Doctoral Dissertation

Construction of a biosensor *Daphnia magna* for the detection of environmental estrogens via integration of the human estrogen receptor α

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Abstract

Environmental estrogens have a variety of adverse effects including increased risks for cancer and abnormalities in reproductive tissues. Current screening methods are mostly limited to yeast or mammalian cell assays which lack tissue diversity, and to transgenic fish which employ the respective fishes' own estrogen receptors. Water fleas (*daphnia*) have a long history in aquatic testing and now lend themselves to applications as biosensor due to recent progress in the development of genome editing tools.

I aimed to develop a transgenic line of the water flea *Daphnia magna* that functions as estrogen biosensor for both natural and synthetic estrogens via the human estrogen receptor alpha (hERα). For this I designed plasmids to express hERα in *daphnia* (EF1α-1:*ESR1*) and to report estrogenic activity via red fluorescence (ERE:*mcherry*). I adjusted the level of protein expression by implementing different lengths of EF1α-1 3'UTR.

After confirmation of functionality of the plasmids by microinjection into wild type *daphnia* embryos, I joined the two plasmids and added a TALE site. This biosensor plasmid was integrated into the *D. magna* genome using TALEN and the resulting line was named estrogen sensor (ES) line.

Exposure of ES line *daphnia* to Diethylstilbestrol (DES) and 17β-Estradiol (E2) showed that the ES line could reliably detect DES at concentrations as low as 0.5 mg/L, and only at 4 mg/L for E2.

In conclusion, a functional estrogen biosensor line of *D. magna* was established. This is the first time a human gene was expressed in *daphnia*, showcasing potential for further research of the interaction between environmental factors and human genes in this organism.

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Abbreviations

ADaM	Aachener Daphnien Medium
AU-rich	sequence rich in adenine and uracil
bp	base pairs
°C	degree Celsius
CTEF	Corrected Total Embryo Fluorescence
D. magna	*Daphnia magna*
D. pulex	*Daphnia pulex*
DNA	deoxyribonucleic acid
EF1a-1	*Elongation factor 1a-1*
ER	estrogen receptor
ERE	estrogen response element
GPER	G-protein coupled estrogen receptor
h	hour
hpi	hours post injection
kb	kilobases
kPa	kilopascal
mM	millimolar
mRNA	messenger RNA
n	sample number
NHEJ	non-homologous end joining
NIES	National Institute for Environmental Studies
nm	nanometre
nt	nucleotide

ORF	open reading frame
PCR	polymerase chain reaction
RFI	relative fluorescence increase
RNA	ribonucleic acid
RNAi	RNA interference
s	second
TALEN	transcription activator-like effector nuclease
UTR	untranslated region

1 Introduction

Environmental estrogens

Environmental estrogens are a group of endocrine disrupting chemicals (EDCs) that can mimic hormones and therefore alter hormone signalling in the body. They have been associated with increased risks of cancer, abnormalities in reproductive tissues in fish and mammals (Ma 2009), reduced sperm count and quality in humans (Li et al. 2009) and altered sexual behaviour in fish (Van den Belt et al. 2004). Put simply, estrogen and EDCs form a complex with estrogen receptors (ER), the resulting complex interacts with Estrogen Response Elements (ERE) on the genome and activate transcription of downstream genes(Eyster 2016; Klinge 2001; Nilsson et al. 2001).

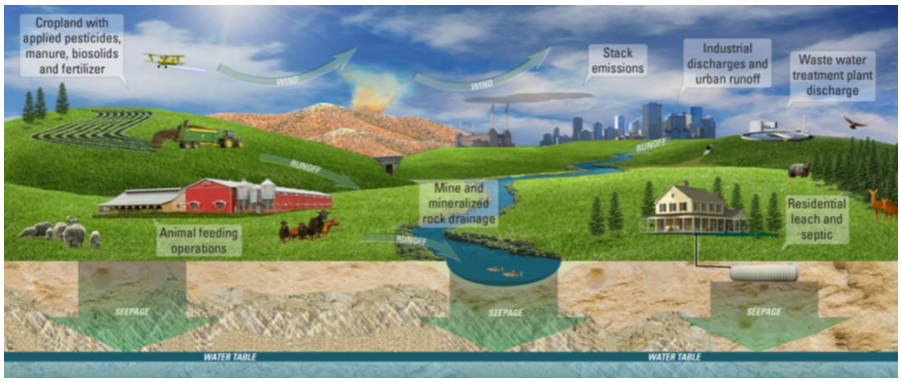

Figure 1. Sources of EDCs

EDCs can enter the environment from animal husbandry, agriculture, rock drainage, stack emissions, industrial discharges, waste water treatment plant discharges, and residential leach and septic, among other sources. Picture source: Journey et al. 2013

EDCs from human excrement and industrial discharge cannot be removed in most waste water treatment plants (WWTP) since contact

time is limited due to the high volume throughput in most WWTPs. Effective tertiary processes exist, but retrofitting is usually cost prohibitive since they require land for scale-up and will negatively impact economics of the WWTPs (Beecher 2013; Ting and Praveena 2017).

Aside from WWTP discharge, EDCs can also enter the environment from a variety of other sources including but not limited to animal husbandry, agriculture, and industrial leeches as shown in Figure 1 (Adeel et al. 2017; Journey et al. 2013).

Mechanism of estrogen signalling

The way both natural and synthetic estrogens affect humans is by affecting hormone signalling.

Put simply, as shown in Figure 2 natural estrogens like E2 can enter a cell, bind to and activate the estrogen receptor α (ERα), initiating genomic actions like transcription activation on estrogen response elements (EREs).

