequenced transcriptomes and performed pairwise comparisons to detect differentially expressed genes, and analysed significant GO terms and KEGG pathways to see how the gene regulation has changed. Besides genomic analyses, we also looked at how mating behaviour (copulation frequency and duration, as well as probability of post-copulatory sucking behaviour) and sexual anatomy (gonad size and morphology of the male copulatory organ called stylet) has changed.

We observed that the female-selected lines seemed to have responded the most at the genomic level. For example, the number of significantly differentially expressed transcripts was largest between the female-selected lines and the control lines. These changes seemed to involve downregulation of testes-biased genes. In addition, we observed the highest number of SVs in the female-selected lines, which could be related to changes in recombination rate. In contrast, the male-selected lines seemed to have responded the most at the phenotypic level, since we observed a decrease in the ovary size and body size in the male-selected lines, as well as behavioural changes that may be related to changes in the ejaculate. Both sex-specific selection regimes showed evidence of alterations in the shape of the stylet. Based on these results, we can conclude that our worms have indeed responded to the sex-limited selection in a way that is generally consistent with our expectations from other young sex chromosome systems. The evidence of a decrease in the testes function in the female-selected lines resembles adaptation towards gynodioecy, and the evidence of a decrease in the ovary size in the male-selected lines resembles adaptation towards androdioecy.

Key words: Sex chromosome, Sexual conflict, *Macrostomum lignano*, Experimental evolution, Genomics of sex

Classification system and/or index terms (if any)

Supplementary bibliographical information		**Language:** English
ISSN and key title		**ISBN** Print: 978-91-8039-201-3 PDF: 978-91-8039-202-0
Recipient's notes	**Number of pages:** 226	Price
	Security classification	

Signature  Date 2022-03-21

Splitting the sexes

The birth and senescence of sex chromosomes

Aivars Cīrulis

LUND
UNIVERSITY

Cover illustration by Nauris Cinovics and Aivars Cīrulis

Faculty of Science
Department of Biology

ISBN 978-91-8039-201-3 (print)
ISSN 978-91-8039-202-0 (PDF)

Printed in Sweden by Media-Tryck, Lund University
Lund 2022

"Whereas many people just accept the ready-made answers provided by the powers that be, spiritual seekers are not so easily satisfied. They are determined to follow the big question wherever it leads, and not just to places you know well or wish to visit."

Prof. Yuval Noah Harari, Homo Deus

Table of Contents

List of papers **9**

Author contributions **11**

Abstract **13**

Popular science summary **15**

Introduction and background **17**

Why sex? 17

Hermaphroditism and the evolution of separate sexes 18

Sex determination 21

Sexual selection and sperm competition 27

Sexual selection and sperm competition in hermaphrodites 28

Sexual conflict and the evolution of sex differences 29

Sexual conflict in hermaphrodites 30

Escaping intralocus sexual conflict 31

Aims of the thesis **33**

General materials and methods **35**

Macrostomum lignano 35

Genome 35

Reproduction 36

Worm cultures 39

RNA extraction and sequencing 41

DNA extraction and sequencing 42

Mating assay 43

Results and discussion **45**

Paper I. Effects of sex-limited experimental evolution on a hermaphrodite transcriptome 45

Paper II. Effects of sex-limited experimental evolution on a hermaphrodite genome 47

Paper III. Effects of sex-limited experimental evolution on hermaphrodite sexual anatomy 49

Paper IV. Effects of sex-limited experimental evolution on mating behaviour in a hermaphrodite....51
Paper V. Effects of degenerated sex-limited chromosomes on non-reproductive traits....52
Overall patterns in the evolution of gonochorism and sex chromosomes....53
Origin of gonochorism leads to sexual specialization....53
Accumulation of sexually antagonistic loci....55
Changes in recombination rate around the sex-determining region....55
Y – an evolutionary dead end?....56
Conclusions and future perspectives....57
References....59
Acknowledgments....69

List of papers

I. **Cirulis, A.,** Nordén, A. K., Churcher, A., Ramm, S., Zadesenets, K., Abbott, J. K. Effects of sex-limited experimental evolution on a hermaphrodite transcriptome. *Manuscript*

II. **Cirulis, A.,** Ramnath, V., Abbott, J. K. Effects of sex-limited experimental evolution on a hermaphrodite genome. *Manuscript*

III. **Cirulis, A.,** Qinyang, L., Pranter, R., Abbott, J. K. Effects of sex-limited experimental evolution on hermaphrodite sexual anatomy. *Manuscript*

IV. **Cirulis, A.,** Majvall, M., Abbott, J. K. Effects of sex-limited experimental evolution on mating behaviour in a hermaphrodite. *Submitted*

V. **Cirulis, A.,** Hansson, B., Abbott, J. K. Effects of degenerated sex-limited chromosomes on non-reproductive traits. *Submitted*

Author contributions

I. JKA and SAR conceived the study and AKN designed and performed the extractions with input from JKA. KZ performed the karyotyping. AMC performed the bioinformatics with input from **AC** and JKA. **AC** and JKA performed additional statistics. **AC** and AKN wrote the manuscript with input from all the authors.

II. JKA conceived the study and **AC** designed and performed the extractions with input from JKA. VR performed the bioinformatics and statistics with input from **AC** and JKA. **AC** wrote the manuscript with input from all the authors.

III. JKA conceived the study and **AC** designed and performed the laboratory work with input from QL. **AC** analysed the data with input from RP. **AC** and JKA performed the statistics. **AC** wrote the manuscript with input from all the authors.

IV. JKA conceived the study and **AC** designed and performed the laboratory work. **AC** and MM analysed the video data. **AC** and JKA performed the statistics. **AC** wrote the manuscript with input from both authors.

V. **AC** conceived and led the study. **AC**, JKA and BH wrote the manuscript.

Authors: Aivars Cīrulis (AC), Jessica K. Abbott (JKA), Bengt Hansson (BH), Anna K. Nordén (AKN), Allison M. Churcher (AMC), Steven A. Ramm (SAR), Vignesh Ramnath (VR), Kira Zadesenets (KZ), Martin Majvall (MM), Qinyang Li (QL), Robin Pranter (RP).

Abstract

The evolution of gonochorism from hermaphroditism can be gradual by increasing investment in one sex role while decreasing in the other, or rapid through the fixation of sex-role sterility mutations, eventually leading to the evolution of sex chromosomes. It is expected that the transition will involve a temporary state of gynodioecy or androdioecy as the mutations are not expected to take place at the same time. If the first mutation is a dominant female-sterility mutation, later accompanied by a recessive male sterility mutation, then an XY sex chromosome system evolves, while the opposite combination of mutations will result in a ZW system. Later on sexually antagonistic (SA) genes can be linked to the newly established sex-determining regions on the sex chromosomes. This is followed by recombination arrest in the region, so that the inheritance pattern is sex-limited for all these sex-specific genes. However, the lack of recombination leads to degeneration of the genetic content on the sex-limited chromosomes, since recombination is important for repairing mutations. Nevertheless, recombination arrest does not necessarily mean a dead-end for the sex-limited chromosomes. As our understanding of the very early stages of sex chromosome evolution is mainly based on theory and comparative evidence, we developed a system which we hoped would make it possible to observe in real time what happens after the acquisition of a new sex-determining gene. We used a previously established green fluorescent protein (GFP) line of the simultaneous hermaphrodite *Macrostomum lignano*. We used the GFP locus as a dominant sterility mutation, which is inherited in a Mendelian fashion. By allowing the GFP allele to be inherited only through sperm, we created male-limited selection lines (resembling the early stages in XY chromosome evolution), and by allowing the GFP allele to be inherited only through egg cells, we created female-limited selection lines (resembling the early stages in ZW chromosome evolution). We also created control lines, where the inheritance pattern was equally mixed. After tens of generations, we investigated how these lines have responded on the level of the genome, the transcriptome, and the phenotype. We sequenced genomes and analysed changes in SNP frequency and structural variant (SV) distribution in pairwise comparisons to see changes across the genome, but particularly on the scaffold where the GFP is located. We also sequenced transcriptomes and performed pairwise comparisons to detect differentially expressed genes, and analysed significant GO terms and KEGG pathways to see how the gene regulation has changed. Besides genomic analyses, we also looked at how mating behaviour (copulation frequency and duration, as well

as probability of post-copulatory sucking behaviour) and sexual anatomy (gonad size and morphology of the male copulatory organ called stylet) has changed.

We observed that the female-selected lines seemed to have responded the most at the genomic level. For example, the number of significantly differentially expressed transcripts was largest between the female-selected lines and the control lines. These changes seemed to involve downregulation of testes-biased genes. In addition, we observed the highest number of SVs in the female-selected lines, which could be related to changes in recombination rate. In contrast, the male-selected lines seemed to have responded the most at the phenotypic level, since we observed a decrease in the ovary size and body size in the male-selected lines, as well as behavioural changes that may be related to changes in the ejaculate. Both sex-specific selection regimes showed evidence of alterations in the shape of the stylet. Based on these results, we can conclude that our worms have indeed responded to the sex-limited selection in a way that is generally consistent with our expectations from other young sex chromosome systems. The evidence of a decrease in the testes function in the female-selected lines resembles adaptation towards gynodioecy, and the evidence of a decrease in the ovary size in the male-selected lines resembles adaptation towards androdioecy.

Popular science summary

In evolutionary biology sexes can be seen as reproductive strategies. If only one of the strategies is present in a single individual, this results in two separate sexes, referred to as males and females. If both strategies are combined into a single individual, then this individual is called a hermaphrodite. The male and female reproductive strategies spur from the size and motility of the sex cells they represent, where females create large and immobile egg cells, while males compete to fertilize eggs through the production of small and motile, but numerous sperm.

We are interested in how two separate sexes can evolve from a hermaphrodite ancestor. Two main mechanisms have been suggested. The first is that the evolution of two sexes results gradually when some individuals in the hermaphrodite ancestor start to lose the characteristics from one sex, and instead specialize in the other sex. The second mechanism can be more rapid, if a sex determining gene evolves that determines male or female phenotype. Sex chromosomes are the main mechanism by which two sexes are determined (in species with genetic sex determination). In humans, the *SRY* gene located on the Y chromosome determines male sex (XY) and the lack of it determines female sex (XX). However, in other species sex can be determined in other ways. For example, in birds sex is determined by a female-specific chromosome called W, where females are (ZW) and males are (ZZ). Although scientists have developed theories to explain the evolution of sex chromosomes, however as the best-studied sex chromosomes are usually millions of years old, we cannot directly study how they actually evolved in the first place.

To contribute to the understanding of the evolution of separate sexes via sex chromosomes, we started an experimental evolution experiment to study this process in real time. For that we used a transgenic line of a hermaphroditic worm *Macrostomum lignano*, which has a green fluorescent protein (GFP) gene inserted in the genome. We then used this GFP as a sex-determining gene, meaning that in the experimental male populations, it is inherited only through sperm (like the Y chromosome in humans. For the experimental female populations, we allow the GFP to be inherited only through the egg cells (like the W chromosome in birds). We can do this by mating one GFP worm with regular worms, because under the near-UV light the GFP worms glow green and we can distinguish them from the normal worms. After the mating we separate the worms to let them lay eggs to produce the next generation. In the experimental female populations, we keep only the GFP worms to lay eggs so that their offspring have inherited the GFP gene

through egg cells. In the experimental male populations, we take the regular worms for egg laying, so that the GFP offspring inherited the gene through sperm. After many generations of selection, we expect that experimental male worm populations will become better at being male, and lose their female function since it is not needed anymore, and vice versa for the experimental female populations. Moreover, as there are male and female-specific genes determining male and female sex organs around the genome, these genes are expected to relocate on the chromosome where the sex-determining gene is located, since in this way those genes can be inherited together creating male (Y) and female (W) specific chromosomes over the course of millions of years of their evolution.

After tens of generations we indeed observed that the male-selected populations became more specialized in the male role, and vice versa with the female-selected populations. We observed that the male-selected lines started to invest more in mating and reduced their ovary size, while the female-selected lines reduced investment in the testes and mated less. We saw many differences in the genome, where many genes have changed either in their sequence or their expression level. More importantly, we saw differences close to the GFP gene, consistent with predictions about sex chromosome evolution. In the thesis we discuss different evolutionary mechanisms which could explain these changes in more detail, but our most important conclusion is that it is possible to evolve sex differences in lab animals. Understanding sex differences is not only fundamentally interesting, but can also explain the existence of many medical conditions with different prevalence or severity between males and females, and in the long term, can lead to the development of more effective treatments for these ailments.

Introduction and background

Why sex?

There are two leading definitions of sex, where one defines sex as any exchange of genetic material (including, for example, horizontal gene transfer) between two or more individuals, while the other is defined by the presence of meiosis (also known as meiotic sex) (Beukeboom & Perrin, 2014). Meiosis is a process of halving the chromosome number, which also includes recombination, so that diploid cells produce different haploid gametes, which later will form a zygote (Sherratt & Wilkinson, 2009). The first definition includes prokaryotic "sex", but, as we study eukaryotes, I will use the word sex to refer to meiotic sex hereafter.

Scientists believe that sex has originated only once 1.5 billion years ago in basal eukaryotes. And it seems to be a highly adaptive strategy as asexual lineages seem to be evolutionarily short-lived (Beukeboom & Perrin, 2014). This is despite the fact that sex seems to be very costly. Beukeboom and Perrin (2014) have listed many examples. First, there are physiological costs of meiosis, as it is a slower process than mitosis. Secondly, there can be a recombination load (breakdown of beneficial gene combinations). Thirdly, there are many mating costs such as mate searching and competition, sexually transmitted diseases, predation risks etc. Finally, there is the two-fold cost of sex, where there is a cost to producing separate sexes (especially males). This cost stems from the fact that two parents are needed for offspring production instead of just one, even though males often provide very little in the way of resources to the developing offspring. So why has meiotic sex evolved and stayed as an evolutionarily stable strategy despite these pitfalls and the fact that all else being equal, an asexual mutation should take over a population (Sherratt & Wilkinson, 2009)?

Firstly, Beukeboom and Perrin (2014) argue that sex evolved in an isogamous and automictic ancestor, in which case there was basically no cost to evolving sex, as gamete production and fusion in automictic species takes place within the same individual. Secondly, based on the empirical evidence that sexual reproduction is common, there must be solid evolutionary benefits for having sex.

Indeed, there are two major benefits of having sex, which have experimental support. Traditionally scientists have believed that sexual reproduction has evolved to purge deleterious mutations and bring together beneficial ones (Sherratt & Wilkinson, 2009). Recombination creates new genotypes on which selection can

act, thus facilitating creation of high-fitness genotypes (Aggarwal *et al.*, 2015). However, relatively recently it has been shown that this benefit may not have been the main source of selection originally favouring the evolution of sex. There is a growing body of evidence which suggests that DNA repair is the main reason for the evolution of meiotic sex (Mirzaghaderi & Hörandl, 2016). In different organisms, there can also be other benefits of sex – including protection from selfish genetic elements and parasites, rejuvenation (resetting the epigenome and maintaining ploidy throughout generations, restoring cell size and telomere length after asexual reproduction), as well as stress resistance through formation of seeds and spores (Sherratt & Wilkinson, 2009; Beukeboom & Perrin, 2014).

Hermaphroditism and the evolution of separate sexes

After the evolution of meiotic sex, the evolution of syngamy (fusion of two cells) paved the way for the evolution of sex differences through alterations between ploidy levels. Mating types can be considered the first "sex" difference, where isogamic cells can fuse only between different mating types (Rhen & Crews, 2007). Later the evolution of anisogamy (i.e. different-sized gametes) independently and repeatedly has taken place under disruptive selection on gamete size and number, apparently through incorporation of gamete size genes into the mating-type locus (Bachtrog et al., 2014; Charlesworth & Charlesworth, 2010). It has been shown that both partners can increase fitness if one produces large and sessile (female) gametes, increasing zygote survival by means of provisioning, and the other produces small and mobile (male) gametes. Meanwhile, intermediates cannot compete in terms of mobility and abundance with the small male gametes, or in terms of provisioning with the large female gametes (Bachtrog et al., 2014; Beukeboom & Perrin, 2014; Parker, 2014). That is the reason why anisogamy creates two reproductive strategies - male and female. These strategies are distributed in nature in varied ways - simultaneous and sequential hermaphroditism, androdioecy (males and hermaphrodites), gynodioecy (females and hermaphrodites) and two separate sexes (gonochorism or dioecy or heterothallism), as well as trioecy (females, males and hermaphrodites) (Avise, 2011; Bachtrog et al., 2014; Beukeboom & Perrin, 2014; Schärer & Ramm, 2016). Competition between gametes (haploid selection) could drive transitions between hermaphroditism and gonochorism (Scott *et al.*, 2018).

Hermaphroditism is the ancestral state in plants, while gonochorism is the ancestral state in animals (Tanurdzic & Banks, 2004; Avise, 2011; Muyle *et al.*, 2021), however multiple independent transitions from hermaphroditism to gonochorism have taken place. The knockout of male or female function could arise gradually by decreasing investment in one sex role, or by the fixation of at least two separate mutations, where one causes male-sterility (producing females) and the other female-sterility (producing males). However, it is unlikely that both mutations will arise simultaneously; therefore, an intermediate androdioecious or gynodioecious step is usually expected. Gynodioecy is more common, presumably

because it reduces the risk of inbreeding. Specialization in the female sex role may also be more favourable because female function is usually resource-limited to a greater extent than male function, due to larger investment in egg cell production and offspring care (Bachtrog *et al.*, 2014). The most common mutation becoming fixed is a recessive male-sterility mutation. In this case, a later-occurring dominant female-sterile mutation will drive the evolution of an XY system. In contrast, if the initial mutation is a dominant male-sterility mutation, then a ZW system should evolve, by later acquiring a recessive female-sterile mutation. These mutations can only spread if they increase fitness in the sex-limited function by at least twofold. Because sexual selection is often stronger in males than in females, it is therefore thought that female-sterility mutations should fix more easily than male-sterility mutations, meaning that the XY system should arise more easily than the ZW (Ming *et al.*, 2011; Beukeboom & Perrin, 2014). Most of the research studying the evolution of two separate sexes through the evolution of sex chromosomes has been done on plants (Avise, 2011). Thus, we are the first ones to apply an experimental evolution approach in a hermaphroditic animal in an attempt to determine if we can detect an evolutionary response consistent with current theory on sex chromosome evolution. The initial steps of sex chromosome evolution are difficult to study, as many new sex-chromosome systems are already several million years old (Charlesworth, 2019; Martin *et al.*, 2019; Veltsos *et al.*, 2019; Rafati *et al.*, 2020).

So far, I have only considered diploid systems. But separate sexes can evolve in haploid organisms as well, where sex evolves from mating types, where the mating-type locus first evolves into a sex-determining locus containing genes coding for anisogamy. The sex-determining locus then expands and evolves into U and V sex chromosomes. Later XY or ZW system can evolve through temporary epigenetic sex determination (Beukeboom & Perrin, 2014).