Estrogenic compounds might also interact with ERβ, membrane bound ER (mER) and/or G-protein coupled ER (GPR30), activating or inactivating them depending on their properties. ERβ activation will also initiate genomic actions, though of different genes than ERα. On the other hand, mER GPR30 initiate a signal cascade of second messengers (Gogos et al. 2015).

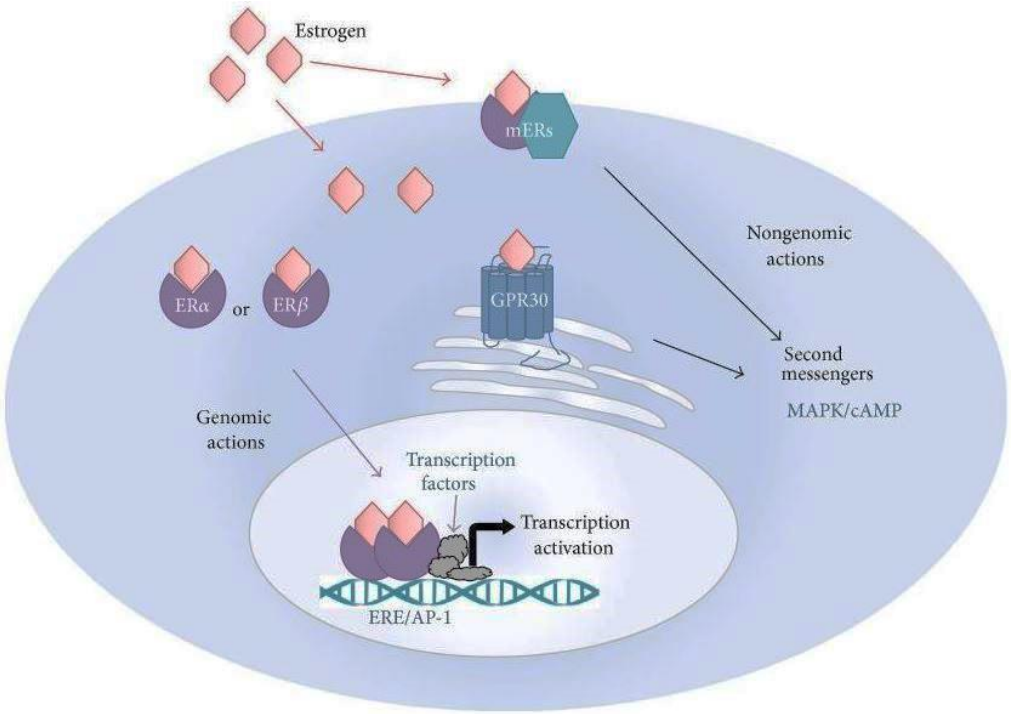

Figure 2. Activation of estrogen receptors

Estrogen bind to and activate ERα, ERβ, membrane bound ER (mER) and/or G-protein coupled ER (GPR30). The first two initiate genomic actions (transcription activation), the latter two initiate nongenomic actions (signal cascade of second messengers).
Picture source: Gogos et al. 2015

EDCs that affect estrogen signalling can therefore potentially affect all estrogen responsive pathways. As shown in Figure 3 that can lead to a wide variety of diseases depending on the effect of specific EDCs including but not limited to reproductive cancers, problems with the nervous system and cardiovascular diseases (Scognamiglio et al. 2016).

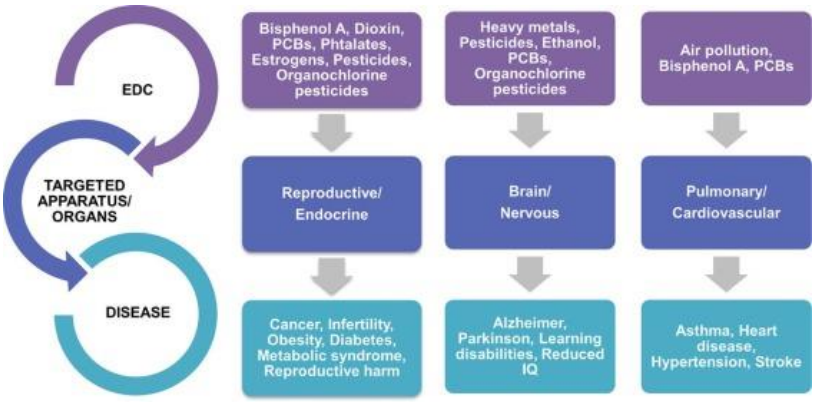

Figure 3. Possible effects of EDCs on humans

Picture source: Scognamiglio et al. 2016

Existing methods to detect environmental estrogens

Existing screening and testing systems mostly use yeast and mammalian cell cultures (Legler et al. 1999; Leskinen et al. 2005), which are limited in that they cannot represent tissue diversity nor chemical absorption. Non-aquatic biosensor organism like fruit flies (Thackray et al. 2000) are difficult to expose to estrogenic compounds in the same way aquatic life forms can be exposed especially over the whole life cycle of the animal. In biosensor fish species like zebrafish or medaka on the other hand, scientists often make use of their own estrogen-responsive metabolic pathways like using the regulatory elements from estrogen induced gene instead of human ERE. In one example shown in Table 1, Choriogenin H was used (Kurauchi et al. 2005; Lee et al. 2002), but vitellogenin is also a popular focus (Chakraborty et al. 2011; Chen et al. 2010; Van den Belt et al. 2004).

Table 1. Estrogen biosensor overview

Receptor	**Reporter**	**Organism**	**Source**
hERα	ERE:*Luc*	Human cell lines	(Legler et al. 1999)
hERα	ERE:*GFP*	Drosophila	(Thackray et al. 2000)
hERα	ERE:*Luc*	Yeast cell lines	(Leskinen et al. 2005)
mER	**(Choriogenin H regulatory elements)**:*GFP*	Medaka	(Kurauchi et al. 2005)
zER	**ERE-zvtg1**:*GFP*	Zebrafish	(Chen et al. 2010)
zER	ERE:*GFP*	Zebrafish	(Gorelick and Halpern 2011)
XVE with estrogen receptor ligand binding domain	LexAop:*YFG*	Drosophila	(Kuo et al. 2012)
hERα	ERE:*mCherry*	Waterflea	(presented here)

Short list of present estrogen biosensor cell lines and estrogen biosensor organisms. Endogenous elements from the respective organism are marked in **bold**.

Particularly in zebrafish it is popular to make use of the endogenous zebrafish ERs (Chen et al. 2010; Gorelick and Halpern 2011), which is of course optimal to find the effects of EDCs on these fish but suboptimal when I want to investigate possible effects on humans since several EDCs have been shown to interact differently with hERs than they do with zERs (Gorelick et al. 2014; Menuet et al. 2002).

A non-representative short overview over some of the recent estrogen biosensor cell lines and species is given in Table 1, to showcase the differences in implemented estrogen receptor and responsive elements.

Daphnia magna **anatomy and life cycle**

Daphnia are small planktonic crustaceans, belonging to the class of branchiopoda and the order of cladocera. They live mostly in quiet fresh waters where cladocera, together with rotifers and copepods, account for most of the zooplankton. Adult *daphnia* reach 0.5 - 6 mm in length, with *D. magna* on the upper end with generally 4 - 6 mm in length.

Figure 4. Female adult *D. magna*

Picture source: Hajime Watanabe. Bar = 500 µm.

Daphnia have a chitinous exoskeleton, their carapace, which must be molted to allow for physical growth. During development this happens between the instar stages, in adults in between reproductive cycles, roughly every 72 hours.

Daphnia have nine pairs of appendages, from front to back: small first antennae (antennule, a sensory organ), much larger second antennae (for locomotion), maxillae (jaws) and mandibles (for food processing), and five thoracic appendages (filtration) (see Figure 5). Together they form an apparatus for feeding and respiration.

As filter feeders, *daphnia* ingest mainly unicellular algae as well as various sorts of organic detritus including protists and bacteria. Movement of the thoracic appendages produces a current throughout the carapace, bringing small food particles into the digestive tract while bigger particles are kept out. Food particles then move through the digestive tract until excretion through the anus.

Water current from the movement of thoracic appendages also supplies fresh oxygen to the shell valves and inner walls of the carapace, where the main gas exchange happens. Hemolymph is pumped throughout the body cavity by a relatively simple heart (Paul et al. 2004; Pirow et al. 1999).

Daphnia have two modes of reproduction. They usually asexually produce parthenogenetic eggs after molting, though after certain environmental cues resting eggs can be produced too. This follows the asexual production of diploid males, which are needed to fertilize the haploid eggs. Here I will focus on parthenogenesis.

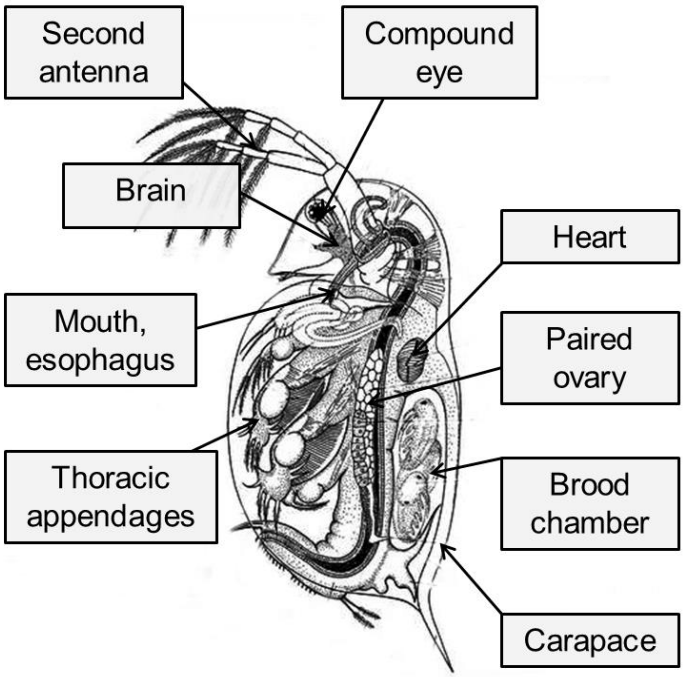

Figure 5. *Daphnia* anatomy

Picture source: Ebert 2005 (edited)