So, what are the pros and cons of evolving separate sexes? There are two main hypotheses. The first is the trade-off hypothesis (Bachtrog *et al.*, 2014), where it is best to invest more in the male function because it is less costly and one can obtain more progeny from multiple mates compared to being fertilized alone (Bateman, 1948; Leonard, 1993). This could lead to a conflict between the sex roles within an individual, if investment in one sex role leads to decreased fitness in the other, or if maintaining two reproductive strategies is particularly costly (Heath, 1977). In such cases, a reproductive advantage could be achieved by specializing in one or the other sex role, thus leading to the evolution of separate sexes. Hermaphroditism becomes unstable at high mating rates as higher investment in male role to increase one's fitness drives even higher levels of sperm competition (i.e. when sperm from different males compete to fertilize the same egg). This leads to a scenario where individuals who invest exclusively in the male role (pure males) will outcompete others with a lower investment in the male role, thus selecting for investment in the female role among its hermaphroditic mates, thus leading to the evolution of gonochorism (Santi *et al.*, 2018). The second hypothesis is that the evolution of separate sexes alleviates inbreeding, as it is possible to escape the negative effects

of deleterious recessive alleles (Bachtrog *et al.*, 2014). This is unlikely to be the only explanation, however, since selfing (inbreeding) can also expose these mutations to natural selection, thus enhancing purging from a population (Noël *et al.*, 2019).

The fact that not all species have separate sexes - indeed most sexually reproducing organisms are hermaphrodites (Schärer, Janicke, & Ramm, 2015) - shows that there must be advantages to hermaphroditism as well, since the total fitness gained by a hermaphrodite must be larger than for a male or a female in order for hermaphroditism to be evolutionarily stable (Michiels, 1998). The main expected advantage of simultaneous hermaphroditism is reproductive assurance. This is clearly seen in the distribution of hermaphroditism in nature. Hermaphroditism is extremely common in plants (94% of flowering plants (Renner & Ricklefs, 1995)), it is also present in fungi (Nieuwenhuis, 2012; Cirulis, 2016) and mainly in immobile or low mobility animals (Ascidiaceae, Bryozoa, Entoprocta and Porifera). This lends support to the conclusion that it is best to possess both sex functions if the chances of meeting a partner are low (Weeks *et al.*, 2006; Bachtrog *et al.*, 2014; Beukeboom & Perrin, 2014). This applies to species with low population densities or parasitic lifestyles (Schärer, 2009; West, 2009). Moreover, it has been shown that selfing can limit accumulation of deleterious mutations especially if sexual selection is low (Noël *et al.*, 2019). Simultaneous hermaphrodites also do not suffer from the two-fold cost of sex. The main advantage of sequential hermaphroditism is thought to be that male or female function can be expressed at specific ages depending on environmental or social stimuli, when reproductive value could be increased via sex change. Thus it is common in species with indeterminate growth (fishes, invertebrates and plants) (West, 2009). In many sequentially hermaphroditic species, females are larger than males. In such cases, it is expected that success as a male should be higher when young, and success as a female should be higher at older ages, as larger females can produce more eggs (this pattern is mainly seen in invertebrates). The opposite pattern can occur if there is much sperm competition present, if the benefit of egg-bearing is decreasing over time, or when larger males are able to defend a nesting site, and monopolize more females (this pattern is mainly seen in fishes) (West, 2009). The outcome depends on which sex benefits more, however it is expected that protogynous (females first) mating systems create a larger sex ratio bias (West, 2009) and thus could be less evolutionarily stable. Note that simultaneous hermaphrodites can adjust their investment in both sex roles (sex allocation) according to social and environmental cues (Santi *et al.*, 2018; Ramm *et al.*, 2019), thus not wasting too much resources without a need in male function and instead allocating to the female function, if needed (Heath, 1977) and vice versa.

Sex determination

One might think that sex determination should be a highly conserved process; however, sex determination can be very plastic, as the mechanisms of sex determination are very diverse depending on different genetic and environmental factors that can vary even within the same species (Bachtrog *et al.*, 2014; Beukeboom & Perrin, 2014; Natri *et al.*, 2019)!

The first sex determining system I will describe is genetic sex determination (GSD) through male (XY) or female (ZW) heterogamety. It is the most common system in animals and plants, with XY being more common than ZW (Saunders *et al.*, 2018). As you probably already know, in our own species sex is genetically determined by the presence or absence of the Y chromosome, where the sex-determining gene (SRY) is located. This means that the genotype of the sperm determines the sex of the offspring, resulting in XY males or XX females. That is how sex is determined in mammals, beetles, several flies, reptiles, fishes, frogs and plants (Bachtrog et al., 2014; Charlesworth & Mank, 2010; Ming et al., 2011). Interestingly, the Y chromosome has been lost in many species with an XY system, resulting in XO males. In fact, the very first sex chromosome to be discovered was chromosome X in an XO system (*Pyrrhocoris apterus*) back in 1891 (Beukeboom & Perrin, 2014). While in other species multiple X and Y chromosomes are present (Gruetzner *et al.*, 2006; Ming *et al.*, 2011).

Sex is determined in a similar way in the ZW system, where the heterogametic sex is female with Z and W chromosomes, whereas males are ZZ. ZW systems are found in birds, Lepidoptera, Trichoptera or caddis flies, crustaceans, parasitic flatworm *Schistosoma mansoni*, many reptiles, some amphibians, fishes and plants (Bachtrog et al., 2014; Charlesworth & Mank, 2010; Ellegren, 2011; Parsch & Ellegren, 2013). Thus, the egg cell genotype determines the sex of the offspring. Similarly, to the XY system, the W chromosome may also be lost in ZW systems, which results in ZO females. (Pennell et al., 2015), and due to autosome fusion or translocation, resulting in neo-sex chromosome evolution, multiple sex chromosomes can also be observed (Ellegren, 2011). Furthermore, both heterogametic systems can coexist within the same species on rare occasions, observed in fish, frogs, houseflies, and midges (Scott *et al.*, 2018). But although they are the most common, XY and ZW systems are not the only possibilities – there are many other different types of sex determination.

Besides heterogamety, genetic sex determination known as UV sex chromosome systems are found in haploid organisms (some mosses, liverworts, algae and fungi). There are also other ways to genetically determine sex. One example is polygenetic systems, where many independent genes have additive effects in determining sex (common in fishes and flowering plants). Sex can even be determined by the whole genome – for example via haplodiploidy (e.g. Hymenoptera) or paternal genome elimination (in many scale insects), where only maternal genome is passed on. Genetic sex determination can also include cytoplasmic factors (e.g. in some insects

and flowering plants), and monogeny, where a particular female gives rise only to sons or daughters (found in some flies and crustaceans) (Bachtrog *et al.*, 2014; Beukeboom & Perrin, 2014).

Apart from GSD, there is also environmental sex determination (ESD). Sex can be determined by various environmental factors - temperature (common in reptiles and fishes), nutrient availability, maternal control, photoperiod (several marine arthropods), social factors (e.g. green spoon worms, common in fishes and limpets, as well as in some plants), pH and seasonality (Bachtrog *et al.*, 2014; Beukeboom & Perrin, 2014). Or a mixture of these factors, such as in a shrimp species, where sex is determined by the interaction of photoperiod and temperature (McCabe, 1994). Interestingly, in many fish, amphibian and lizard species extreme temperatures can override their heterogametic GSD (Bull, 1983). ESD seems to be favoured in long-lived species, where the sexes have different environmental optima and when environmental conditions change in a predictable manner, reducing the risk of skewed population sex ratios (Bachtrog *et al.*, 2014; Beukeboom & Perrin, 2014). Social sex determination is favoured when mating opportunities are limited or when relative fitness gains as a male or female are different depending on the partner (Beukeboom & Perrin, 2014). The diversity of sex-determining systems is also driven by selfish genetic elements and endosymbionts (*Wolbachia, Arsenophonus, Cardinium, Rickettsia, Spiroplasma, Microsporidium, Nosema, Microbotryum violaceum*), including mitochondria and chloroplasts, which can skew sex ratios (sex ratio distorters) (Bachtrog *et al.*, 2014; Beukeboom & Perrin, 2014). Fisherian sex-ratio selection, ploidally-antagonistic selection, sexually-antagonistic and haploid selection on alleles linked to the sex-determining locus have been shown to be important drivers influencing the evolution of sex determination (Scott *et al.*, 2018). However, indirect selection on recessive deleterious mutations, genetic drift, pleiotropic benefits and inversions also play a role (van Doorn, 2014; Natri *et al.*, 2019). The overall need to equalize sex ratio, when spatial and temporal changes in environment result in skew, favours a transition towards GSD, as GSD is more robustly able to sustain equal sex-ratios (van Doorn, 2014). Furthermore the transition from ESD to GSD seems to be more common in animals (Charlesworth & Mank, 2010), and could become even more so during ongoing climate change.

No matter what kind of sex determination has evolved, two classes of transcription factors control sex differentiation: DM-domain proteins in all animals, by binding to the DNA in the minor groove, and MADS-box proteins in all angiosperms by binding to specific DNA sequences (CArG-boxes) (Beukeboom & Perrin, 2014). Thus, sex determination can actually be seen as a threshold trait, where there is always some interaction between genes and environment. If the variance in phenotypic sex is entirely explained by genotype, then we call it GSD, and if by environment only, ESD. However, in-between these extremes there are many mixed systems. Moreover, within the same species there can be local adaptations, such that in some populations there is GSD, while in others ESD. Thus

sex is not determined at conception, but at the moment when differentiation of sexual tissues begin, influenced by genes and biological (e.g. sex hormones, temperature, pH, photoperiod) and/or social environment (Beukeboom & Perrin, 2014).

Sex chromosome evolution

In haploid organisms, sex evolves from mating types, where the mating-type locus first evolves into a sex-determining locus containing genes coding for anisogamy. The sex-determining locus later expands and evolves into U and V sex chromosomes (e.g. in Volvocales) (Beukeboom & Perrin, 2014). U and V sex chromosomes evolve quickly as there is a selection pressure within both sexes to halt recombination (Bachtrog *et al.*, 2011). Later XY or ZW systems can evolve through temporary epigenetic sex determination during transitions in ploidy (Beukeboom & Perrin, 2014).

Sex chromosome evolution is often faster than autosome evolution, due to less efficient purifying selection and increased rates of adaptation (Dufresnes *et al.*, 2020). The evolution of a new sex chromosome can be divided into six stages (fig. 1) (Ming *et al.*, 2011). First, sex chromosomes usually start as a pair of autosomes acquiring major gene(s) involved in sex determination by mutation, usually duplications of downstream gonadal factors undergoing transcriptional rewiring (Rafati *et al.*, 2020). They may establish more easily in a region of low recombination such as the pericentromere (Xue *et al.*, 2021), transposable element rich regions or other regions displaying heterochiasmy (Edvardsen *et al.*, 2022). After a sex-determining function is acquired, the second step is a complete recombination arrest, which is thought to be achieved by recombination modifiers, inversions, acquisition of sexually antagonistic (SA, beneficial for one sex, but detrimental for the other) alleles and transposable elements (Beukeboom & Perrin, 2014; Furman *et al.*, 2020). Thirdly, the newly established sex-determining region (SDR) can also expand over time, creating distinct strata, if it acquires an increasing number of SA autosomal alleles by translocations (van Doorn, 2014; Coelho *et al.*, 2019), thus possibly resolving sexual antagonism (Anderson *et al.*, 2020). Male-beneficial genes can accumulate on the Y and V chromosomes, and female-beneficial ones on the W and U (Beukeboom & Perrin, 2014; Immler & Otto, 2015). However, the SDR may also expand due to genetic drift, heterozygote advantage and/or meiotic drive (Coelho *et al.*, 2019; Sigeman, 2021). In the end, the non-recombining sex chromosomes (Y, W, U and V) usually contain two regions – a pseudo-autosomal region, where recombination is still possible, and a sex-specific region under recombination arrest. The sex-specific region, due to suppressed recombination and smaller effective population size, inevitably starts to lose genes and acquire transposable elements, repetitive, and organellar DNA (stage 4). Various phenomena contribute to this process, including a higher mutation rate (especially in male-specific sex chromosomes), stronger background selection, linkage effects, genetic drift, and weaker purifying selection resulting in limited

adaptive evolution (Bachtrog, 2013; Beukeboom & Perrin, 2014; van Doorn, 2014; Immler & Otto, 2015; Peneder *et al.*, 2017). Due to the accumulation of repetitive DNA, sex-limited chromosomes become heterochromatic to silence them, facilitating further recombination suppression and creating an epigenetic conflict between the need to silence transposable elements and expressing genes (Muyle *et al.*, 2021). Over time, the sex-limited chromosomes can become very small due to deletions (stage 5) and may eventually be lost (stage 6) (fig. 1).

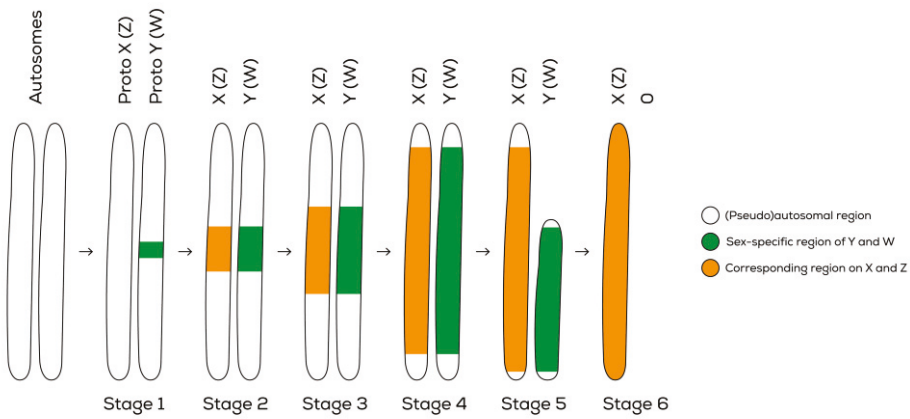


Figure 1 Sex chromosome evolution following six stages. (1) origin of a sex-determining gene, (2) recombination suppression, (3) development of a sex-specific region, (4) sex chromosomes become heteromorphic due to accumulation of repetitive elements, (5) Y and W chromosomes become smaller due to deletions, (6) Y and W chromosomes can eventually be lost.

However, the dynamics of sex chromosome evolution differ widely between species. Some species lack recombination between the X and Y entirely (Piergentili, 2010; Borodin *et al.*, 2012), while in others, such as emu for example, the Z and W have failed to evolve large non-recombining regions, potentially due to acquired sex-specific gene expression on autosomes (Vicoso *et al.*, 2013b). Therefore, if the evolution of sex-specific gene expression evolves before sex chromosome differentiation, it can potentially protect sex-specific chromosomes from degeneration and their loss or turnover, as linkage of sex-specific genes is not needed. Genetic exchange between non-recombining regions of sex chromosomes may also occur through gene conversion (Peneder *et al.*, 2017), delaying degeneration. In some species sex reversal (e.g. production of XY females) can increase recombination between the sex chromosomes, thus delaying differentiation and decay of the Y (Beukeboom & Perrin, 2014).

Similar to the sex-limited chromosomes, the X and Z chromosomes have a lower effective population size (3/4) compared to the autosomes, and restricted recombination in the heterogametic sex. Because of this, these chromosomes also

experience weaker selection and a larger effect of genetic drift, resulting in lower gene densities due to the accumulation of repetitive elements, retrogenes and pseudoretrogenes. These chromosomes contain ampliconic gene structures with male-specific functions and reduced standing genetic variation at neutral sites, similar to the sex-limited chromosomes (Ellegren, 2011; Beukeboom & Perrin, 2014). In addition, they are expected to significantly contribute to speciation by coding for reproductive barriers via transition of sex-chromosome systems (Dufresnes *et al.*, 2020) or due to generally faster evolution than autosomes (Ellegren *et al.*, 2012).

XY and ZW systems

When XY and ZW systems evolve, they may encounter a problem – there is a double dosage of the X or Z in one of the sexes. Thus, when the Y and W chromosomes degenerate, dosage compensation (i.e. mechanisms to equalize gene expression between the sexes) is often necessary on the X and Z. However, in many cases the dosage compensation is incomplete, leading to sex differences in phenotype. The most convenient way to achieve dosage compensation would be upregulation of the X or Z in the heterogametic sex (found in Diptera and *Anolis*). However, many species do not follow this pattern, e.g. in mammals one of the X's is inactivated instead and the other is then upregulated in both sexes, while in *C. elegans* partial downregulation of both Xs takes place in females (Muyle *et al.*, 2021). Meanwhile ZW systems seem to lack global dosage compensation (Vicoso *et al.*, 2013a).

Moreover, gene dosage is not the only conflict experienced on these chromosomes. Due to this twofold difference in X or Z copy number between the sexes, the effects of dominant and recessive alleles will differ between the sexes, as recessive alleles will always have an effect in the heterogametic sex, while in the homogametic sex they will be overridden by dominant alleles. Thus, it is expected that dominant female beneficial alleles can lead to X feminization, while recessive male beneficial alleles can lead to masculinization of the X. The opposite patterns are expected on the Z, therefore representing a tug-of-war on these chromosomes, where the end result will depend on the fitness cost to the opposite sex (Rice, 1984; Beukeboom & Perrin, 2014). Moreover, not only the gene content can be sex-biased (Long *et al.*, 2012); the epigenome of these chromosomes has been shown to be biased, where the X chromosome has feminized gene expression, and the Z has masculinized expression (Beukeboom & Perrin, 2014; Mank *et al.*, 2014). These chromosomes are therefore expected to be a hotspot for SA traits, although empirical evidence for this hypothesis is currently mixed (Sayadi *et al.*, 2019; Hitchcock & Gardner, 2020; Manat, 2021). Nevertheless, accumulation of sperm competition genes and female (male) fertility and growth genes is expected on the X (or Z) (Beukeboom & Perrin, 2014). Hitchcock and Gardner (2020) predict that three additional factors will determine sex-bias on the X and Z: dosage compensation, mean parental age, and assortative mating. The combination of increased dosage compensation, paternal age, and decreased inbreeding can be

responsible for the observed masculinization of the X and vice versa (Hitchcock & Gardner, 2020). They also predict that initial fixed X-linked alleles should be more female biased in case of Y degeneration and vice versa for ZW (Hitchcock & Gardner, 2020). It has also been proposed that the silencing of sex chromosome meiotic drive contributes to the decay of Y and W (Jaenike, 2001). Meiotic-drive and SA genes linked to sex chromosomes can also result in switches between XY and ZW systems (Beukeboom & Perrin, 2014).