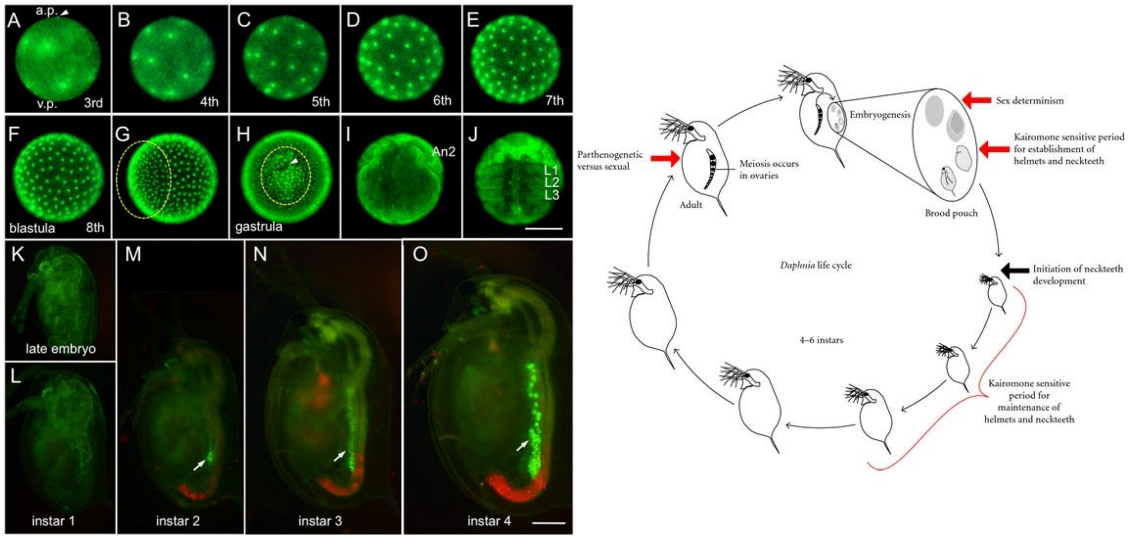

Figure 6. *Daphnia* maturation and parthenogenetic life cycle

Picture source: left Kato et al. 2012 (scale bar 100 µm in A–J; 250 µm in K–O), right Harris et al. 2012

Ovulated eggs are placed in the brood chamber and start developing immediately. After about one day, the embryos hatch (Figure 6 A – J); they remain in the brood chamber for about two more days (Figure 6 K) until they are released into the environment and start swimming (Figure 6 L). The juvenile *daphnia* undergo several instars (Figure 6 L – O) before producing parthenogenetic eggs themselves. The first clutch is usually released after 5 – 7 days, with clutch sizes in the double digits for *D. magna*. Adult females can produce a clutch of eggs ever three to four days until death, in the laboratory they can live for over two months.

Genetic manipulation in *Daphnia magna*

Genetic manipulation of *daphnia* only became possible in recent years thanks to sequenced genomes of *D. pulex* and *D. magna*. I would like to present a partial timeline of their sequencing and subsequent genetic manipulation methods. Unless otherwise stated, *D. magna* was the species used, and microinjection the delivery method.

When I started working on this project as a PhD student in 2013, development of gene manipulation methods had just started. As can be seen in Table 2 the vast majority of genome editing techniques were still in development at that time and would be published in subsequent years, like knock-out and later knock-in via TALEN and CRISPR/Cas in 2014 – 2017.

I began by developing a method to transiently overexpress protein in *D. magna* based on 3' UTR length (Torner et al. 2014). This method has since become standard in our laboratory, as well as the method I developed for fluorescence quantification in *daphnia*. Both will be presented in detail in Chapter 2.

I then proceeded to apply this method to develop an estrogen biosensor daphnia, which will be presented in detail in Chapter 3 and 4.

Table 2. Timeline of genetic manipulation in *D. magna*

1999	*D. pulex* mitochondrial genome published (Crease 1999)
2005	Expressed sequence tags (EST) published (Watanabe et al. 2005)
2006	Viral transgenesis of embryonic cell culture (Robinson et al. 2006)
2007	DNA microarray (Watanabe et al. 2007)
2010	Electroporation for transient expression (Kato et al. 2010)
2011	*D. pulex* genome published (Colbourne et al. 2011)
	RNAi (Kato et al. 2011b)
	Genome published (Orsini et al. 2011)
2012	Random integration by nonhomologous recombination (Kato et al. 2012)
2013	RNAi for *D. pulex* (Hiruta et al. 2013)
2014	Transient hormone reporter assay (Asada et al. 2014)
	Knock-out via CRISPR/Cas9 (Nakanishi et al. 2014)
2015	RNAi by feeding for *D. pulex* (Schumpert et al. 2015)
	Knock-out via TALEN (Naitou et al. 2015)
	Knock-in via TALEN and homologous recombination (Nakanishi et al. 2015)
2016	Knock-in via TALEN and non-homologous end joining (Nakanishi et al. 2016)
2017	Knock-in via CRISPR/Cas9 and non-homologous end joining (Kumagai et al. 2017b)
	Reporter assay for gene expression (Nong et al. 2017)

The crustacean *daphnia* and its potential as biosensor

The crustacean *Daphnia*, as keystone species of freshwater ecosystems, has been used in water quality assessments for many decades (Gersich et al. 1986; Martins et al. 2007) and been studied even longer (Metschnikoff 1884). High growth rate and high fecundity combined with cheap and easy rearing make them an excellent organism for screening.

In non-estrogenic invertebrates like *D. magna*, EDC toxicity is presumed to not be based in hormonal imbalance but to be caused by oxidative stress (Heger et al. 2015), and potentially by interfering with vitellogenesis (Hannas et al. 2011).

This prompted me to establish a transgenic *Daphnia* with hERα and a fluorescence reporter gene to detect estrogens without interference of innate hormone metabolism.

Aim of this study

The goal of this study was to realize an estrogen biosensor *D. magna*.

This was accomplished by investigation of the effect of different lengths of EF1α-a 3' UTRs sequences on expression (Chapter 2), which I implemented in the design of an estrogen biosensor (Figure 7). This biosensor was integrated as a plasmid into the genome of *D. magna* using a set of previously established TALENs (Naitou et al. 2015) (Chapter 3). Then I exposed the resulting estrogen sensor line (ES line) to Diethylstilbestrol (DES), a synthetic estrogen, and 17β-Estradiol (E2), a natural estrogen, to showcase its response phenotype and sensitivity (Chapter 4).