As sexual selection is often stronger in males, dominant mutations on the Z are expected to be more male-biased compared to dominant female-biased mutations on the X. This is one reason to expect higher levels of sexual conflict on the Z than X, with females being pulled comparatively farther from their phenotypic optimum. Can this be the reason why some species switch between XY and ZW systems (amphibians, angiosperms, Coleoptera, Hemiptera, lizards, snakes, teleost fish and tephritids (Scott *et al.*, 2018))? Moreover, both diploid heterogametic systems can occasionally be found within the same species, for example in frogs, houseflies, midges and fishes (Scott *et al.*, 2018), indicating a possible transition happening in real time. It has been speculated that female heterogamety may be more prone to cytoplasmic sex-ratio distorters, because bacteria can target male-determining eggs during oogenesis, as the sex of the offspring is already known. This implies that XY systems may provide better protection against meiotic drive (Beukeboom & Perrin, 2014), thus favouring XY systems over ZW. For example, Wolbachia infection can lead to extremely female-biased populations and loss of the W chromosome, which can result in a transition to an XY system (Beukeboom & Perrin, 2014). Moreover, Bateman suggested another benefit of XY systems already almost a century ago. He speculated that the loss of the Y, and therefore males, would be less harmful than the loss of the W (i.e. females), causing male heterogamety to be favoured overall, as a few remaining males can more easily sustain population growth by increasing sexual activity, compared to females (Bateman, 1948). Similarly, the Z chromosome has a higher mutation rate than the W (Ellegren & Fridolfsson, 1997), meaning that ZW systems could limit female fitness more than XY. However, Blaser, Grossen, Neuenschwander and Perrin (2013) predict faster degeneration and turnover of XY systems due to higher mutation rates in males and stronger sexual selection, resulting in lower effective population size of Y and more selective sweeps.

Compared to the W, the Y chromosome is expected to degenerate faster, largely because it is present in males, which usually means a lower effective population size, higher mutation rate because of more sex cell divisions, and stronger sexual selection, which leads to faster selective sweeps of SA alleles (Beukeboom & Perrin, 2014). However, it also has a potential for more efficient limitation of deleterious mutations (Noël *et al.*, 2019).

Young Y chromosomes are predicted to carry many deleterious mutations, while in old Y chromosomes purifying selection becomes strong enough to prevent the fixation of new deleterious mutations, meaning that mutation accumulation declines over time (Bachtrog, 2008; Hughes *et al.*, 2012). Bachtrog (2008) has shown in a

model that degeneration of the Y chromosome slows down over time. In the first few millions of generations Muller's ratchet and background selection causes rapid degeneration of the Y, leaving around 500-1000 genes, after which genetic hitchhiking (rather than Muller's ratchet or background selection) is the main force shaping the Y for millions of generations to come (Bachtrog, 2008). The few genes left on the Y are so essential for male fertility, that selection is strong enough to oppose drift at this stage, and these genes are retained thanks to intra-Y recombination at palindromic sites (Beukeboom & Perrin, 2014). A good example is the fact that since humans and Rhesus monkeys split 25 million years ago, no single gene has been lost from strata 1-4 on the Y (Hughes *et al.*, 2012). The decay of the Y could be stopped by increased recombination during sex reversal in some species and by replacing degenerated genes by their homologs on the X via X-Y transposition (Beukeboom & Perrin, 2014). The rate of gene acquisition on *Drosophila* Y is eleven times higher than gene loss, thus its gene content is actually increasing (Koerich *et al.*, 2008). The Y chromosome can also acquire genes from autosomes, after which they can undergo duplications and acquire new mutations (Bissegger *et al.*, 2020). A study of 22 Diptera species showed that most genes on old Y chromosomes have been hijacked from autosomes, and have then undergone convergent evolution acquiring male-specific functions (Mahajan & Bachtrog, 2017). A similar scenario has happened in mouse (Soh *et al.*, 2014). Therefore, sequence acquisition from autosomes can be seen as a normal part of Y chromosome evolution (Wang *et al.*, 2013) and survival.

Moreover, sex chromosomes can occasionally fuse with autosomes, creating neo-sex chromosomes. Y chromosomes fuse most frequently and that could be explained by slightly deleterious fusions with male-biased mutation rate and biased reproductive sex ratios (e.g. polygamy), which are fixed due to drift. In addition, the fact that Y and W chromosomes contain many more repetitive sequences than the X and Z increases the probability of fusions through nonhomologous recombination (Pennell et al., 2015). Thus, chromosome fusions can make sex chromosomes the largest chromosomes in the genome and delay their loss, as well as increase raw genetic material, which can later be co-opted for sex-specific benefits.

Sexual selection and sperm competition

Back in the 19th century, Darwin observed that male birds are often strikingly different from females of the same species, and that many of these differences are not directly related to reproduction. Many male birds have evolved long and colourful feathers, attributes that can be easily seen and even encumber flight, thus leading to a higher predation risk. This fact seemed to contradict the theory of natural selection; however, he eventually realized that apart from the struggle to survive, there is also a struggle in finding a partner willing to mate, as there are other

possible partners out there. Therefore, he introduced the concept of sexual selection, which can have an opposite direction to that of natural selection (Darwin, 1859). Darwin later elaborated his concept by providing an explanation that these burdensome traits are selected by the opposite sex (mainly females via intersexual selection), or they increase mating success in the environment of male-male competition for access to females (intrasexual selection) (Darwin, 1871). It is established that males have a higher intensity of sexual selection than females in many species and is ultimately explained by anisogamy, the fact that males produce far more gametes than females do, thus there is a more intense competition between males to fertilize the few available female gametes (Pélissié *et al.*, 2012; Marais *et al.*, 2018). This strong sexual selection imposed on males drives male-biased genes to evolve faster (Beukeboom & Perrin, 2014; Ingleby *et al.*, 2015; Veltsos *et al.*, 2017). Moreover it can play a crucial role acting as purifying selection against deleterious mutations and in this case having the same direction that of natural selection by benefitting the population as a whole (Bonel *et al.*, 2018).

In promiscuous species, competition for inseminating multiple partners should be high. Aside from pre-copulatory competition such as competing for a mate, post-copulatory competition for fertilization also takes place. Therefore, in animals with frequent promiscuous matings, males are predicted to enhance sperm number and quality, especially if the group size is large (Edward *et al.*, 2015). Furthermore, males can transmit seminal fluid proteins and other substances to influence female metabolic and reproductive biology (e.g. stimulate egg laying, sperm storage and feeding) and even affect progeny, as well as to negatively impact competitor sperm (Sirot *et al.*, 2015). Males can also use other strategies (e.g. forced or traumatic insemination) for their benefit. All these tactics fall under the term sperm competition. Sperm competition is costly, limiting investment in other fitness components. It can also have a detrimental effect on the mating partner (for example, prohibiting remating or lowering longevity), resulting in sexual conflict (Edward *et al.*, 2015; Sirot *et al.*, 2015).

Sexual selection and sperm competition in hermaphrodites

Darwin doubted the existence of sexual selection in hermaphrodites (Darwin, 1871). Nevertheless, sexual selection does take place in hermaphrodites; however, it is generally shifted to the post-copulatory arena compared to gonochorists. The reason could be that to avoid mating conflicts, hermaphrodites tend to mate reciprocally (Schärer & Pen, 2013) and then exhibit post-copulatory sexual selection such as in obligatory outcrossing and simultaneously hermaphroditic sea worm *Macrostomum lignano*. Interestingly it has been shown that sexual selection on the male side in a hermaphroditic snail acts as purifying selection against deleterious mutations and in this case sexual selection thus have the same direction that of natural selection by benefitting the population as a whole (Bonel *et al.*, 2018).

The signs of sexual selection in hermaphrodites can be observed in their courtship structures and behaviour, sperm competition, and sexual polymorphism (Leonard, 2006). We searched for these types of signs in *M. lignano* undergoing sex-limited experimental evolution. We studied, how sex-limited experimental evolution has affected mating behaviour (Paper IV), sexual anatomy (Paper III) and genomics (Paper I and II).

Similarly, as in promiscuous gonochoric species, also in hermaphrodites sexual selection can act pre- and post-copulation. Post-copulatory competition seems to be especially important in simultaneous hermaphrodites (Ramm, 2017; Bonel *et al.*, 2018). Therefore, as in males, male function of hermaphrodites is shown to increase testes size and therefore sperm production as well as seminal fluid protein production in prostate glands, especially if the group size is large (Ramm *et al.*, 2019). In *M. lignano* seminal fluid proteins have been shown to reduce sucking behaviour of mating partners, where worms seem to suck out the received ejaculate from their sperm receiving organ called antrum. Therefore the seminal fluid proteins act in a way so that the mating partners would not remove the received ejaculate (Patlar *et al.*, 2020). As in gonochorists, also in hermaphrodites sperm competition can be costly and can result in sexual conflict (Charnov, 1979).

Sexual conflict and the evolution of sex differences

Differing reproductive roles and investment, as well as differences in life histories between males and females, can create a different fitness optimum for a certain trait. As most of the genome is shared (and in hermaphrodites - all of it), differing evolutionary interests between reproductive strategies (male or female) can create a sexual conflict, which is a special case of intragenomic conflict.

Sexual conflict takes place if a trait expressed in one sex increases fitness while decreasing it in the other. The term sexual conflict or sexual antagonism was first introduced by Parker (1979). There are two main types of sexual conflict - intralocus sexual conflict and interlocus sexual conflict.

Intralocus sexual conflict occurs when a locus experiences sex-specific selection pressures, meaning its optimal allele expression differs between the sexes. Therefore, because this locus is shared between the sexes, it creates a genetic tug-of-war in offspring, where female-beneficial alleles present in sons will decrease their fitness, and vice versa for male-beneficial alleles in daughters. In hermaphrodites, this means that if an allele makes an individual better in the female sex function, while simultaneously making it worse in the male sex function, this could be considered a SA allele causing a genetic constraint.

In contrast, interlocus sexual conflict is a conflict between different loci coding for traits involved in interactions between the sexes, where the highest fitness outcome for one mating partner (usually male) is achieved at the expanse of the

other mating partner (usually female) (Schenkel *et al.*, 2018). The most extreme sexual conflict observed is sexual cannibalism in spiders (Schneider, 2014). However, mating frequency is used as a classical example. From the perspective of a male, nearly unrestricted mating rate is the optimal strategy for reaching maximum fitness, however for a female, the benefits of unlimited mating are restricted owing to considerably higher investment per gamete, resulting in fewer gametes (Bateman, 1948), hence females are expected to be choosier on average (Henshaw *et al.*, 2015). Increased mating rate can be harmful to females, especially if males have forceful insemination tactics, which lead to injuries, as in e.g. the bed bag (Reinhardt *et al.*, 2003). To undo the harm, females can evolve protective behavioural, physiological, and sometimes even morphological mechanisms used to minimize the fitness gains of males (Perry & Rowe, 2015). As in host-parasite interactions consisting of constant antagonistic co-evolution of invasion and defence mechanisms, between the sexes there can also be an ongoing arms race as a result of sexual conflicts over mating rate. The best example is bedbugs, where males do not inseminate females through vagina, but instead through forcefully stabbing the abdomen, thereby leading to a ~25% reduction in female lifespan, primarily due to infections (Reinhardt *et al.*, 2003). In response, female bedbugs have evolved a new organ called the spermalege, which activates sperm, digests seminal fluid and protects from the traumatic mating-induced infections and water loss (Reinhardt *et al.*, 2015). A similar type of adaptation has been observed in hermaphroditic snail *Eobania vermiculata*, which has evolved physiological resistance to love-dart substances exerted by the mating partner's male role (Lodi & Koene, 2017). This type of arms race between the sexes can lead to co-evolution of male and female traits with quite unpredictable outcomes. Evolution of new and complex sexual traits in a population can sometimes lead to reproductive isolation, thus resulting in speciation (Chapman, Arnqvist, Bangham, & Rowe, 2003; Gavrilets, 2014; Pennell & Morrow, 2013). Sexual conflict also helps to maintain standing genetic variation in populations, as the fitness of SA alleles is context-dependent (Nordén, 2017; Dutoit *et al.*, 2018). The net result of sexual conflict need therefore not always be negative on the population or species level.

Sexual conflict in hermaphrodites

Charnov (1979) was the first person to note that hermaphrodites experience sexual conflict as well. He suggested that sexual conflict can possibly drive the evolution of different offensive male and defensive female strategies, such as forceful and traumatic copulation with manipulative seminal fluids on the one hand, while on the other, non-random use of the received sperm (Charnov, 1979).

It has long been known that female fertility is far more limited than male fertility due to different investment per gamete and usually higher costs related to reproduction (Bateman, 1948). Since hermaphrodites cannot specialize in a particular sex role, hypothetically they could harbour substantial intralocus sexual

conflict, representing constraints on fitness gains in a given sex role. Interlocus conflict is also highly expected if hermaphrodites would tend to prefer the male role rather than the female role, as in doing so, they would increase their fitness through fertilization of many partners and escape the burden of high female mating costs (Schärer, Janicke, & Ramm, 2015). Interlocus sexual conflict could favour a transition to separate sexes if the resulting male-biased investment causes higher levels of sperm competition, which in turn drives the evolution of pure males, which then selects for the evolution of pure females, finally leading to the evolution of separate sexes (Santi *et al.*, 2018).

Escaping intralocus sexual conflict

To escape the fitness costs for both sexes resulting from sexual conflict, it has to be resolved. There are five possible mechanisms of sexual conflict resolution that have been discussed in the literature: sex-specific gene expression through cis- or trans-regulatory changes, evolution of sex chromosomes, alternative splicing, imprinting, and selection of offspring sex based on the phenotype of the mating partner (Parsch & Ellegren, 2013; Pennell & Morrow, 2013). Evolution of sex chromosomes is arguably the most effective one (Pennell & Morrow, 2013), as sex-specific genes can be linked to the Y, W, U and V chromosomes to ensure sex-limited expression, thus resolving intra-locus sexual conflict (Paper V). If a gene is still needed in both sexes, but at different expression levels, it can be retained on an autosome, but an extra copy could be translocated to a sex-limited chromosome via duplication (Griffin, 2015). Alternatively, sex-specific transcription factors could evolve to activate genes on autosomes in a sex-specific way. We have studied sex chromosome evolution and the origins of sex-biased gene expression by performing sex-limited experimental evolution on a simultaneous hermaphrodite described below.

Aims of the thesis

The main aim of the thesis was to observe the initial steps of XY and ZW sex chromosome evolution from a hermaphroditic ancestor by performing sex-limited experimental evolution, as manipulative experiments are lacking in the field. An additional aim was to learn more about sex-specific and sexually biased traits, and if they exhibit sexual conflict in the simultaneous hermaphrodite *M. lignano*. Lastly, we wanted to learn about different dynamics taking place during the life history of sex chromosomes in the tree of life.

In **Paper I**, we examined how the transcriptome of *M. lignano* has responded to the sex-limited experimental evolution over more than 20 generations. Specifically, we wanted to see if previously annotated testes- and ovary-specific genes are respectively enriched or diminished in expression in male- versus female-selected lines. We also wanted to see which biochemical pathways have been affected the most, and show the largest degree of differences between the male- and female-selected worms.

In **Paper II**, we complemented the expression data with DNA sequencing to look for changes in allele frequencies between selection regimes. We also hoped to identify specific loci that may have sexually antagonistic effects. We expected that the GFP locus, by mimicking a sex-determining locus, could potentially evolve reduced recombination and acquire nearby sex-biased genes.

In **Paper III**, we investigated whether the selection regimes differed in sex allocation and the male insemination organ (the stylet). We expected that male-selected lines would increase testes size and decrease ovary size, and vice versa for the female-selected lines. Changes in the stylet (especially in the male-selected lines) were also expected, since male copulatory organs evolve rapidly in the animal kingdom.

In **Paper IV**, we looked at how sexual behaviour has changed due to the sex-limited experimental evolution. We measured mating frequency and duration as well as post-copulatory sucking behaviour. We expected the male-selected lines to increase mating investment and also sucking behaviour, since they do not need to be fertilized. Meanwhile, a clear decrease in the mating frequency was expected for the female-selected lines, since a few matings a day should be enough to fertilize the 1-2 eggs that are normally laid per day.

In **Paper V**, we wanted to see what happens after the heteromorphic sex chromosomes are established. More specifically, if highly degraded sex chromosomes, such as the Y in many animals but also the W, U and V in different clades, are able to affect other complex traits besides sex determination and fertility. Thus, we performed an extensive literature review, where we show that these chromosomes potentially can affect traits in all organ systems even if they are highly degraded. Moreover, we discuss how the sex-limited chromosomes can resist further degradation and even acquire new genes.

General materials and methods

Macrostomum lignano

M. lignano (Macrostomorpha, Rhabditophora, Platyhelminthes, Lophotrochozoa) is a free-living, transparent, regenerating, obligatory outcrossing and simultaneously hermaphroditic flatworm of small size (1.5 mm in length). It has the ability to stick itself using its adhesive tail to the intertidal fine sand in its natural habitat of the Northern Adriatic and Aegean Seas (Ladurner, Schärer, Salvenmoser, & Rieger, 2005; Ladurner, Rieger, & Baguñà, 2000; Zadesenets et al., 2016). It has two black eyes, and because it feeds on diatoms, the worm looks brownish under a microscope (fig. 2). It has a short generation time (18 days (Schärer & Ladurner, 2003)) and is relatively long-lived, with a median lifespan of 205 days (Mouton *et al.*, 2009). It is easily cultured and manipulated in the laboratory, thus taking all of this together - it is an ideal model organism for our research.

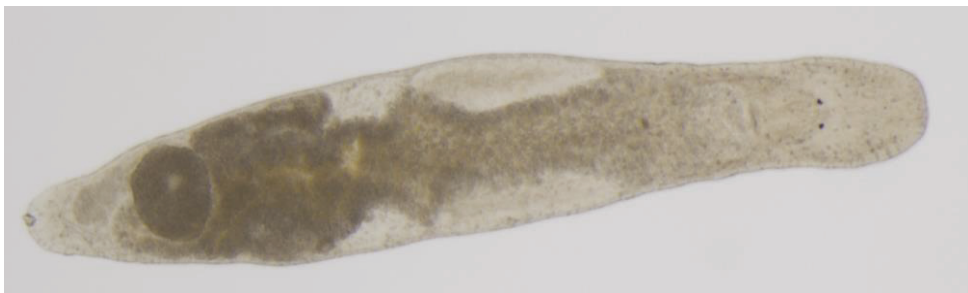


Figure 2 *Macrostomum lignano*.