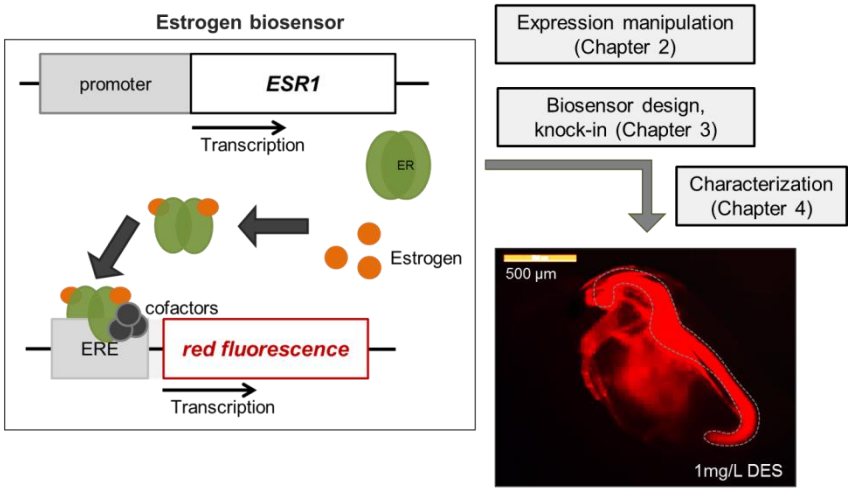

Figure 7. Project overview

Design of an estrogen biosensor consisting of *ESR1*, encoding human estrogen receptor (ER) α. In the presence of estrogens and cofactors, ERα will bind to estrogen response elements (EREs) and initiate downstream transcription of a red fluorescence protein. This construct is to be integrated into the *D. magna* genome, resulting in an ES line (right, bar = 500 µm)

To my best knowledge this is the first time a human gene was expressed in *Daphnia*, showcasing the potential to test the interaction of different human genes and environmental stimuli relatively directly, and therefore determine potential health impacts in both animals and humans.

2 Adjusting expression levels of endogenous proteins in the crustacean *Daphnia magna*

2.1 Introduction

It can be of utter importance to adjust the expression levels of genes to a desired amount, be it for overexpression or to avoid toxicity. In this chapter I focus on the 3' untranslated region for this. 5' and 3' UTRs surrounding a gene's ORF have been shown to be essential for post-transcriptional regulation, including modulation of transport and therefore an mRNAs subcellular localization (Irier et al. 2009), of translation efficiency (Irier et al. 2009; Sandberg et al. 2008; Subramaniam et al. 2011) and of stability (Alonso 2012; Wu and Brewer 2012). The regulation of UTRs is mediated by binding of RNA binding proteins or complementary noncoding RNA specific to motifs within the UTRs (Mazumder et al. 2003; Wilkie et al. 2003). Well-studied examples are AU rich regions, sequences rich in adenine and uracil, within the 3'UTR which increase the degradation rate of mRNAs (Barreau et al. 2005; Zubiaga et al. 1995). However, while there have been hints that 3'UTRs play an important role in gene regulation (Kato et al. 2011a), UTRs optimal for protein expression in *daphnia* have not yet been elucidated. I tested UTRs of one constitutively expressed *daphnia* gene, *elongation factor 1a-1* (*EF1a-1*), by linking them to an ORF encoding DsRed2 protein that functions as a reporter of gene expression. The resulting chimeric RNAs were injected into *D. magna* eggs so that DsRed2 fluorescence could be monitored during embryonic development. 3' UTR sequences leading to higher and lower red fluorescence can then be implemented in the later biosensor construct.

DsRed2, a red fluorescent protein (a mutant form of DsRed from *Discosoma sp.*), was utilized here for its high signal-to-noise ratio, fast maturation and its distinctly different spectral properties compared to *daphnia* embryos' autofluorescence (excitation and emission maxima of DsRed2 are 563 and 582 nm respectively, well outside the range of green autofluorescence). DsRed2 is also highly soluble and therefore it is likely it will not affect the embryo negatively unless in extreme concentrations.

We noticed before that microinjection of mRNAs of DsRed2 with *EF1a-1* 3' UTR sequences transcribed with either T7 or SP6 RNA polymerase showed noticeably different fluorescence strengths at the same concentration. Both T7 and SP6 RNA polymerase come from bacteriophages and utilize different promotors which have been characterized in depth since the 1980s (Brown et al. 1986; Lee and Kang 1993; Schenborn and Mierendorf 1985). Usually polymerases transcribe until a terminator sequence, though they have been known to stop after certain RNA loops or when blocked by DNA-binding proteins (Molodtsov et al. 2014; Pavco and Steege 1991).

In this chapter, my goal was to develop a useful tool for protein overexpression in *D. magna*. This was accomplished by testing the effects of different lengths of *EF1a-1* 3' UTR sequences on protein translation from microinjected mRNA.

2.2 Methods

Daphnia strain

I used a wildtype line of *D. magna,* the NIES clone (obtained from the National Institute for Environmental Studies, NIES; Tsukuba, Japan). This line has been maintained in our laboratory for many years. It is healthy and reproduces quickly.

Daphnia culture conditions

80 neonates of NIES *daphnia* (under 24 h old) were cultured in 5 L AdaM (Klüttgen et al. 1994) at 22–24 °C under a light/dark cycle of 16/8 h. The culture medium was renewed once a week. *Daphniids* were fed every day with 5 mg of *chlorella vulgaris* (Nikkai Center, Tokyo, Japan) during the first week. After maturation, offspring was removed daily and adults were fed with 10 mg *chlorella* per day.

All methods regarding animal use were carried out in accordance with the relevant guidelines and regulations.

In vitro synthesis of DsRed2 mRNAs

The vector pRCS21(EF1α-1 UTR) had been generated previously using *D. magna EF1a-1* cDNA sequence obtained from the *D. magna* EST database (Watanabe et al. 2005).

To mimic dynamics of *D. magna EF1α-1* mRNAs in embryos, I amplified 73 nt and 522 nt of 5'UTR and 3'UTRs from the *EF1α–1* gene and subcloned them into the SacI/SmaI, NotI/EcoT22I sites, respectively, of pRCS21 which encodes for DsRed2 (Kurokawa et al. 2006). In addition, I added a *Xenopus* β-globin leader sequence to the 5' end of the mRNA because it efficiently mediates cap-dependent translation and does not appear to contain any sequence elements or RNA structures that might significantly affect translation otherwise (Kozak 1994; Krowczynska and Brawerman 1986). In a region surrounding the start AUG codon, I utilized the Kozak sequence to optimize translation initiation (Kozak 1989).

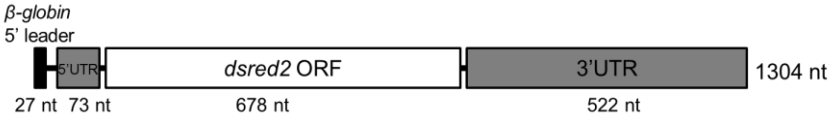

Figure 8. Structure of DsRed2 mRNA

For SP6 transcribed, capped-RNA, I linearized the plasmid with EcoT22I and used this as a template for subsequent *in vitro* transcription with SP6 RNA polymerase (mMessage mMachine Kit, Life Technologies, Carlsbad, USA).

To prepare exclusively full-length and truncated RNAs respectively, I prepared templates with T7 promoter sequence instead of SP6 promoter sequence by PCR, using a forward primer (5'-TAATACGACTCACTATAGGGGAATACAAGCTACTTGTT-3') to introduce a T7 promoter sequence, and conducted *in vitro* transcription with T7 RNA polymerase (mMessage mMachine Kit, Life Technologies). All of the transcribed RNAs were poly-adenylated with a Poly(A) Tailing Kit (Life Technologies) and purified by using an RNeasy Mini Kit (QIAGEN GmbH,

Hilden, Germany) and phenol-chloroform extraction. The RNA samples were stored at -80 °C until injection.

Sequencing

I used RNA synthesized by SP6 polymerase for cDNA synthesis (SMART RACE cDNA Amplification Kit; Clontech, Palo Alto, CA, USA). Then I cloned the PCR fragments corresponding to full-length RNA s and shorter RNA s into TOPO vectors (TOPO TA Cloning Kit, Life Technologies) and introduced them into chemically competent Escherichia coli cells (XL10-Gold). For each sequence reaction using Big Dye Terminator v3.1 Cycle Sequencing Kit (Life Technologies), I used 150–200 ng of the purified plasmids with 1.5 pmol of the forward primer (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') or the reverse primer (5'-GAGCGGATAACAATTTCACACAGG-3'). The reaction volume was 5 μL. I purified the reaction products by ethanol/sodium acetate precipitation and washed them with 80 % ethanol. The dry pellet obtained was resuspended in 20 μL of Hi-Di formamide (Life Technologies), denatured at 95 °C for 2 min, and subsequently sequenced with an ABI 3100 genetic analyzer (Life Technologies).

Microinjection

Microinjection was conducted according to established procedures (Kato et al. 2011b). In short, adult *D. magna* with empty brood chambers were selected and observed until ovulation started. Then *D. magna* were transferred to ice-chilled M4 medium (Elendt and Bias 1990) containing 80 mM sucrose (M4-suc) and dissected to collect the eggs within the

brood chamber. The eggs were stored in ice-chilled M4-suc medium until injection to slow the hardening of the egg membrane. Microinjection was performed within 1 hour post ovulation (hpi) for the same reason. Successfully manipulated eggs were transferred to fresh medium and were cultured in a 96-well plate at 23 ± 1 °C. From each clutch of eggs, 2 – 3 eggs that were not injected served as control for development. Injected solutions contained Lucifer Yellow (1 mM; LY, Life Technologies) to quantify the injection volume 1 h after microinjection.

Photography of fluorescent embryos

One hour after microinjection, I transferred the manipulated eggs and non-injected control eggs to fresh M4-sucrose medium placed on a micro slide glass (Matsunami, Osaka, Japan). For normalization of the injection volume, I recorded the green fluorescence intensity from LY with the help of a colour digital camera (Leica DC500) mounted on a Leica M165C fluorescence microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) equipped with a 480-nm excitation and a 510-nm barrier filter. The pictures were taken under 80× magnification with 100 % aperture, 4.4 s exposure, 1.5 gain, 1.0 saturation, and 0.7 gamma. The red fluorescence intensity of embryos was recorded 24 h after microinjection from a dorsal view, employing a 545-nm excitation and a 620-nm barrier filter. Corresponding pictures were taken under 90× magnification, with 100 % aperture, 1.0 s exposure, 1.5 gain, 1.5 saturation, and 0.7 gamma.

Relative fluorescence intensity (RFI)

Fluorescence intensities were calculated from pictures using ImageJ based on methods developed in previous studies (Burgess et al. 2010). For quantifications, I used the following formula:

Total-embryo fluorescence = sum of the intensities of all pixels of one embryo

Background fluorescence = mean intensities per pixel for a region close to the embryo.

Corrected Total-Embryo Fluorescence (CTEF) = total-embryo fluorescence – (number of pixels of the selected embryo x mean of three background fluorescence measurements)

As the green fluorescence 1 h after injection caused by LY varies according to the injected volume in relation to the size of the egg, it was important to normalize the injection volume. To do so, sample injections of 1 mM LY were conducted and the CTEF of green fluorescence 1 h after injection was calculated. To account for different egg sizes all individual CTEFs were normalized to a size of 10^4 pixels before their average was calculated. The resulting average value was defined as standard injection volume.