Genome

The diploid genome consists of eight chromosomes, where one pair of them is noticeably larger, however different karyotypes (e.g. 2n=9 or 10) also exist, mainly in inbred laboratory populations. The large chromosome is a fused duplicate of the three smaller chromosomes. That means that the regular karyotype is a hidden tetraploid and each duplication of this chromosome represents a whole genome duplication, creating penta- (2n=9) and hexaploid (2n=10) individuals. Worms with additional chromosomes do not exhibit noticeable abnormalities in the phenotype

and are fertile (Zadesenets *et al.*, 2017). In addition to the recent whole genome duplication and flexible karyotype in the laboratory, the DNA sequence itself is very complex, where ¾ of the genome contains simple and long tandem repeats and transposon sequences. The DNA methylation level is low (Wasik *et al.*, 2015), however many transcripts are trans-spliced (Wudarski *et al.*, 2017), thus increasing transcriptome complexity. The enormous amount of minisatellites could explain genomic instability through meiotic mutability (Wasik *et al.*, 2015). The haploid genome size is 502 Mbp (Wudarski *et al.*, 2017) and predicted gene content is 20'000 genes (Wasik *et al.*, 2015). A recent bioinformatics study using previously published transcriptomes has identified 2547 long intergenic non-coding RNAs (lincRNAs) transcripts, most of which showed specific expression patterns, and they are thought to be important for the regeneration capabilities (Azlan *et al.*, 2020).

Reproduction

Male reproductive organs include spermatozoa with bristles, male genitalia, and testes, which make 6% of the body area and are located in the central region anterior to smaller paired ovaries on each side of the gut (fig. 2). Gonad size correlates with the amount of active cells and thus gonadal productivity (Schärer, Sandner, & Michiels, 2005). The male genitalia consist of a male copulatory organ called the stylet (fig. 3), a copulatory bulb, muscular and false seminal vesicles that contain sperm, and prostate gland cells that produce seminal fluid (Arbore et al., 2015; Ladurner et al., 2005; Schärer & Ladurner, 2003). It has already been found that the gene *Mlig-sperm1* is exclusively expressed in the testes and is necessary for normal gonad and sperm development (Grudniewska *et al.*, 2018). Also three testis-limited transcripts (MLRNA110815_6628.2, MLRNA110815_7008 (involved in spermatogenesis), MLRNA110815_9973.1 (affects testes, seminal vesicles and vas deferens)) and other male-biased transcripts have been identified, for example three *Mlig-stylet* genes, which are required for the differentiation of the male copulatory apparatus (Arbore *et al.*, 2015; Lengerer *et al.*, 2018; Ramm *et al.*, 2019) (Paper I). Sexual selection in *M. lignano,* as in other simultaneous hermaphrodites, appears to be shifted towards post-copulatory episodes rather than on the likelihood of mating itself, thus genital morphology and testis size play a very important role in male mating success (Ramm, 2017). At least 10% of the transcriptome is socially sensitive in expression. Thus with an increasing mating group size and therefore increased potential for sperm competition, allocation to the male role increases, where every third gonad-specific or seminal fluid gene changes in expression (Ramm *et al.*, 2019). Phenotypically worms start to contain more active spermatogenic cells and increase testes size, as well as the rate of sperm production (Giannakara, Schärer, & Ramm, 2016; Schärer & Ladurner, 2003). Also the stylet changes in size in response to mating group size (Janicke & Schärer, 2009). It also exhibits male bias, when small or starved. Testes can be fully developed before any sign of ovaries can be seen (Vizoso & Schärer, 2007).

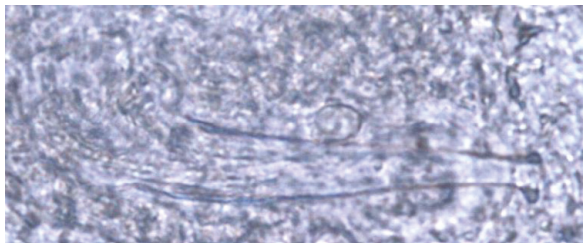


Figure 3 Morphology of the stylet of *M. lignano*. The opening, which is inserted in the female antrum has a shoe like shape, where the ending increasis in width on one side.

Female reproductive organs include paired ovaries located posterior to the testes and female genitalia located anterior to the male genital opening and stylet. The female genitalia consist of cellular valve, ciliary tuft, ciliated vagina, shell and cement glands and female antrum, which retains received sperm (fig. 4) (Ladurner et al., 2005). Eggs develop in a sequence starting posterior to the ovaries and moving towards the female antrum from where they are later being laid (Schärer & Ladurner, 2003) at a rate of approximately one egg per day (Schärer et al., 2005). With an increased group size, half of the ovary-specific candidate transcripts change in expression, where almost all of them are downregulated, which goes in accordance with the observation that ovaries tend to decrease in size (Ramm *et al.*, 2019). Six ovary egg-limited transcripts (MLRNA110815_1618.1, CPEB (MLRNA110815_2640) (necessary for egg maturation), MLRNA110815_4558, MLRNA110815_7498, MLRNA110815_7725.2, MLRNA110815_16738) and other female-biased transcripts have been identified (Arbore *et al.*, 2015; Lengerer *et al.*, 2018; Ramm *et al.*, 2019) (Paper I).

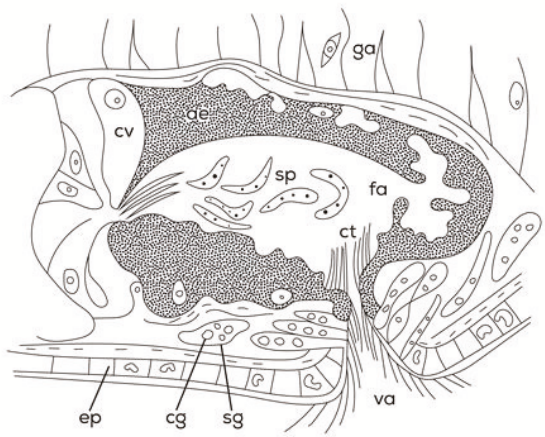


Figure 4 Female reproductive organs of *M. lignano*. ae, antral epithelium; cg, cement glands; ct, ciliary tuft; cv, cellular valve; ep, epidermis; fa, female antrum; ga, gastrodermis; sg, shell glands; sp, sperm; va, vagina (adapted from Ladurner et al. (2005)).

These worms copulate frequently – around 20 times in an hour (Paper IV) with up to ten different mates during a day (Janicke & Schärer, 2009). The worms seem to be unable to distinguish new from old partners (Sandner & Schärer, unpublished data). A partner is approached by circling it, and then a ten-second reciprocal copulation follows, in which both individuals insert their stylet into the partner's female antrum (Schärer et al., 2004). The worms cannot self-fertilize (Vizoso, Rieger, & Schärer, 2010). After approximately every third copulation (Paper IV), the worm performs a sucking behaviour on itself by placing its head on its female genital opening, seemingly trying to remove the received ejaculate (fig. 5) potentially removing partner's seminal fluid proteins and sperm. This is thought to be the reason why the sperm has evolved bristles to anchor itself in the female antrum (Vizoso, Rieger, & Schärer, 2010). Worms have been found to be able to use received sperm from previous matings for up to two weeks (Marie-Orleach *et al.*, 2016). If a worm has mated with numerous individuals, this time window allows for sperm competition (both in nature and in the laboratory). Mating frequency increases with increasing group and testes size, influencing sperm transfer success, which depends on the stylet morphology as well. Thus male-biased individuals, as expected, have more mating partners and their sperm transfers are more successful (Janicke & Schärer, 2009), thus siring more offspring (Marie-Orleach *et al.*, 2016). Individuals in larger groups not only become more male-biased in their sex allocation, but they also produce longer sperm, are larger and perform less sucking, probably due to smaller seminal vesicles in the partners transferring smaller ejaculates. Interestingly, experimental evolution under monogamy did not produce changes in sex allocation; however, stylet shape changed and sperm became shorter (Janicke, Sandner, Ramm, Vizoso, & Schärer, 2016). Trade-offs in sex allocation have been tricky to find, however in stressful environments some trade-offs can be observed (Schärer et al., 2005).

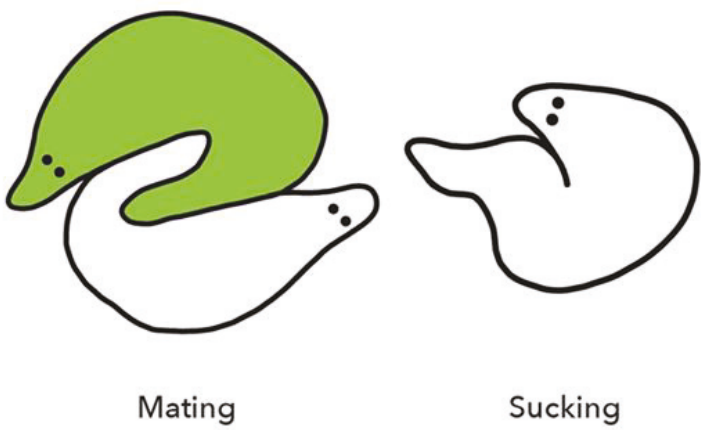


Figure 5 Mating behaviour in *M. lignano*.

It has been shown that virgin worms are smaller and have smaller absolute testes size, thus resulting in more female-biased sex allocation (Schärer & Janicke, 2009). They mate earlier, for a longer time, and more frequently, as well as sucking less. This is expected, as they have not been mated before, and are unlikely to want to remove the partner's sperm. However, the difference in the sucking frequency depends not only on the worm itself, but on the mating status of the partner, where the virgin focal worm sucks less if mated with a virgin partner (Marie-Orleach *et al.*, 2013). Presumably this is because virgin individuals transfer larger ejaculates since they have more stored autosperm and seminal fluid, and it is well established in several species that seminal fluid can manipulate the recipient's behaviour (Marie-Orleach *et al.*, 2013). Indeed, it has been recently found that two seminal fluid proteins termed suckless-1 and suckless-2 decrease sucking frequency in the recipient (Patlar *et al.*, 2020). However, a different study found that one seminal fluid protein Mlig-pro63 increases suck behaviour in the receiving partner, which seems to be contrary to the previous findings, as well as to the donor's own interests (Weber *et al.*, 2020). The function of this protein is therefore currently unclear. Interestingly, many sexual characteristics such as testes and seminal vesicle size, copulation and sucking behaviour depend on the genotype of the mating partner (Marie-Orleach *et al.*, 2017). Female fitness has a considerably higher heritability than that of male under certain conditions (Nordén, 2017), thus the effects of experimental evolution should be mainly observed in the female-selected lines.

Worm cultures

In our laboratory, we have three green fluorescent protein (GFP) populations collectively known as BAS1, and six wild-type populations collectively known as LS2. In both the GFP and wild-type stocks, each population consists of two subpopulations with a 10% migration rate between them every generation to avoid inbreeding. The worms are kept in glass Petri dishes of 100 individuals, meaning that each previously described population consists of 200 individuals. The LS2 wild-type line was collected in Italy in 2011 (Zadesenets *et al.*, 2016), while the outbred BAS1 GFP transgenic line was created by crossing two other lines – an outbred wild-type population called LS1 with the inbred HUB1 GFP transgenic line (Nordén, 2017). The GFP marker is believed to be located on the large chromosome, and it is usually inherited as a dominant allele in Mendelian fashion. It is ubiquitously expressed and is excited by a near-UV light source (CoolLED pE-100, CoolLED Ltd., England) and can be observed using a stereoscope (Nikon) (Marie-Orleach *et al.*, 2014).

For the experimental evolution lines, we created genetically variable start-up populations by crossing BAS1 lines homozygous for the GFP with wild-type LS2 lines. Then the heterozygous GFP offspring were backcrossed to LS2 to obtain GFP (-/-) worms used as source populations, together with GFP (+/-) worms used to found the sex-limited selection populations (fig. 6).

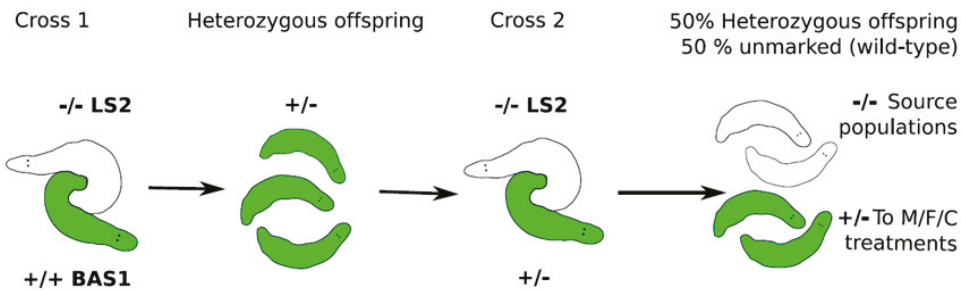


Figure 6 Worms for the experimental evolution experiment were obtained crossing BAS1 line with LS2 line and then the offspring backcrossing with LS2 (Nordén, 2017).

In May of 2014, we started twelve sex-limited selection populations (four female-limited, four male-limited and four control) heterozygous for the GFP gene, allowing experimental evolution of heterogametic sex chromosome systems by artificially selecting on the GFP as a sex-determining locus. Therefore, the female-selected lines inherit the GFP gene only through egg cells, resembling a W chromosome, while the male-selected lines inherit the GFP gene only though sperm, thus resembling a Y chromosome (fig. 7). Controls are an equal mixture of both selection regimes (half of the population is handled in the same way as the male-selected lines, and the other in the same way as the female-selected lines). A new generation is produced every five weeks, where in the first week experimental lines are crossed with wild-type worms from their respective source populations to produce offspring, which inherit the GFP gene via sperm or eggs only. The mating is carried out by putting one worm from the experimental evolution line together with two wild-type worms from the respective source population in 24-well tissue culture plates (TPP, Trasadingen, Switzerland), for 48 groups in total. This mating ratio of 1:2 between the evolved worms and wild-type worms is chosen to allow sperm competition to take place. In the second week, worms are isolated for egg laying in new 24-well plates. In the female-limited selection lines, it is the focal GFP worm that lays eggs, so that the GFP gene will be inherited through eggs only, and in the male-limited selection lines, the wild-type worms lay the eggs, so that the GFP gene will be passed on through the received sperm from the GFP worm only. In the third week, we remove the egg-laying worms, and allow the eggs to hatch. Finally, in the fourth week we collect all the progeny and allow them to grow until the next generation, which is started by selecting only the GFP-positive offspring.

All worms are kept in Guillard's f/2 medium (32‰ salinity) (Andersen, Berges, Harrison, & Watanabe, 2005), fed *ad libitum* with diatom *Nitzschia curvilineata* (Heterokontophyta, Bacillariophyceae) (Schärer et al., 2005; Wudarski et al., 2017) in incubator (Snijders Scientific, Netherlands) at 20 °C on a 14:10 h light:dark cycle with 60% humidity.

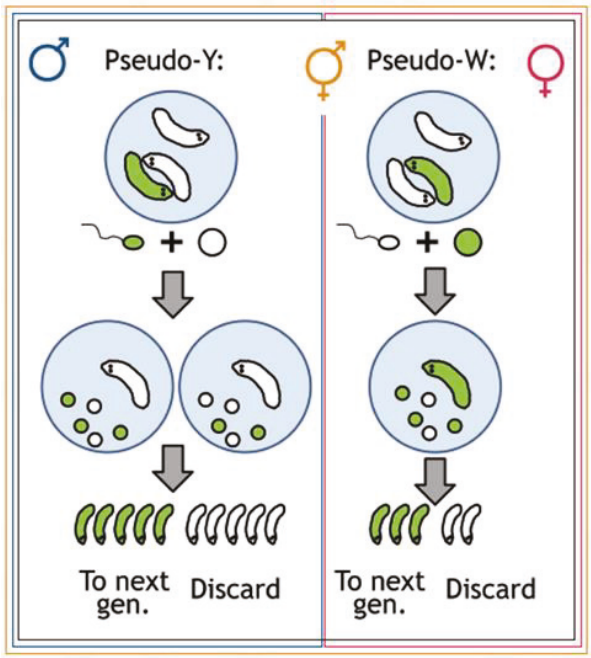


Figure 7 We have attempted to mimic the early stages of sex chromosome evolution by subjecting simultaneous reciprocally mating hermaphrodite *M. lignano* to sex-limited selection using a GFP marker as a sex-determining locus.

RNA extraction and sequencing

Next-generation sequencing technologies paved the way for RNA sequencing, of which the most popular are Illumina systems (Goodwin *et al.*, 2016). RNA sequencing is used to roughly infer gene expression by quantifying RNA levels. Before sequencing cDNA libraries are made from extracted RNA samples and barcoded by adding a tag (known sequence) to know the identity of the sample later on. RNA sequencing is applied in virtually all fields of biology and is a very common technique in molecular ecology. I myself have studied the RNA content of extracellular vesicles from lung cancer cell cultures (Line *et al.*, 2014), but this time we studied whole body gene expression from *M. lignano* worms undergoing sex-limited experimental evolution (Paper I). Below is a short description of methods we used.

Worms were homogenized in RLT buffer using a pipette and stored in a - 80°C freezer until extractions. RNA was extracted using RNeasy Mini Kit (Qiagen, Germany) and treated with DNase I before being eluted in nuclease-free water following manufacturer's recommendations for RNA purification from animal tissues. Before sending for sequencing to Science for Life Laboratory (SciLifeLab)

SNP&SEQ Technology Platform in Uppsala, Sweden, RNA quantity and quality was assessed with Agilent 2100 BioAnalyzer (Agilent Technologies, USA). The quality of raw sequencing data was examined with Nextflow (version 19.07.0) (Ewels *et al.*, 2019) and adapter-contaminated reads trimmed using Trimmomatic (version 0.36). We created a *de novo* transcriptome assembly running Trinity (version 2.4.0) on RNA-seq data from three ancestral GFP line samples. Transcript reads were aligned against this newly-created ancestral GFP line transcriptome assembly using Salmon (version 0.9.1) to quantify transcript expression (Patro *et al.*, 2017). We chose to use a *de novo* assembled transcriptome instead of an existing genome assembly, because by using this approach, we did not "lose" as much data due to multi-mapping reads for downstream differential expression analysis. The *de novo* assembly could also be used to get a clearer picture of the relationships between samples than from the genome-based alignment strategy. Differentially expressed transcripts between selection regimes (male-limited, female-limited and control) were identified with the EdgeR R package (Robinson *et al.*, 2010). Transcripts that had less than 0.5 CPM in a minimum of three samples were excluded from the analysis.

DNA extraction and sequencing

The method of DNA sequencing is used for determining DNA nucleotide sequence. It was developed it in the 70s (Sanger *et al.*, 1977) and received the Nobel prize in Chemistry 1980 and is still used today and called Sanger sequencing. The method is precise, however it is mainly used for sequencing small fragments, as it is very slow compared to next-generation sequencing technologies such as Illumina. I have used it myself for sequencing PCR products, and it is still used for that purpose here in the department for both teaching and research. The first next-generation sequencing method, called Roche 454 Life Sciences pyrosequencing, was launched in 2005 (Margulies *et al.*, 2005) and increased the efficiency several hundred times (Schuster, 2008) as well as lowering the costs. It could also be used to determine cytosine methylation status, however it ceased to exist in 2016 (Skvortsova *et al.*, 2018). Another method called ion semiconductor (Ion Torrent, Thermo Fisher Scientific) is considered a third-generation sequencing method as it does not require PCR amplification (Goodwin *et al.*, 2016; Rowe *et al.*, 2017). However, for pooled DNA sequencing we used the most popular technique in the market called Illumina, the same as for our pooled RNA sequencing. I describe the method we used in short in the following paragraph.

200 pooled individuals per population were collected at generation 46 from among the worms that were not chosen at random to produce the next generation. They were kept in clean f/2 medium and starved for a day. Then they were collected in Eppendorf tubes filled with 96% ethanol and homogenized with a pipette. After

that they were stored at -80°C until extraction. DNA extractions were done using the MagAttract HMW DNA Kit (Qiagen, Hilden, Germany). DNA quality and quantity was assessed with NanoDrop™ Spectrophotometer (Thermo Fisher Scientific) at OD 260/280, Qubit dsDNA BR assay kit (Life Technologies), and by running an agarose gel. After ensuring the quality of the samples, they were sent for library preparations (Illumina TruSeq PCR-free) and 2x150bp S4 lane sequencing with the Illumina NovaSeq6000 (Illumina Inc., San Diego, CA, USA) at Science for Life Laboratory (NGI Stockholm, Sweden).

Mating assay

Mating assays to study mating behaviour of *M. lignano* were originally developed by Schärer *et al.* (2004). We performed mating assays using an attached camera (Nikon DS-Fi3) to a stereoscope connected to a computer. The mating assay was carried out by putting together one experimentally evolved worm with two LS2 worms. To be able to tell the experimental evolution worms apart from the wild-type LS2 worms, they were coloured with two blue food dyes. Experimental evolution focal worms were individually marked in a 60-well HLA Terasaki plate (Greiner Bio-One™, Frickenhausen, Germany) using a colour solution (table 1), where the final concentration of the Grand Bleu [E131, E151] (Les Artistes—Paris) dye is 2.04 mg/ml, and 1.6% of the liquid blue colour dye (Dr. Oetker) for approximately a day. The LS2 mating partners were also isolated in well plates filled with f/2 and algae as a food source, but without the colour solution. For limiting evaporation, the empty space on the plate was filled with wet tissue paper, then closed with a lid. The next day individual worms were washed in 1 mL f/2. After that six focal worms were filmed simultaneously by creating six droplets on a single silicone-covered slide (Sigmacote®), where the worm of interest was mated with two wild-type LS2 worms (each worm was pipetted onto the slide with 1.5 µL f/2, resulting in three worms per droplet). All three selection regimes were represented with two mating groups on each slide to avoid confounding factors. To limit evaporation, Vaseline was applied around the slide, leaving small gap for the air. After that two HERMA photo stickers (Filderstadt, Germany) were attached to each side of the slide. Then a coverslip was placed on top. This creates somewhat squeezed droplets, which allows the worms to be easily observed. By using NIS Elements software, the worms were recorded for 2 h. Used slides were cleaned with precision wipes and reused. The mating videos which were obtained were analysed by speeding them up eight times in VLC media player, creating a 15 min movie. Then we counted the matings and sucking behaviour for one worm of interest at a time. Data was analysed in R (version 3.5.1 (R Core Team, 2020)), where statistical significance for matings was assessed by creating linear mixed effects models, while

for the sucking behaviour, we used generalized linear mixed models, with significance testing using ANOVA.

Table 1.
Colour solution used to colour worms for the mating assay.

INGREDIENT	AMOUNT (µL)
3.4 mg/ml Grand Bleu [E131, E151] (Les Artistes—Paris) in f/2	15
4 ml/50 ml liquid colour blue (Dr. Oetker) in f/2	5
algae in f/2	3
f/2 with a worm of interest	2

Results and discussion

Paper I. Effects of sex-limited experimental evolution on a hermaphrodite transcriptome

In the first study I investigated the transcriptomic response to the sex-limited selection after more than 20 generations. Sex organ-biased gene expression makes it possible for males and females to create differentiated structures from the rest of the body despite having the same genome, and the same is true of hermaphrodites. Thus we particularly looked into genes previously described as sex organ-candidates (Lengerer *et al.*, 2018; Weber *et al.*, 2018; Ramm *et al.*, 2019).

By performing pooled RNA-seq of worms and pairwise comparisons on the transcript counts, we found that the female-selected lines have diverged the most, showing three times the number of differentially expressed transcripts compared to the male-selected lines (1175 vs 318). Interestingly, most of these transcripts were downregulated compared to the controls. 234 transcripts which were differentially expressed in the female-selected lines compared to both other selection regimes could be considered female-specific. Similarly, 84 transcripts which were differentially expressed in the male-selected lines compared to the other two selection regimes can be regarded as male-specific. The three transcripts in the middle are particularly interesting, since they are different between all the selection regimes (fig. 8), where they have the highest expression in the female-selected lines and the lowest in the male-selected lines, thus can be considered female-line-specific.

Looking into more details of these significantly differentially expressed transcripts, we checked if they overlap with previously identified sex organ candidates (Ramm *et al.*, 2019). We found that testes candidates were overrepresented compared to the ovary candidates, which is not surprising, since selection on male traits is often stronger (Grath & Parsch, 2016; Veltsos *et al.*, 2017). The most interesting finding was that testes-biased genes were mainly downregulated in the female-selected lines (table 2). This finding is consistent with evolution towards gynodioecy by reducing investment in the non-rewarding male role. These results are also in line with previously measured fitness differences, which found that the female-selected lines had become better at performing in the female role, and worse in the male role, compared to the other selection regimes (Nordén, 2017).

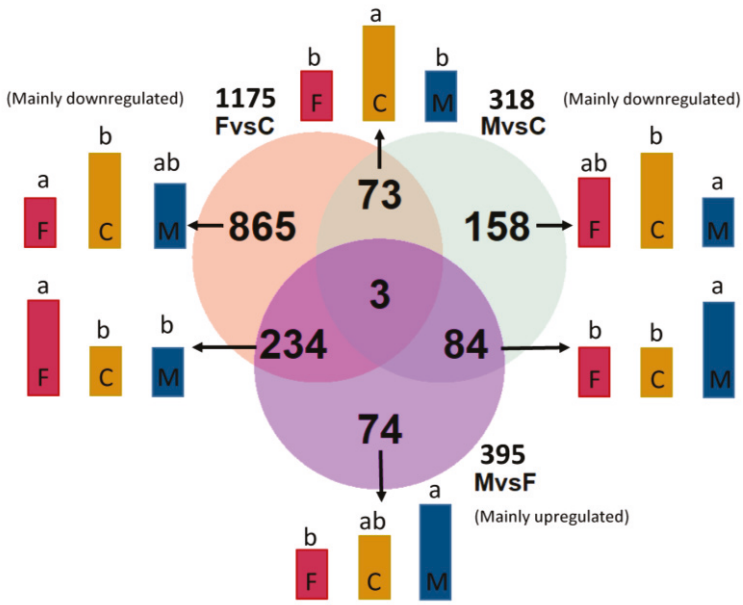


Figure 8 Venn diagram for significantly differentially epressed transcripts between the selection regimes.

Additionally, by performing GO term and KEGG pathway analyses, we found overrepresentation of transcripts involved in metabolism (Paper I). This is not surprising, since sex differences in metabolism are widespread and may be influenced by sex chromosomes (Link & Reue, 2017; Ellison & Bachtrog, 2019; Abbott *et al.*, 2020).

Table 2.
Differentially expressed transcripts of putative sex organ genes (adapted from Paper I)

ORGAN	UPREGULATED*	DOWNREGULATED*	P-VALUE†
Male- versus female-selected lines			
Testis (3736)	13	4	0.049
Ovary (366)	1	2	N/A
Tail (406)	0	0	N/A
Gonad (likely ovary-biased) (136)	2	1	N/A
Female-selected versus control lines			
Testis (3736)	12	53	2.786e-07
Ovary (366)	2	1	N/A
Tail (406)	6	1	0.125
Gonad (likely ovary-biased) (136)	0	2	N/A
Male-selected versus control lines			
Testis (3736)	4	17	0.007
Ovary (366)	1	2	N/A
Tail (406)	1	0	N/A
Gonad (likely ovary-biased) (136)	1	0	N/A

Paper II. Effects of sex-limited experimental evolution on a hermaphrodite genome

Because we found evidence of a transcriptomic response, we also wanted to investigate how allele frequencies have changed in our sex-limited experimental evolution lines, and more particularly if the GFP scaffold has changed after 46 generations of selection. To do that we carried out pool-seq of 200 worms per replicate from each selection regime.

Quality control of the samples before and after trimming with Trimmomatic (Bolger *et al.*, 2014) was performed with FastQC. Then the reads were mapped to the *M. lignano* reference genome (vMlig_3_7) (Wudarski *et al.*, 2017) with bwa mem (Li & Durbin, 2009). We used quasibinomial generalized linear models (Wiberg *et al.*, 2017) to identify consistent allele frequency differences.

We found that more than 20'000 single nucleotide polymorphisms have changed in their frequency ($q < 0.05$) in all pairwise comparisons and that a few of them were located on the GFP scaffold (Paper II). All of these single nucleotide polymorphisms are unique between the pairwise comparisons on the GFP scaffold, and most of them have changed in the male-selected lines (fig. 9). This is consistent with results from other species showing that Y chromosomes generally evolve faster than W chromosomes (Paper V).

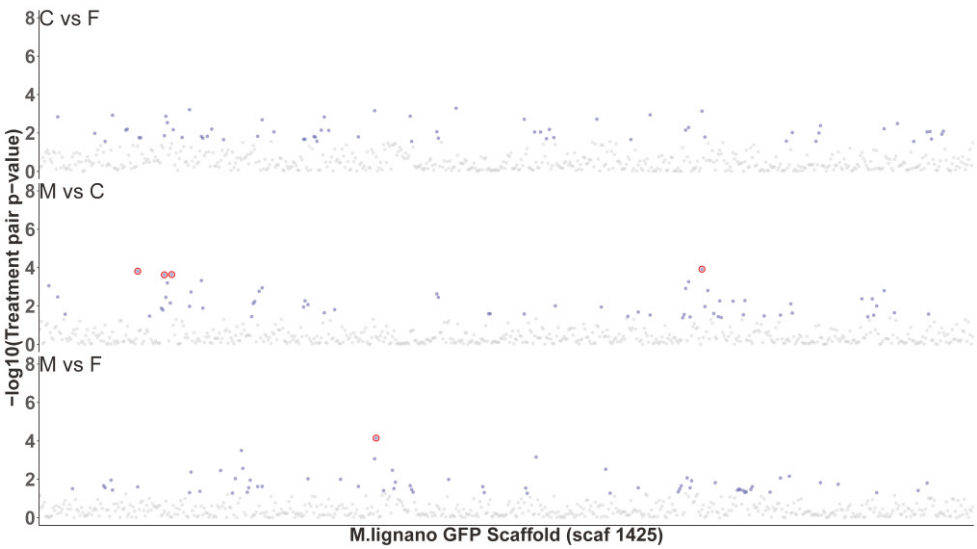


Figure 9 Manhattan plot of allele frequency differences among the GFP scaffold. Significant ones are circled red (Paper II).

We also checked for structural variants, and found that almost all of the structural variant classes are overrepresented in the female-selected lines and underrepresented in the male-selected lines (fig. 10). This could indicate that the female-selected lines have increased recombination rate and/or male-selected lines have increased purifying selection. Both of these explanations are consistent with previous results in the literature (Paper II).

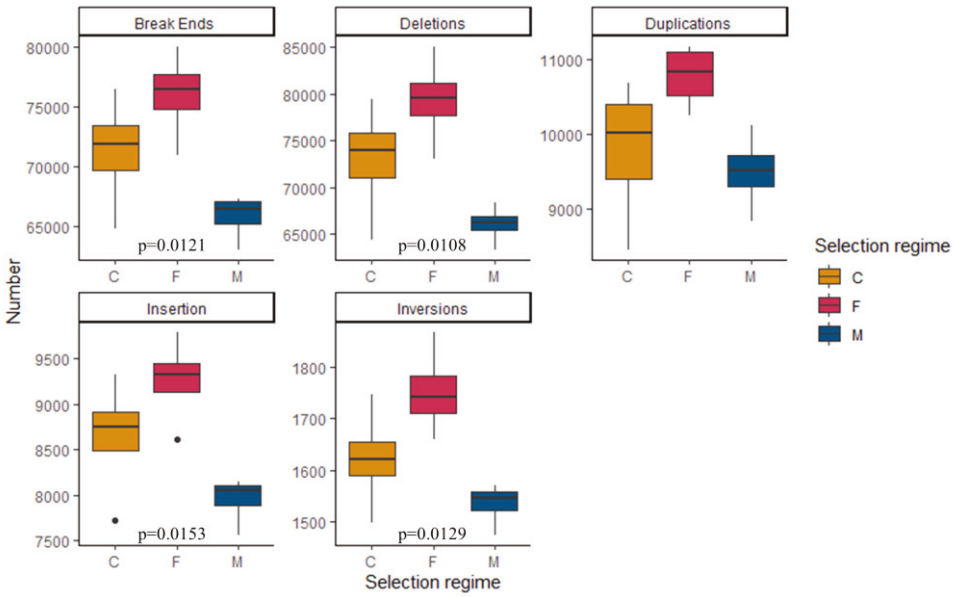


Figure 10 Incidence of different structural variant classes among the selection lines. Significant differences are indicated with p-values (one-way ANOVA) (Paper II).

Finally, we also found that the coverage of the mitochondrial scaffold was significantly higher in the male-selected lines, suggesting that the male-selected lines have evolved higher mitochondrial density (Paper II). This is consistent with our findings of changes in the expression of transcripts related to metabolism (Paper I) and increased sexual activity in the male-selected lines (Paper IV), since mitochondria are responsible for energy production. Moreover, by performing enrichment analyses for the significant SNPs, we found that many GO terms not only relate to metabolism and locomotion, but also to response to stimuli (Paper II), possibly explaining the observed differences in the sucking behaviour (Paper IV) and developmental anatomy, possibly explaining the observed differences in the stylet and body and ovary size (Paper III).

Paper III. Effects of sex-limited experimental evolution on hermaphrodite sexual anatomy

Besides the genomic changes we discovered in the first two papers, what were the actual differences in sex-related phenotypes? To answer this question, we measured body and gonad size, as well as the morphology of the stylet after more than 50 generations of selection. The gonad and body size measurements were taken after exposing worms either to their regular feeding conditions or after one week of starvation in mating groups of three.

We found that the male-selected lines had reduced body and ovary size in regular feeding conditions, but that the difference disappeared under the starvation treatment (fig. 11, 12). A previous study of these selection lines found that higher male fitness was indeed associated with a smaller body size (Nordén, 2017). These findings resemble the evolution of androdioecy, as the male-selected lines seem to have acquired a male-beneficial body size, and reduced investment in the non-rewarding female role. Interestingly, we also observed that our selection lines had reduced plasticity in gonad and body size, since they show more similar size between the feeding treatments compared to the controls, which more significantly reduced the size of all traits when experiencing starvation (fig. 11, 12). This implies that the loss of plasticity in these traits could be an adaption to the experimentally imposed sex roles in the male and female selection lines. Because they are selected under regular feeding conditions, they have presumably canalized their optimal size towards this environment (Paper III).

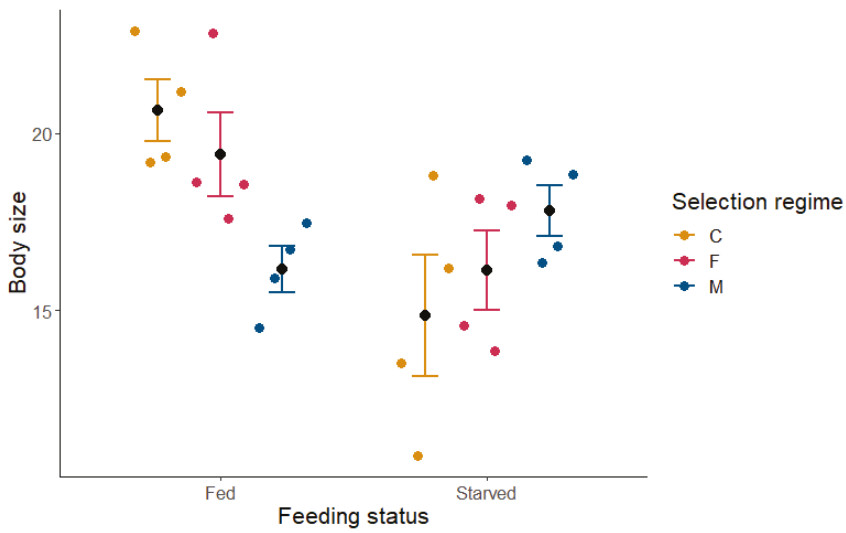


Figure 11 Differences in body size (mm^2/100 +/- SE) between the selection regimes. The only significant difference between the selection regimes is in the fed conditions, where the male-selected lines differ from controls (p=0.03) (Paper III). Points represent population means.

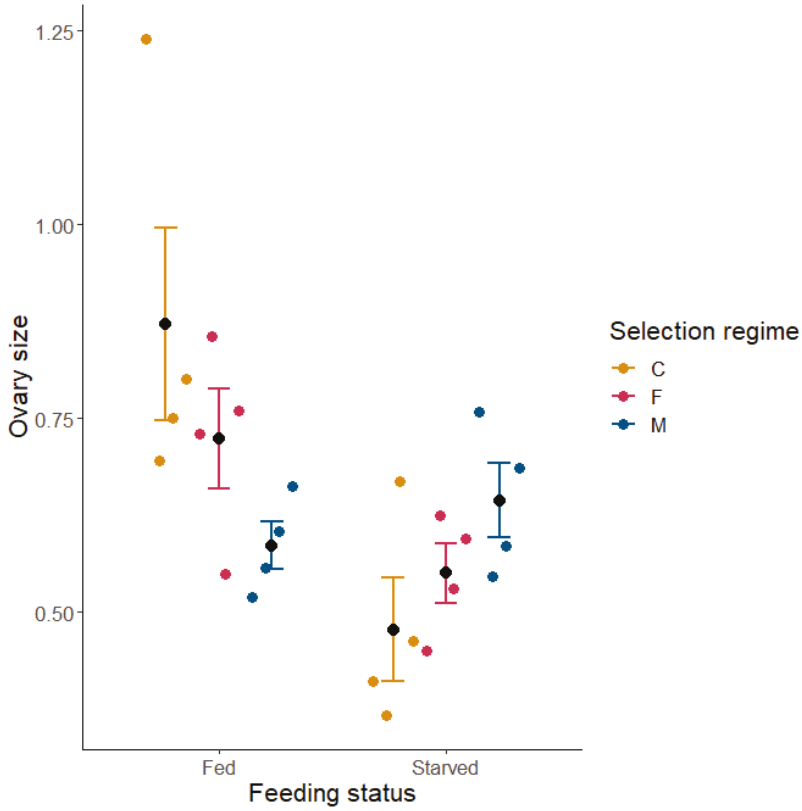


Figure 12 Differences ovary size (mm^2/100 +/- SE) between the selection regimes. The only significant difference between the selection regimes is in the fed conditions, where the male-selected lines differ from controls (p=0.01) (Paper III). Points represent population means.