For all sample injections, a correction factor was calculated so that their individual CTEF per 10^4 pixels could be adjusted to the standard one. This correction factor was then used to adjust the resulting fluorescence intensities at 24 h to the standard injection volume.

Additionally to that, a new relative value was developed to avoid errors due to different light intensities of exchanged halogen lamps as well as due to individual differences in auto-fluorescence between embryos from

different mothers, as these often had significantly different strengths of auto-fluorescence.

> Relative fluorescence intensity (RFI) = CTEF (injected embryo) / CTEF (uninjected embryo of the same age and of the same mother as the injected embryo)

Opposed to CTEF which is given in form of pixel intensity per 10^4 pixels, RFI is unit free.

In order for a quantitative fluorescence value of a given sample injection to be used in this study, at least two injections were carried out on separate days and from each, at least two injected eggs and one uninjected control had to be quantified successfully at 24 h. Therefore, all used fluorescence values have a sample number of $n \geq 4$.

2.3 Results

I tested the effect of truncating the *D. magna* native EF1α-1 3'UTR at different lengths. We found previously that transcription of DsRed2 with this UTR with different RNA polymerases (SP6 and T7) lead to different fluorescence strength, caused presumably by the different lengths of transcripts.

So first, I sequenced shorter SP6 polymerase transcribed mRNAs as well as full length mRNAs transcribed with T7 polymerase. With one exception, the shorter-length RNA s had an average length of 0.967 ± 0.015 kb (n = 42) and that of the full-length RNA s was 1.304 ± 0.001 kb (n = 16; Figure 9).

Since this result might be caused by inhibitory structures or motifs for protein expression, I searched for regulatory motifs in the EF1α-1 3' UTR using RegRNA , Transterm, UTRsite, and AREscore (Grillo et al. 2010; Huang et al. 2006; Jacobs et al. 2009; Spasic et al. 2012) and found two types of present elements that are reported as inhibitory in other organisms. These were AU-rich regions (Barreau et al. 2005; Fan et al. 1997; Zubiaga et al. 1995) and Musashi-binding elements (MBEs) (MacNicol et al. 2011). Three AU-rich regions and one AUUUA motif located within the first AU-rich region were identified. Two MBE candidates, one of which overlapped with the AUUUA motif, were also present in the 3' UTR.

I designed the lengths of EF1α-1 3' UTR in the mRNAs for microinjection accordingly. In addition to the T7 full length transcript and the SP6 mixture of full length and roughly half length transcripts, I also tested shorter versions of the 3'UTR that lack half and all known instability motifs, and an mRNA without any 3'UTR (Figure 10).

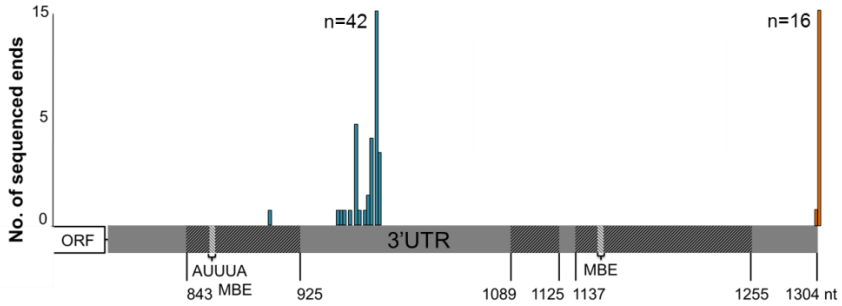

Figure 9. Different lengths of EF1α-1 3' UTRs after transcription with SP6 or T7 polymerase

Structures of AU rich regions of the EF1α-1 3' UTR are indicated in darker grey, specific motifs (AUUUA, MBE) in light grey. Short SP6 transcripts (n=42) shown in blue, full length T7 transcripts (n=16) shown in orange.

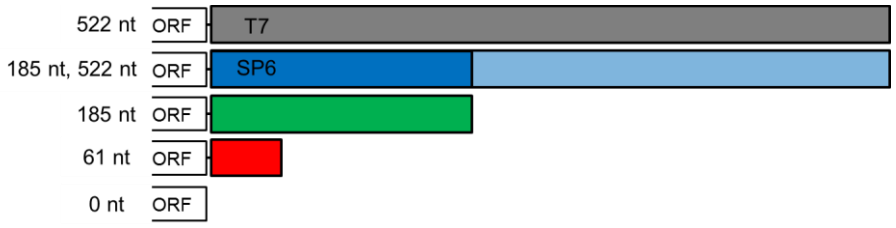

Figure 10. Structure of EF1α-1 3' UTRs of injected mRNAs

1. T7 transcript. Full length 3' UTR (522 nt).
2. SP6 transcript. A mixture of truncated (185 nt) and full length 3' UTRs (522 nt).
3. Length of the shorter SP6 transcripts (185 nt).
4. RNA instability motif free 3' UTR (61 nt).
5. No 3' UTR (0 nt).

When injecting wildtype NIES *daphnia* embryos with mRNAs encoding the fluorescence protein DsRed2 with different lengths of EF1α-1 3'UTR, I confirmed a low translation rate for full length constructs and the

highest fluorescence was detected from the mRNA with the shortest 3'UTR (Figure 11). MRNAs of lengths in between resulted in translation rates in between, supporting the hypothesis that the longer mRNAs have shorter life spans because of their instability motifs. Interestingly, mRNA that entirely lacked the 3'UTR (Figure 11 – white bar) did not increase fluorescence beyond the *daphnia*'s auto-fluorescence.