Finally, we also found changes in the shape of the stylet (fig. 13), by measuring 26 landmarks with equal distances set on the pictures of the stylet, using the Geomorph R package (Baken *et al.*, 2021). However, results regarding the implied differences between male- and female-biased stylets are mixed between different studies, as we discuss in Paper III.

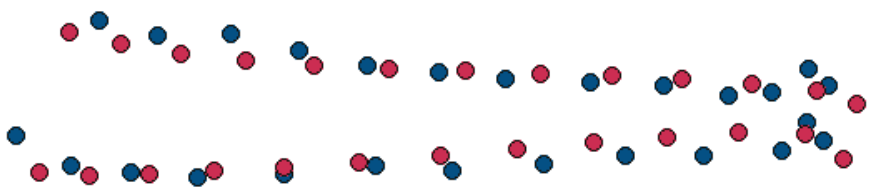


Figure 13 Ten-fold exaggerated stylet differences between male- (blue) and female-selected (red) lines, where shape is significantly different between the selection regimes (p=0.001) (adapted from Paper III).

Paper IV. Effects of sex-limited experimental evolution on mating behaviour in a hermaphrodite

Lastly, we wanted to investigate how the sexual behaviour has changed in our selection lines, as it has also been shown that behaviour can respond rapidly to selection (Lindsay *et al.*, 2019). Therefore, we recorded 2 h long mating videos after more than 40 generations of selection, where each focal worm from one of the selection lines was allowed to mate with two wild-type worms. After that, we counted how many matings the focal worm performed, how many post-mating sucking behaviours the focal worm performed, as well as how many times the wild-type mating partner performed a sucking behaviour after mating with the focal worm. Besides counting the number of matings, we also measured mating duration to later be able to see if there were any relationships between mating frequency, duration and the probability of observing sucking response in the focal worm and/or its partner. Frequency can be seen as a form of quantitative strategy, while duration can be seen as related to quality of insemination, since a longer duration allows the transfer of more ejaculate.

We observed that the male-selected lines mated more often and for a longer duration, in accordance with our predictions, although the difference was not statistically significant (Paper IV). Besides that, we found a significantly increased sucking frequency in the wild-type mating partners of the male-selected lines (fig. 14), which is opposite to our initial expectations. In the paper we discuss several possible explanations and come to the conclusion that the most plausible is that this result is due to conflict over fertilization. The wild-type mating partners come from a much larger mating group of 100, and therefore are expected to be more male-biased in their sex allocation than even the male-selected lines, and therefore primarily motivated to mate in order to donate sperm to the focal worm, rather than receiving sperm from the male-selected partner (Paper IV). However, the differences in stylet shape between selection regimes (Paper III) might also partly explain this result, if a more pointed stylet results in damage to the partner and induces sucking. The somewhat ambiguous mating behaviour results for the adaptation to the sex-limited selection could explain, why male-selected lines have not become better at the male role, when fitness was assessed at generation 14 (Nordén, 2017).

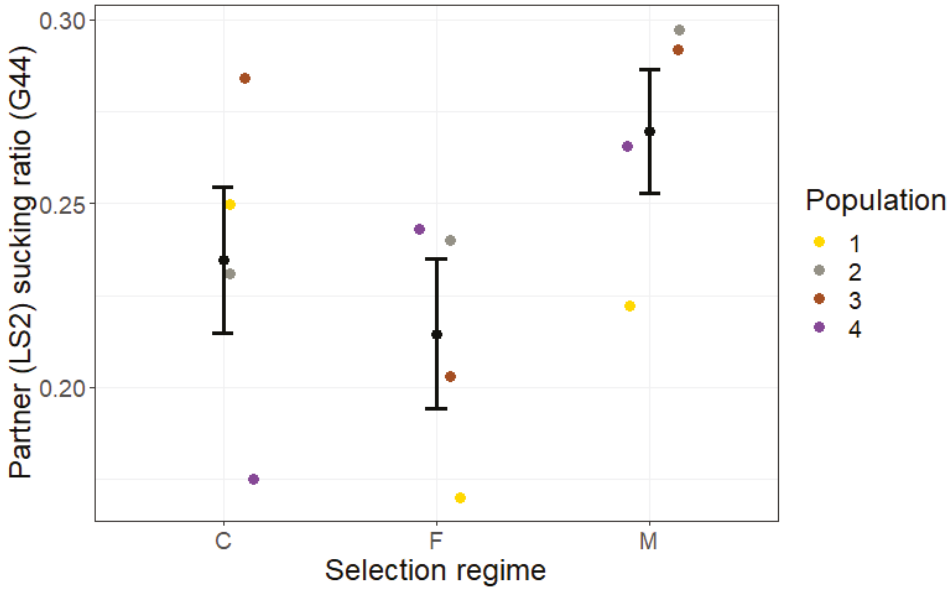


Figure 14 Wild-type partner's probability of sucking after matings with experimental evolution lines at generation 44 (p=0.018) (Paper IV).

Paper V. Effects of degenerated sex-limited chromosomes on non-reproductive traits

Besides the initial stages of sex chromosome evolution, I wanted to investigate what happens later on. When sex chromosomes evolve they become the only consistent difference between the sexes, and thus they become the main source of sex differences, either directly or indirectly. After tens of millions of years, sex chromosomes tend to degenerate and lose most of the genes that were present on the original autosome which they evolved from. Most of these genes initially are not sex-related, however over time sex chromosomes become more specialized. For example, in humans, the Y mainly contains the sex-determining gene and testes genes. Nevertheless, I wanted to know how much of an effect the Y has on sex differences observed in humans and other species beyond the primary sex characteristics. We therefore performed an extensive literature review including the Y chromosome as well as other sex-limited chromosomes: W, U and V. We found that many more traits have been shown to be affected by the Y than we had expected. Despite the fact that testosterone has traditionally been regarded as the main cause of sex differences in mammals (Jost, 1954), we found that the Y can affect virtually all organ systems independently of the effect of testosterone (table 3). We therefore encourage more research on sex-limited chromosomes and their effects on sex

differences in future, despite the fact that they are often difficult to study due to repetitive content and lack of recombination.

Table 3.
Some of the affected traits by Y in mammals (adapted from Paper V)

ORGAN SYSTEM	EXAMPLE
Nervous system	Dopamine system
Cardiovascular system	Lipid profile
Immune system	Autoimmunity
Other	Cancer

Overall patterns in the evolution of gonochorism and sex chromosomes

Origin of gonochorism leads to sexual specialization

As discussed in the introduction, the evolution of gonochorism can be directly linked to the evolution of sex chromosomes, when a sex-determining gene arises on a pair of autosomes (i.e. the GFP gene in our setup). However, the evolution of gonochorism can also be gradual by progressively evolving sex-biased sex allocation. In practice, this means that some hermaphroditic individuals become better at male sex function (and worse at female sex function) in a population, while others begin to specialize in the opposite direction. Below, I discuss how our results relate to predictions about the initial stages in the evolution of gonochorism, specifically what evidence we have that our selection lines have become more and more sexually specialized, and which mechanisms seem to be driving this adaptation.

Our results show that sex-specific phenotypic traits can evolve rapidly, especially male related traits (Paper III; Paper IV), which has also been shown in previous research in other species (Parsch & Ellegren, 2013; Veltsos *et al.*, 2017; Kulikov *et al.*, 2020). One of the proposed ways for rapid adaptive evolution to new environments or selection pressures is a plasticity-first scenario. This theory postulates that phenotypic plasticity precedes genetic evolution, by enabling initial adaptation via already existing gene pathways, allowing populations to acquire a new niche. After this, selection starts to work on these pathways in a constant environment, resulting in the evolution of genetic canalization to fix a trait at its optimal value, and reducing the trait's variability in reaction norms (Levis & Pfennig, 2016). Consistent with this scenario, when we measured a phenotypically plastic trait as body size, we observed reduced plasticity in size under different resource budgets. Specifically, the male-selected lines did not change their size between food treatments, while the controls changed the most (Paper III). It

therefore seems that the male-selected lines may have canalized their body size to its optimal value under the normal *ad libitum* feeding conditions, and have lost the ability to plastically adjust their size after one week of starvation. Conversely, the control lines showed the largest size in the fed conditions and the smallest size in the starved conditions, which suggests that they plastically adjust their size to the available resource budget (Paper III). Despite this, our transcriptomic results were not clearly in line with the plasticity-first scenario. When we compared the direction and magnitude of change in our significantly differentially expressed transcripts with the phenotypically plastic changes in expression observed in Ramm *et al.* (2019), there was no correlation (if anything, the correlation was negative; fig. 15) (Paper I). Therefore, our support for the plasticity-first scenario in our experimental evolution lines is mixed overall.

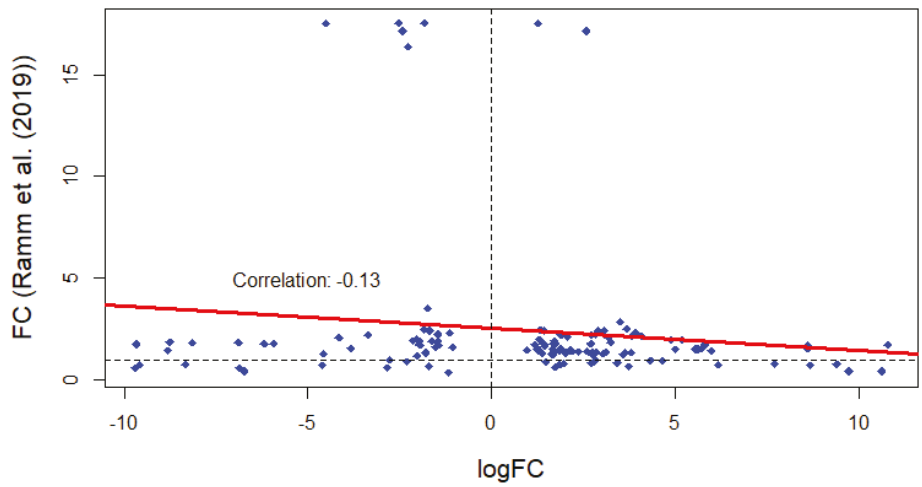


Figure 15 Correlation of fold changes (FC) between our significant transcripts, which match phenotypically plastic transcripts of Ramm *et al*. (2019) (Paper I).

Leaving aside the role of phenotypic plasticity in the evolution of gonochorism, it is clear that there was evidence of substantial genetic adaptation to our selection regimes, but that the response was not symmetric in the male-selected and female-selected lines. For example, in our transcriptome results, we found that testes gene candidates were downregulated in the female-selected lines, but ovary gene candidates were not downregulated in the male-selected lines (Paper I). Similarly, a previous fitness assay found that only the female-selected lines had increased in female fitness and decreased in male fitness, while the male-selected lines were no different from the control lines (Nordén, 2017). In contrast, when it comes to phenotype, we found that the male-selected lines changed the most, displaying considerable behavioural and morphological adaptation to the male role (Paper III;

Paper IV). We can therefore conclude that genomic divergence (Paper I) does not necessarily parallel phenotypic divergence (Paper III; Paper IV), and that it is important to look at different types of data to get a more complete picture of the nature of the response to selection.

Nevertheless, overall our results are consistent with a gradual evolution of gonochorism, where the male-selected lines underwent adaptation to androdioecy and the female-selected lines underwent adaptation to gynodioecy by becoming more sexually specialized.

Accumulation of sexually antagonistic loci

According to the canonical model of sex chromosome evolution, after the evolution of a sex-determining gene, sexually antagonistic alleles are expected to fix close to the sex-determining gene, thus increasing the size of the sex-determining region and favouring cessation of recombination via opposite selection pressures between the sexes and accumulation of genetic differences between the X and Y (Z and W). We have some evidence consistent with fixation of sexually antagonistic alleles from previous phenotypic data (Nordén, 2017). The observed fitness increase in the female role and decrease in the male role in the female-selected lines could be the result of fixation of sexually antagonistic alleles, mediated by differences in metabolism (Paper I; Paper II). Body size may also be a sexually antagonistic trait in our study species (Paper III) as it has been shown that male fitness is higher for smaller individuals (Nordén, 2017). However, we cannot say for certain at this stage that these results are caused by fixation of sexually antagonistic loci rather than parallel fixation of separate female-benefit and male-detriment alleles (or vice versa), since the annotation of the genome is rather poor. It is also not possible to determine whether sexually antagonistic alleles show higher rates of fixation around the GFP locus, since there were so few significant single nucleotide polymorphisms on the GFP scaffold (Paper II). Translocation of sexually antagonistic loci to the GFP scaffold is also unlikely to have occurred over the short timescale of the experiment. Our results are therefore inconclusive overall with respect to accumulation of sexually antagonistic loci around the new sex-determining locus.

Changes in recombination rate around the sex-determining region

As discussed above, cessation of recombination is expected to evolve around the sex-determining region. The increased total number of structural variants in the female-selected lines is consistent with increased recombination rate throughout the genome, presumably because recombination facilitates adaptation via standing genetic variation (Aggarwal *et al.*, 2015). However, when we looked at the GFP scaffold specifically (Paper II), there was no evidence of an increased number of structural variants in the female-selected lines compared to the other two selection

regimes. This suggests that although recombination rate on the GFP scaffold may not be reduced in the female-selected lines compared to the controls, it is reduced compared to the rest of the genome in this selection regime, consistent with selection for recombination arrest around the sex-determining locus. Another possibility is that the accumulation of differences around the sex-determining locus is inhibited by the very plastic nature of *M. lignano*. Although we discussed the plasticity-first scenario above, it has also been proposed that plasticity can inhibit genetic evolution (Santi *et al.*, 2018). This "genetic inhibition" hypothesis posits that if a plastic response is sufficient to achieve the optimal phenotype, then selection for genetic adaptation will be weak. and that is what we see in *M. lignano*, that phenotypically plastic traits (sex allocation) have a lower heritability under experimental evolution than non-plastic traits (stylet) (Janicke *et al.*, 2016). The lack of an obvious evolutionary response on the GFP scaffold may therefore be a result of phenotypic plasticity inhibiting a genetic response, although again we cannot be sure that the timescale of the experiment is not sufficient for changes in recombination rate to evolve around the sex-determining region.

Y – an evolutionary dead end?

Due to the evolution of recombination arrest on the sex-limited chromosomes, they are expected to degenerate, paving the way to the evolution of heteromorphic sex chromosomes, which can greatly differ in size and gene content. Moreover, sex-limited chromosomes, especially Y chromosomes, can be entirely lost. The enormous differences in gene content between heteromorphic sex chromosomes have an important potential role in the evolution of sex differences, as the genomes of males and females become more and more different over time (Paper V). Since our experimental evolution setup was designed to mimic the early stages of sex chromosome evolution, it cannot be used to make inferences about ancient and highly degenerated sex chromosome systems. We therefore turned to the literature to try to answer the question whether the final stages in sex chromosome evolution represent an evolutionary dead-end.

In our review we searched for all examples of sex differences which had been found to be caused by the sex-limited chromosomes, specifically excluding the primary sexual traits since it is already known that this is one of their main functions. With this extensive literature review we could clearly show that recombination cessation does not necessarily mean the inevitable end for these chromosomes, and demonstrated that scarce gene content does not necessarily limit effects on the rest of the body. Specifically, we could show that the Y chromosomes of mammals and *Drosophila* influence surprisingly many (and various) traits despite low gene content. We could also show that traits can be affected not only by sex-linked protein-coding genes, but also by genes coding for non-coding RNAs or heterochromatin. They are important for sexual specialization and may play a potentially important role in alleviating intralocus sexual conflict (Paper V).

Conclusions and future perspectives

This is the first experimental evolution experiment of its kind, where we have imposed sex-limited evolution on a simultaneous hermaphrodite in order to attempt to observe the initial steps of sex chromosome evolution. We used a combination of phenotypic assays and genomic approaches and indeed found some genomic differences on the scaffold where the pseudo-sex-determining GFP gene is located. In addition, we found patterns of adaptive changes consistent with the evolution of gynodioecy in the female-selected lines and androdioecy in the male-selected lines. Although we were successful in detecting evidence of the evolution of sexual specialization, our evidence for structural changes consistent with those expected on nascent sex chromosomes (Bachtrog *et al.*, 2014; Beukeboom & Perrin, 2014) is very limited, probably because many more generations are needed to observe larger genetic changes around the GFP gene.

M. lignano has been a great model organism for the research project since it is a non-self-fertilizing hermaphrodite, which is very easy to maintain in the laboratory with a short generation time. The previously created transgenic GFP line (Wudarski *et al.*, 2017) allowed us to infer sex-limited inheritance with easy phenotyping, which has not shown any negative side-effects. The fact that *M. lignano* is transparent allowed us to easily measure inner organs. Previously established techniques in this species allowed us to confidently measure sexual traits and compare results to similar research. All this resulted in the possibility to observe sexual specialization evolving at all the levels of study.

Nevertheless, for the future experiments in the field, I have three main suggestions. For future studies aiming to use experimental evolution to study the evolution of separate sexes, I suggest using a sexually dimorphic species with a haploid UV sex chromosome system, because these chromosomes are expected to show faster evolution, since both sexes select for a difference (U in females and V in males) (Beukeboom & Perrin, 2014). Genetic changes consistent with theory and comparative data may therefore be possible to detect more easily within such a system. Secondly, I suggest using an artificial sex-determining gene located in a genomic region which already has lower recombination rates, such as the pericentromere (Xue *et al.*, 2021), transposable element rich region or other regions exhibiting heterochiasmy (Edvardsen *et al.*, 2022). As structural changes can occur more rapidly in such cases. Last, but not least, it could be valuable to carry out experiments using model organisms which are not reciprocally mating, since

reciprocal mating and egg-trading are modes of reproduction which are predicted to stabilize hermaphroditism, and thus may hamper the evolution of separate sexes (Schärer & Pen, 2013; Henshaw *et al.*, 2015). The possibility of developing new empirical models of early sex chromosome evolution through experimental evolution may be challenging, but it is not impossible!

Additionally, I would like to propose a new idea which has been inspired by the work I carried out during my thesis, but is not directly related to it. As previously mentioned, it has been suggested that the X chromosome is a hotspot for sexually antagonistic traits, however the evidence is currently mixed (Sayadi *et al.*, 2019; Hitchcock & Gardner, 2020; Manat, 2021). I suggest that the X chromosome in species with random X chromosome inactivation (i.e. mammals) could perhaps be used as a signal for genetic diversity, and therefore be a hot-spot for sexual signalling traits instead. For example, coat colour in female cats is determined through X-chromosome inactivation, where different colour patches reveal parental differences in X-linked colour loci (Niemi & Hao, 2019). In this case, X-linked traits are essentially co-dominant, and could be used as a proxy for genetic diversity. Other studies show that we can detect MHC-derived pheromones unconsciously, and that these pheromones have the potential to influence our reproductive decisions to benefit the genetic makeup of our offspring (Wedekind *et al.*, 1995). Why then could other traits not work in the same way? In the case of cat coat colour, both genetic dissimilarity and diversity could be observed in female cats by male cats. Having a different coat colour from oneself, and/more colourful fur could serve as an indicator trait for genetic differences on other chromosomes, including immune genes. This mechanism may not be very important in cats specifically, which are highly promiscuous. However, the same logic will apply to any other trait in any other animal with random X chromosome inactivation which could be detected in a similar way by the sexual partner. X- or Z-linked traits causing detectable phenotypic differences could therefore serve as potentially important indicator traits for partner genetic diversity or compatibility, especially in monogamous or long-lived species, where such effects should be of greater importance.

References

Abbott, J.K., Chippindale, A.K. & Morrow, E.H. 2020. The microevolutionary response to male-limited X-chromosome evolution in *Drosophila melanogaster* reflects macroevolutionary patterns. *J. Evol. Biol.* **33**: 738–750.

Aggarwal, D.D., Rashkovetsky, E., Michalak, P., Cohen, I., Ronin, Y., Zhou, D., *et al.* 2015. Experimental evolution of recombination and crossover interference in Drosophila caused by directional selection for stress-related traits. *BMC Biol.* **13**: 101.

Andersen, R., Berges, J., Harrison, P. & Watanabe, M. 2005. Recipes for freshwater and seawater media. In: *Algal Culturing Techniques* (R. A. Andersen, ed), pp. 429–538. Elsevier, Amsterdam.

Anderson, N.W., Hjelmen, C.E. & Blackmon, H. 2020. The probability of fusions joining sex chromosomes and autosomes. *Biol. Lett.* **16**: 20200648.

Arbore, R., Sekii, K., Beisel, C., Ladurner, P., Berezikov, E. & Schärer, L. 2015. Positional RNA-Seq identifies candidate genes for phenotypic engineering of sexual traits. *Front. Zool.* **12**: 14.

Avise, J.C. 2011. *Hermaphroditism: A Primer on the Biology, Ecology, and Evolution of Dual Sexuality*. Columbia University Press, New York.

Azlan, A., Halim, M.A. & Azzam, G. 2020. Genome-wide identification and characterization of long intergenic noncoding RNAs in the regenerative flatworm *Macrostomum lignano*. *Genomics* **112**: 1273–1281.

Bachtrog, D. 2008. The temporal dynamics of processes underlying Y chromosome degeneration. *Genetics* **179**: 1513–1525.

Bachtrog, D. 2013. Y-chromosome evolution: emerging insights into processes of Y-chromosome degeneration. *Nat. Rev. Genet.* **14**: 113–124.

Bachtrog, D., Kirkpatrick, M., Mank, J.E., McDaniel, S.F., Pires, J.C., Rice, W.R., *et al.* 2011. Are all sex chromosomes created equal? *Trends Genet.* **27**: 350–357.

Bachtrog, D., Mank, J.E., Peichel, C.L., Kirkpatrick, M., Otto, S.P., Ashman, T., *et al.* 2014. Sex determination: Why so many ways of doing it? *PLOS Biol.* **12**: e1001899.

Baken, E.K., Collyer, M.L., Kaliontzopoulou, A. & Adams, D.C. 2021. geomorph v4.0 and gmShiny: Enhanced analytics and a new graphical interface for a comprehensive morphometric experience. *Methods Ecol. Evol.* **12**: 2355–2363.

Bateman, A.J. 1948. Intra-sexual selection in Drosophila. *Heredity (Edinb).* **2**: 349–368.

Beukeboom, L.W. & Perrin, N. 2014. *The evolution of sex determination*. Oxford University Press.

Bissegger, M., Laurentino, T.G., Roesti, M. & Berner, D. 2020. Widespread intersex differentiation across the stickleback genome – The signature of sexually antagonistic

selection? *Mol. Ecol.* **29**: 262–271.

Blaser, O., Grossen, C., Neuenschwander, S. & Perrin, N. 2013. Sex-chromosome turnovers induced by deleterious mutation load. *Evolution (N. Y).* **67**: 635–645.

Bolger, A.M., Lohse, M. & Usadel, B. 2014. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* **30**: 2114–2120.

Bonel, N., Noël, E., Janicke, T., Sartori, K., Chapuis, E., Ségard, A., *et al.* 2018. Asymmetric evolutionary responses to sex-specific selection in a hermaphrodite. *Evolution (N. Y).* **72**: 2181–2201.

Borodin, P.M., Basheva, E.A., Torgasheva, A.A., Dashkevich, O.A., Golenishchev, F.N., Kartavtseva, I. V., *et al.* 2012. Multiple independent evolutionary losses of XY pairing at meiosis in the grey voles. *Chromosom. Res.* **20**: 259–268.

Bull, J.J. 1983. *Evolution of sex determining mechanisms*. The Benjamin/Cummings Publishing Company, Inc.

Chapman, T., Arnqvist, G., Bangham, J. & Rowe, L. 2003. Sexual conflict. *Trends Ecol. Evol.* **18**: 41–47.

Charlesworth, D. 2019. Young sex chromosomes in plants and animals. *New Phytol.* **224**: 1095–1107.

Charlesworth, D. & Charlesworth, B. 2010. Evolutionary biology: The origins of two sexes. *Curr. Biol.* **20**: R519–R521.

Charlesworth, D. & Mank, J.E. 2010. The birds and the bees and the flowers and the trees: Lessons from genetic mapping of sex determination in plants and animals. *Genetics* **186**: 9–31.

Charnov, E.L. 1979. Simultaneous hermaphroditism and sexual selection. *Proc. Natl. Acad. Sci. U. S. A.* **76**: 2480–2484.

Cirulis, A. 2016. Separating the sexes : sexual conflict and how to resolve it. Uppsala University.

Coelho, S.M., Mignerot, L. & Cock, J.M. 2019. Origin and evolution of sex-determination systems in the brown algae. *New Phytol.* **222**: 1751–1756.

Darwin, C. 1859. *On the origins of species by means of natural selection, or the preservation of favoured races in the struggle for life*.

Darwin, C. 1871. *The descent of man, and selection in relation to sex*. D. Appleton.

Dufresnes, C., Litvinchuk, S.N., Rozenblut-Koscisty, B., Rodrigues, N., Perrin, N., Crochet, P.-A., *et al.* 2020. Hybridization and introgression between toads with different sex chromosome systems. *Evol. Lett.* **4**–**5**: 444–456.

Dutoit, L., Mugal, C.F., Bolívar, P., Wang, M., Nadachowska-Brzyska, K., Smeds, L., *et al.* 2018. Sex-biased gene expression, sexual antagonism and levels of genetic diversity in the collared flycatcher (Ficedula albicollis) genome. *Mol. Ecol.* **27**: 3572–3581.

Edvardsen, R.B., Wallerman, O., Furmanek, T., Kleppe, L., Jern, P., Wallberg, A., *et al.* 2022. Heterochiasmy and the establishment of *gsdf* as a novel sex determining gene in Atlantic halibut. *PLoS Genet.* **18**: e1010011.

Edward, D.A., Stockley, P. & Hosken, D.J. 2015. Sexual conflict and sperm competition. *Cold Spring Harb. Perspect. Biol.* **7**: a017707.

Ellegren, H. 2011. Sex-chromosome evolution: recent progress and the influence of male and female heterogamety. *Nat. Rev. Genet.* **12**: 157–166.

Ellegren, H. & Fridolfsson, A.-K. 1997. Male–driven evolution of DNA sequences in birds. *Nat. Genet.* **17**: 182–184.

Ellegren, H., Smeds, L., Burri, R., Olason, P.I., Backström, N., Kawakami, T., *et al.* 2012. The genomic landscape of species divergence in *Ficedula* flycatchers. *Nature* **491**: 756–760.

Ellison, C. & Bachtrog, D. 2019. Recurrent gene co-amplification on Drosophila X and Y chromosomes. *PLoS Genet.* **15**: e1008251.

Ewels, P.A., Peltzer, A., Fillinger, S., Alneberg, J., Patel, H., Wilm, A., *et al.* 2019. nf-core: Community curated bioinformatics pipelines. *bioRxiv* 610741.

Furman, B.L.S., Metzger, D.C.H., Darolti, I., Wright, A.E., Sandkam, B.A., Almeida, P., *et al.* 2020. Sex chromosome evolution: So many exceptions to the rules. *Genome Biol. Evol.* **12**:750-763.

Gavrilets, S. 2014. Is sexual conflict an "'engine of speciation'"? *Cold Spring Harb. Perspect. Biol.* **6**: a017723.

Giannakara, A., Schärer, L. & Ramm, S.A. 2016. Sperm competition-induced plasticity in the speed of spermatogenesis. *BMC Evol. Biol.* **16**: 60.

Goodwin, S., McPherson, J.D. & McCombie, W.R. 2016. Coming of age: Ten years of next-generation sequencing technologies. *Nat. Rev. Genet.* **17**: 333–351.

Grath, S. & Parsch, J. 2016. Sex-biased gene expression. *Annu. Rev. Genet.* **50**: 29–44.

Griffin, R. 2015. The genetic architecture of sexual dimorphism. Uppsala University.

Grudniewska, M., Mo, S., Grelling, M., Wo, A.H.G., Kuipers, J., Giepmans, B.N.G., *et al.* 2018. A novel flatworm-specific gene implicated in reproduction in *Macrostomum lignano*. *Sci. Rep.* **8**: 3192.

Gruetzner, F., Ashley, T., Rowell, D.M. & Graves, J.A.M. 2006. How did the platypus get its sex chromosome chain? A comparison of meiotic multiples and sex chromosomes in plants and animals. *Chromosoma* **115**: 75–88.

Heath, D.J. 1977. Simultaneous hermaphroditism; cost and benefit. *J. Theor. Biol.* **64**: 363–373.

Henshaw, J.M., Kokko, H. & Jennions, M.D. 2015. Direct reciprocity stabilizes simultaneous hermaphroditism at high mating rates: A model of sex allocation with egg trading. *Evolution (N. Y).* **69**: 2129–2139.

Hitchcock, T.J. & Gardner, A. 2020. A gene's-eye view of sexual antagonism. *Proc. R. Soc. B* **287**: 20201633.

Hughes, J.F., Skaletsky, H., Brown, L.G., Pyntikova, T., Graves, T., Fulton, R.S., *et al.* 2012. Strict evolutionary conservation followed rapid gene loss on human and rhesus Y chromosomes. *Nature* **483**: 82–87.

Immler, S. & Otto, S.P. 2015. The evolution of sex chromosomes in organisms with separate haploid sexes. *Evolution (N. Y).* **69**: 694–708.

Ingleby, F.C., Flis, I. & Morrow, E.H. 2015. Sex-biased gene expression and sexual conflict throughout development. *Cold Spring Harb. Perspect. Biol.* **7**: a017632.

Jaenike, J. 2001. Sex chromosome meiotic drive. *Annu. Rev. Ecol. Syst.* **32**: 25–49.

Janicke, T., Sandner, P., Ramm, S.A., Vizoso, D.B. & Schärer, L. 2016. Experimentally evolved and phenotypically plastic responses to enforced monogamy in a hermaphroditic flatworm. *J. Evol. Biol.* **29**: 1713–1727.

Janicke, T. & Schärer, L. 2009. Determinants of mating and sperm-transfer success in a simultaneous hermaphrodite. *J. Evol. Biol.* **22**: 405–415.

Jost, A. 1954. Hormonal factors in the development of the fetus. *Cold Spring Harb Symp Quant Biol* **19**: 167–181.

Koerich, L.B., Wang, X., Clark, A.G. & Carvalho, A.B. 2008. Low conservation of gene content in the Drosophila Y chromosome. *Nature* **456**: 949–951.

Kulikov, A.M., Sorokina, S.Y.U., Melnikov, A.I., Gornostaev, N.G., Seleznev, D.G. & Lazebny, O.E. 2020. The effects of the sex chromosomes on the inheritance of species-specific traits of the copulatory organ shape in *Drosophila virilis* and *Drosophila lummei*. *PLoS One* **15**: e0244339.

Ladurner, P., Rieger, R. & Baguñà, J. 2000. Spatial distribution and differentiation potential of stem cells in hatchlings and adults in the marine platyhelminth *Macrostomum* sp.: A bromodeoxyuridine analysis. *Dev. Biol.* **226**: 231–241.

Ladurner, P., Schärer, L., Salvenmoser, W. & Rieger, R.M. 2005. A new model organism among the lower Bilateria and the use of digital microscopy in taxonomy of meiobenthic Platyhelminthes: *Macrostomum lignano*, n . sp . (Rhabditophora , Macrostomorpha). *J. Zool. Syst. Evol. Res.* **43**: 114–126.

Lengerer, B., Wunderer, J., Pjeta, R., Carta, G., Kao, D., Aboobaker, A., *et al.* 2018. Organ specific gene expression in the regenerating tail of *Macrostomum lignano*. *Dev. Biol.* **433**: 448–460.

Leonard, J.L. 1993. Sexual conflict in simultaneous hermaphrodites: evidence from serranid fishes. *Environ. Biol. Fishes* **36**: 135–148.

Leonard, J.L. 2006. Sexual selection: Lessons from hermaphrodite mating systems. *Integr. Comp. Biol.* **46**: 349–367.

Levis, N.A. & Pfennig, D.W. 2016. Evaluating “plasticity-first” evolution in nature: Key criteria and empirical approaches. *Trends Ecol. Evol.* **31**: 563–574.

Li, H. & Durbin, R. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**: 1754–1760.

Lindsay, W.R., Andersson, S., Bererhi, B., Höglund, J., Johnsen, A., Kvarnemo, C., *et al.* 2019. Endless forms of sexual selection. *PeerJ* **7**: e7988.

Line, A., Andrejeva, D., Cirulis, A., Abols, A. & Zayakin, P. 2014. 344: Characterisation of RNA content of cancer-derived exosomes and microvesicles. *Eur. J. Cancer* **50**: S82.

Link, J.C. & Reue, K. 2017. Genetic basis for sex differences in obesity and lipid metabolism. *Annu. Rev. Nutr.* **37**: 225–245.

Lodi, M. & Koene, J.M. 2017. Hidden female physiological resistance to male accessory gland substances in a simultaneous hermaphrodite. *J. Exp. Biol.* **220**: 1026–1031.

Long, M., Vibranovski, M.D. & Zhang, Y.E. 2012. Evolutionary interactions between sex chromosomes and autosomes. In: *Rapidly Evolving Genes and Genetic Systems* (R. S. Singh, J. Xu, & R. J. Kulathinal, eds), pp. 101–114. Oxford University Press.

Mahajan, S. & Bachtrog, D. 2017. Convergent evolution of Y chromosome gene content in flies. *Nat. Commun.* **8**: 785.

Manat, Y. 2021. The genomic basis of the response to female-limited X-chromosome evolution. Media-Tryck, Lund.

Mank, J.E., Hosken, D.J. & Wedell, N. 2014. Conflict on the sex chromosomes: Cause, effect, and complexity. *Cold Spring Harb. Perspect. Biol.* **6**: a017715.

Marais, G.A.B., Gaillard, J.M., Vieira, C., Plotton, I., Sanlaville, D., Gueyffier, F., *et al.* 2018. Sex gap in aging and longevity: can sex chromosomes play a role? *Biol. Sex Differ.* **9**: 33.

Margulies, M., Egholm, M., Altman, W.E., Attiya, S., Bader, J.S., Bemben, L.A., *et al.* 2005. Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**: 376–380.

Marie-Orleach, L., Janicke, T. & Schärer, L. 2013. Effects of mating status on copulatory and postcopulatory behaviour in a simultaneous hermaphrodite. *Anim. Behav.* **85**: 453–461.

Marie-Orleach, L., Janicke, T., Vizoso, D.B., David, P. & Schärer, L. 2016. Quantifying episodes of sexual selection: Insights from a transparent worm with fluorescent sperm. *Evolution (N. Y).* **70**: 314–328.

Marie-Orleach, L., Janicke, T., Vizoso, D.B., Eichmann, M. & Schärer, L. 2014. Fluorescent sperm in a transparent worm: Validation of a GFP marker to study sexual selection. *BMC Evol. Biol.* **14**: 148.

Marie-Orleach, L., Vogt-Burri, N., Mouginot, P., Schlatter, A., Vizoso, D.B., Bailey, N.W., *et al.* 2017. Indirect genetic effects and sexual conflicts: Partner genotype influences multiple morphological and behavioral reproductive traits in a flatworm. *Evolution (N. Y).* **71**: 1232–1245.

Martin, H., Carpentier, F., Gallina, S., Gode, C., Schmitt, E., Muyle, A., *et al.* 2019. Evolution of young sex chromosomes in two dioecious sister plant species with distinct sex determination systems. *Genome Biol. Evol.* **11**: 350–361.

McCabe, J. 1994. Reproductive strategies in an aquatic crustacean. Imperial College.

Michiels, N.K. 1998. Mating conflicts and sperm competition in simultaneous hermaphrodites. In: *Sperm Competition and Sexual Selection* (T. Birkhead & A. P. Møller, eds), pp. 219–254. Academic Press, London.

Ming, R., Bendahmane, A. & Renner, S.S. 2011. Sex chromosomes in land plants. *Annu. Rev. Plant Biol.* **62**: 485–514.

Mirzaghaderi, G. & Hörandl, E. 2016. The evolution of meiotic sex and its alternatives. *Proc. R. Soc. B* **283**: 20161221.

Mouton, S., Willems, M., Back, P., Braeckman, B.P. & Borgonie, G. 2009. Demographic analysis reveals gradual senescence in the flatworm *Macrostomum lignano*. *Front. Zool.* **6**: 15.

Muyle, A., Bachtrog, D., Marais, G.A.B. & Turner, J.M.A. 2021. Epigenetics drive the evolution of sex chromosomes in animals and plants. *Philos. Trans. R. Soc. B Biol. Sci.* **376**: 20200124.

Natri, H.M., Merilä, J. & Shikano, T. 2019. The evolution of sex determination associated

with a chromosomal inversion. *Nat. Commun.* **10**: 145.

Niemi, S. & Hao, W. 2019. X marks the spot : How X chromosome inactivation gives females an advantage. *Front. Young Minds* **7**: 134.

Nieuwenhuis, B.P.S. 2012. Sexual selection in fungi. Wageningen University.

Noël, E., Fruitet, E., Lelaurin, D., Bonel, N., Ségard, A., Sarda, V., *et al.* 2019. Sexual selection and inbreeding: Two efficient ways to limit the accumulation of deleterious mutations. *Evol. Lett.* **3**: 80–92.

Nordén, A. 2017. Sex chromosome evolution in a hermaphrodite: Genetic and phenotypic relationships between sex roles. Lund University.

Parker, G.A. 1979. Sexual selection and sexual conflict. In: *Sexual Selection and Reproductive Competition in Insects* (M. S. Blum & N. A. Blum, eds), pp. 123–166. Academic Press.

Parker, G.A. 2014. The sexual cascade and the rise of pre-ejaculatory (Darwinian) sexual selection, sex roles, and sexual conflict. *Cold Spring Harb. Perspect. Biol.* **6**: a017509.

Parsch, J. & Ellegren, H. 2013. The evolutionary causes and consequences of sex-biased gene expression. *Nat. Rev. Genet.* **14**: 83–87.

Patlar, B., Weber, M., Temizyürek, T. & Ramm, S.A. 2020. Seminal fluid-mediated manipulation of post-mating behavior in a simultaneous hermaphrodite. *Curr. Biol.* **30**: 143–149.

Patro, R., Duggal, G., Love, M.I., Irizarry, R.A. & Kingsford, C. 2017. Salmon provides fast and bias-aware quantification of transcript expression. *Nat. Methods* **14**: 417–419.

Pélissié, B., Jarne, P. & David, P. 2012. Sexual selection without sexual dimorphism: Bateman gradients in a simultaneous hermaphrodite. *Evolution (N. Y).* **66**: 66–81.

Peneder, P., Wallner, B. & Vogl, C. 2017. Exchange of genetic information between therian X and Y chromosome gametologs in old evolutionary strata. *Ecol. Evol.* **7**: 8478–8487.

Pennell, M.W., Kirkpatrick, M., Otto, S.P., Vamosi, J.C., Peichel, C.L., Valenzuela, N., *et al.* 2015. Y fuse? Sex chromosome fusions in fishes and reptiles. *PLOS Genet.* **11**: e1005237.

Pennell, T.M. & Morrow, E.H. 2013. Two sexes, one genome: the evolutionary dynamics of intralocus sexual conflict. *Ecol. Evol.* **3**: 1819–1834.

Perry, J.C. & Rowe, L. 2015. The evolution of sexually antagonistic phenotypes. *Cold Spring Harb. Perspect. Biol.* **7**: a017558.

Piergentili, R. 2010. Multiple roles of the Y chromosome in the biology of *Drosophila melanogaster*. *Sci. World J.* **10**: 1749–1767.

Rafati, N., Chen, J., Herpin, A., Pettersson, M.E., Han, F., Feng, C., *et al.* 2020. Reconstruction of the birth of a male sex chromosome present in Atlantic herring. *Proc. Natl. Acad. Sci. U. S. A.* **117**: 24359–24368.

Ramm, S.A. 2017. Exploring the sexual diversity of flatworms: Ecology, evolution, and the molecular biology of reproduction. *Mol. Reprod. Dev.* **84**: 120–131.

Ramm, S.A., Schärer, L., Lengerer, B., Arbore, R., Pjeta, R., Wunderer, J., *et al.* 2019. Sex allocation plasticity on a transcriptome scale: Socially sensitive gene expression in a simultaneous hermaphrodite. *Mol. Ecol.* **28**: 2321–2341.

Reinhardt, K., Anthes, N. & Lange, R. 2015. Copulatory wounding and traumatic insemination. *Cold Spring Harb. Perspect. Biol.* **7**: a017582.

Reinhardt, K., Naylor, R. & Siva-Jothy, M.T. 2003. Reducing a cost of traumatic insemination: female bedbugs evolve a unique organ. *Proc. R. Soc. B* **270**: 2371–2375.

Renner, S.S. & Ricklefs, R.E. 1995. Dioecy and its correlates in the flowering plants. *Am. J. Bot.* **82**: 596–606.

Rhen, T. & Crews, D. 2007. Why are there two sexes? In: *Sex Differences in the Brain: From Genes to Behavior* (J. B. Becker, K. J. Berkley, N. Geary, E. Hampson, J. P. Herman, & E. Young, eds), pp. 3–14. Oxford University Press, New York.

Rice, W.R. 1984. Sex chromosomes and the evolution of sexual dimorphism. *Evolution (N. Y).* **38**: 735–742.

Robinson, M.D., McCarthy, D.J. & Smyth, G.K. 2010. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**: 139–140.

Rowe, G., Sweet, M. & Beebee, T. 2017. *An introduction to Molecular Ecology*, Third Edit. Oxford University Press.

Sanger, F., Nicklen, S. & Coulson, A.R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U. S. A.* **74**: 5463–5467.

Santi, M., Picchi, L. & Lorenzi, M.C. 2018. Dynamic modulation of reproductive strategies in a simultaneous hermaphrodite and preference for the male role. *Anim. Behav.* **146**: 87–96.

Saunders, P.A., Neuenschwander, S. & Perrin, N. 2018. Sex chromosome turnovers and genetic drift: a simulation study. *J. Evol. Biol.* **31**: 1413–1419.

Sayadi, A., Martinez Barrio, A., Immonen, E., Dainat, J., Berger, D., Tellgren-Roth, C., *et al.* 2019. The genomic footprint of sexual conflict. *Nat. Ecol. Evol.* **3**: 1725–1730.

Schärer, L. 2009. Tests of sex allocation theory in simultaneously hermaphroditic animals. *Evolution (N. Y).* **63**: 1377–1405.

Schärer, L. & Janicke, T. 2009. Sex allocation and sexual conflict in simultaneously hermaphroditic animals. *Biol. Lett.* **5**: 705–708.

Schärer, L., Janicke, T. & Ramm, S.A. 2015. Sexual conflict in hermaphrodites. *Cold Spring Harb. Perspect. Biol.* **7**: a017673.

Schärer, L., Joss, G. & Sandner, P. 2004. Mating behaviour of the marine turbellarian *Macrostomum* sp.: these worms *suck*. *Mar. Biol.* **145**: 373–380.

Schärer, L. & Ladurner, P. 2003. Phenotypically plastic adjustment of sex allocation in a simultaneous hermaphrodite. *Proc. R. Soc. B Biol. Sci.* **270**: 935–941.

Schärer, L. & Pen, I. 2013. Sex allocation and investment into pre- and post-copulatory traits in simultaneous hermaphrodites: the role of polyandry and local sperm competition. *Philos. Trans. R. Soc. B Biol. Sci.* **368**: 20120052.

Schärer, L. & Ramm, S.A. 2016. Hermaphrodites. In: *Encyclopedia of Evolutionary Biology* (R. M. Kliman, ed), pp. 212–224. Academic Press.

Schärer, L., Sandner, P. & Michiels, N.K. 2005. Trade-off between male and female allocation in the simultaneously hermaphroditic flatworm *Macrostomum* sp. *J. Evol. Biol.* **18**: 396–404.

Schenkel, M.A., Pen, I., Beukeboom, L.W. & Billeter, J.C. 2018. Making sense of intralocus and interlocus sexual conflict. *Ecol. Evol.* **8**: 13035–13050.

Schneider, J.M. 2014. Sexual cannibalism as a manifestation of sexual conflict. *Cold Spring Harb. Perspect. Biol.* **6**: a017731.

Schuster, S.C. 2008. Next-generation sequencing transforms today's biology. *Nat. Methods* **5**: 16–18.

Scott, M.F., Osmond, M.M. & Otto, S.P. 2018. Haploid selection, sex ratio bias, and transitions between sex-determining systems. *PLoS Biol.* **16**: e2005609.

Sherratt, T.N. & Wilkinson, D.M. 2009. *Big Questions in Ecology and Evolution*. Oxford University Press, New York.

Sigeman, H. 2021. Evolution of sex chromosomes in Sylvioidea songbirds. Media-Tryck, Lund.

Sirot, L.K., Wong, A., Chapman, T. & Wolfner, M.F. 2015. Sexual conflict and seminal fluid proteins: A dynamic landscape of sexual interactions. *Cold Spring Harb. Perspect. Biol.* **7**: a017533.

Skvortsova, K., Iovino, N. & Bogdanović, O. 2018. Functions and mechanisms of epigenetic inheritance in animals. *Nat. Rev. Mol. Cell Biol.* **19**: 774–790.

Soh, Y.Q.S., Alföldi, J., Pyntikova, T., Brown, L.G., Graves, T., Minx, P.J., *et al.* 2014. Sequencing the mouse Y chromosome reveals convergent gene acquisition and amplification on both sex chromosomes. *Cell* **159**: 800–813.

Tanurdzic, M. & Banks, J.A. 2004. Sex-determining mechanisms in land plants. *Plant Cell* **16**: S61–S71.

Team, R.C. 2020. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna.

van Doorn, G.S. 2014. Patterns and mechanisms of evolutionary transitions between genetic sex-determining systems. *Cold Spring Harb. Perspect. Biol.* **6**: a017681.

Veltsos, P., Fang, Y., Cossins, A.R., Snook, R.R. & Ritchie, M.G. 2017. Mating system manipulation and the evolution of sex-biased gene expression in Drosophila. *Nat. Commun.* **8**: 2072.

Veltsos, P., Ridout, K.E., Toups, M.A., Gonzalez-Martinez, S.C., Muyle, A., Emery, O., *et al.* 2019. Early sex-chromosome evolution in the diploid dioecious plant *Mercurialis annua*. *Genetics* **212**: 815–835.

Vicoso, B., Emerson, J.J., Zektser, Y., Mahajan, S. & Bachtrog, D. 2013a. Comparative sex chromosome genomics in snakes: Differentiation, evolutionary strata, and lack of global dosage compensation. *PLoS Biol.* **11**: e1001643.

Vicoso, B., Kaiser, V.B. & Bachtrog, D. 2013b. Sex-biased gene expression at homomorphic sex chromosomes in emus and its implication for sex chromosome evolution. *Proc. Natl. Acad. Sci. U. S. A.* **110**: 6453–6458.

Vizoso, D.B., Rieger, G. & Schärer, L. 2010. Goings-on inside a worm: functional hypotheses derived from sexual conflict thinking. *Biol. J. Linn. Soc.* **99**: 370–383.

Vizoso, D.B. & Schärer, L. 2007. Resource-dependent sex-allocation in a simultaneous hermaphrodite. *J. Evol. Biol.* **20**: 1046–1055.

Wang, Q., Xue, Y., Zhang, Y., Long, Q., Yang, F., Turner, D.J., *et al.* 2013. Genetic basis

of Y-linked hearing impairment. *Am. J. Hum. Genet.* **92**: 301–306.

Wasik, K., Gurtowski, J., Zhou, X., Ramos Mendivil, O., Delás, M.J., Battistoni, G., *et al.* 2015. Genome and transcriptome of the regeneration- competent flatworm, *Macrostomum lignano*. *Proc. Natl. Acad. Sci. U. S. A.* **112**: 12462–12467.

Weber, M., Patlar, B. & Ramm, S.A. 2020. Effects of two seminal fluid transcripts on post-mating behaviour in the simultaneously hermaphroditic flatworm *Macrostomum lignano*. *J. Evol. Biol.* **33**:714-726.

Weber, M., Wunderer, J., Lengerer, B., Pjeta, R., Rodrigues, M., Schärer, L., *et al.* 2018. A targeted in situ hybridization screen identifies putative seminal fluid proteins in a simultaneously hermaphroditic flatworm. *BMC Evol. Biol.* **18**: 81.

Wedekind, C., Seebeck, T., Bettens, F. & Paepke, A.J. 1995. MHC-dependent mate preferences in humans. *Proc. R. Soc. B Biol. Sci.* **260**: 245–249.

Weeks, S.C., Benvenuto, C. & Reed, S.K. 2006. When males and hermaphrodites coexist: A review of androdioecy in animals. *Integr. Comp. Biol.* **46**: 449–464.

West, S. 2009. *Sex Allocation*. Princeton University Press.

Wiberg, R.A.W., Gaggiotti, O.E., Morrissey, M.B. & Ritchie, M.G. 2017. Identifying consistent allele frequency differences in studies of stratified populations. *Methods Ecol. Evol.* **8**: 1899–1909.

Wudarski, J., Simanov, D., Ustyantsev, K., Mulder, K. De, Grelling, M., Grudniewska, M., *et al.* 2017. Efficient transgenesis and annotated genome sequence of the regenerative flatworm model *Macrostomum lignano*. *Nat. Commun.* **8**: 2120.

Xue, L., Gao, Y., Wu, M., Tian, T., Fan, H., Huang, Y., *et al.* 2021. Telomere-to-telomere assembly of a fish Y chromosome reveals the origin of a young sex chromosome pair. *Genome Biol.* **22**: 203.

Zadesenets, K.S., Schärer, L. & Rubtsov, N.B. 2017. New insights into the karyotype evolution of the free-living flatworm *Macrostomum lignano* (Platyhelminthes, Turbellaria). *Sci. Rep.* **7**: 6066.

Zadesenets, K.S., Vizoso, D.B., Schlatter, A., Konopatskaia, I.D., Berezikov, E., Schärer, L., *et al.* 2016. Evidence for karyotype polymorphism in the free-living flatworm, *Macrostomum lignano*, a model organism for evolutionary and developmental biology. *PLoS One* **11**: e0164915.

Acknowledgments

I am grateful for this amazing opportunity to do my PhD in Lund University, Sweden and particularly in the very active department as the Biology department is. I have had amazing experience in my graduate research school **GENECO**. I want to particularly acknowledge people below.

I am particularly grateful to my PhD supervisor **Jessica** for being able to do my PhD here and on this topic. Thank you for providing an open environment and help along the way, especially with statistics and language. Although at some points it has been hard, however we have managed to finish all the things we wanted and I have learned and developed a lot during the process.

I also want to thank my co-supervisors **Bengt** and **Nils** for always attending the meetings, when possible, and providing advice and checking on me. I am additionally thankful to **Bengt** for the interesting SexGen research group meetings we had and the interesting review we wrote together.

I am also thankful for my examiner **Lars-Anders**, who has been very supportive and helped to organize my PhD.

I also want to thank my scientific mentor **Charlie**, who chatted with me and played football and floorball at the department.

I also would like to thank to the head of the unit of Evolutionary Ecology **Jan-Åke** for the support and the great unit Christmas meetings!

I want to thank all the past and present lab and **SexGen** members. **Qinyang**, thanks for being a good colleague and friend. We had some interesting times both at work and outside it. You are my favourite spex actor! **Katrine**, thank you for being such a great lab member. Chatting with you in the lab during the lockdown was very pleasant, and I have enjoyed all the parties with you. **Anna**, thank you for the very nice introduction to the lab and successfully starting this project. **Robin**, thank you for the help with the experiment and the stimulating conversations we had. **Colin**, thank you for leading the SexGen meetings, it is fun to see someone so enthusiastic about mathematical biology and learn something from modelling. **Yesbol**, thank you for being such a good colleague to talk to and the fun we had playing team sports at work. **Martin**, thank you for doing such a great job as a bachelor student! **Vignesh**, thank you for the enormous help with bioinformatics and showing how cool python is! **Damandeep**, thank you for the nice conversations we had in the lab,

I wish you all the best with your PhD. **Simon**, thank you for the drives to the work parties and good luck with your PhD! **Pallavi**, thank you for the help with my initial steps in bioinformatics, much appreciated! **Hanna**, thank you for answering my questions about bioinformatics! **Hongkai**, thank you for the nice conversations and your jojo moves are amazing! **Suvi**, thank you for organizing SexGen meetings and its parties! Also thanks to all the other past and present people.

I want to thank my Half-time opponent and football player **Philip** for the great comments and discussion and also for being such a nice member of the department.

I also want to thank our collaborators and people from the worm and hermaphrodite communities for the scientific exchange in a friendly and relaxed atmosphere! Special thanks to **Steven Ramm and his lab** in Bielefeld, **Lukas Schärer and his lab** in Basel, as well as **Kira Zadesenets** in Novosibirsk.

Additionally, I was supported by the NBIS long-term bioinformatics support. Special thanks to **Allison Churcher**. Computational resources were provided by SNIC on UPPMAX. Additional financial support was provided by Jörgen Lindströms Stipendiefond.

I want to thank my dear friends **Tristan**, **Maria** and **Dona** for being such good friends in the good times and bad, you helped me moving! Thank you for all the fun we had together playing tennis and doing other sports, having Halloween and other parties as well as while playing board games (or chatting with **Maria** during them)! You are a great family and I wish you all the best!

Katja and **Carlos**, thank you for being such great Dominos, the hottest summer in Sweden I will never forget! It was always fun having lunch, doing sports, enjoying nature, playing games and partying with you. Also, chatting with you, **Katja**, during our Mentor Program was amazing, during those fikas! ConDragulations on your PhDs, now it is time to finish mine!

Elsie and **Kristaps**, thank you for being such fun friends, Uppsala never dies!

Violeta, thank you for being such a fun person and sharing aquarium hobby with me!

Jacob, thank you for being such a great colleague and it has always been fun visiting you in Copenhagen!

Ainara, thank you for being so positive and you organize great parties!

I want to thank all the football players at the department for always having a great game and maybe pizza afterwards: **Albert**, **David**, **Hamid**, **Patrik**, **Arne**, **Julio**, **Utku**, **Corin**, **Tamara**, **Amanda** and others!

I also want to thank all the floorball players at the department: **Erik**, **Staffan**, **Åke**, **Gabriel**, **Jorgen**, **Jep**, **Anders**, **Lars**, **Petteri** and others. Those games were always tough!

And thanks to all the other wonderful past and present members at the department: **Leidys**, **Xi**, **Varpu**, **Ivette**, **Margarida**, **Dimitri**, **Humberto**, **Samantha**, **Maarit**, **Julian**, **Nathalie**, **Honor**, **Lokesh**, **Tianhao**, **Jane**, **Vincenzo**, **Victor**, **John**, **Ann-Kathrin**, **Beatriz**, **Linus**, **Mads**, **Reinder**, **Hanna L.**, **Maria S.C.**, **Johannes**, **Jasmine**, **Dag**, **Max**, **Maja**, **Dennis**, **Per**, **Tobias**, **Chon**, **Emily**, **Pierre**, **Micaela**, **Daniel**, **Marie**, **Marita**, **Romain**, **Evelina**, **Sofie** and others.

I also want to thank all my **friends** outside the department both in Sweden and Latvia!

Nauri, liels paldies tev par palīdzību ar attēliem un kopā pavadīto laiku un daudzajiem piedzīvojumiem, kā arī novēlu tev pabeigt doktorantūru!

Vēlos arī pateikties visai savai **ģimenei** par atbalstu manā ilgajā izglītības ceļā, es jūs mīlu!