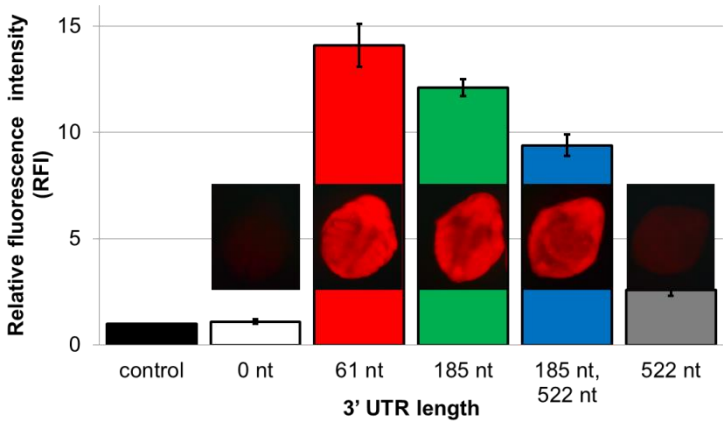

Figure 11. 3'UTR length dependent DsRed2 fluorescence

Relative fluorescence intensities (RFIs) of the embryos injected with truncated mRNAs. 522 nt (grey) and 185 nt, 522 nt (blue) indicate T7- and SP6-products respectively. Uninjected *daphnia* embryos were used as control and their auto-fluorescence was defined as an RFI of 1.

2.4 Discussion

Microinjection of mRNA is a common technique to study gene function (Godinho 2011; Porazinski et al. 2010; Sive et al. 2010; Zhang and Weisblat 2005). It was found that UTRs can regulate mRNA stability, localization, and translation (Falcone and Andrews 1991; Fink et al. 2006). Here I examined the effects of the endogenous EF1α-1 UTRs on protein expression in *D. magna*.

I found that EF1α-1 3' UTRs indeed regulated DsRed2 expression. With quantitative imaging and a reporter I could show full length EF1α-1 3' UTR to be a negative regulator of protein expression in *D. magna* while shortening the 3' UTR increased the reporter expression.

Of course one of the factors affecting protein expression might have been the length of the 3'UTR itself. I recommend testing different lengths of other 3' UTRs or using random sequences in the future, though with total deletion of the 3' UTR no translation could be detected.

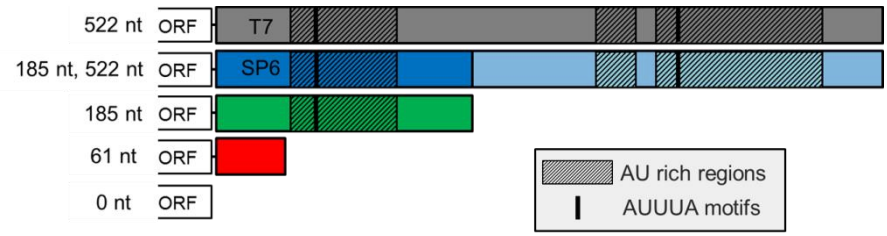

Figure 12. RNA instability motifs within different lengths of EF1α-1 3' UTR

Black striped areas mark AU rich regions, a black line marks AUUUA motifs within them.

My results also suggest the influence of inhibitory motifs on protein expression. I identified AU-rich regions within the EF1α-1 3' UTR, these

are known negative regulators of protein expression in both invertebrates and vertebrates (Zhang et al. 2002). AU-rich regions in 3' UTRs are targets for RNA-binding proteins that initiate degradation and therefore negatively regulate reporter expression.

In contrast, I found that the first 60 nt of the 3' UTR were necessary for translation. I could not identify any known factors influencing mRNA localization or stability this region, but I found a 10 nt element (5'-CCATCCAACC-3') with high RNA accessibility as revealed by a RegRNA 2.0 search (Chang et al. 2013). Therefore I conclude that a yet unknown factor might bind to this element and regulate protein expression. Deletion or mutagenesis of the element should allow confirming the regulatory mode.

In conclusion, I successfully designed an mRNA structure for overexpressing DsRed2 protein ubiquitously in *D. magna* embryos using UTRs of the endogenous EF1α-1 gene. This was the first evaluation of the UTRs of EF1α-1 gene for protein overexpression in *daphnia*. Compared with the generation of transgenic animals, this method was simpler and less time consuming. This method will be useful in analysing the developmental and physiological consequences of gain-of-function of proteins. Also, together with the genome sequences (Colbourne et al. 2011; Orsini et al. 2011), the gene manipulation tools we developed (Kato et al. 2011b, 2012; Nakanishi et al. 2014, 2016) have enabled the use of *D. magna* for ecological, evolutionary, and toxicological genomics and should accelerate new discoveries (Ebert 2005; Hebert 1978; Reynolds 2011).

My findings will be useful for developing new methods for controlled expression of proteins during *D. magna* development. By using appropriate 3' UTRs that control mRNA stabilization in specific organs and tissues, key developmental events can be visualized (Köprunner et al. 2001; Lin et al. 2012). My findings have already been implemented in a variety of studies to improve reporter expression in *D. magna* by

implementing the first 60 nt of the EF1α-1 3' UTR plus its poly(A) signal instead of full length EF1α-1 3' UTR, most of them not yet published (Kumagai et al. 2017b, 2017a).

3 Knock-in of an estrogen biosensor plasmid into the *Daphnia magna* genome

3.1 Introduction

The crustacean *daphnia*, as keystone species of freshwater ecosystems, has been used in water quality assessments for many decades (Gersich et al. 1986; Martins et al. 2007). Since the recent sequencing of the *D. pulex* and *D. magna* genomes (Colbourne et al. 2011; Orsini et al. 2011), a variety of methods for RNA interference (Kato et al. 2011b; Schumpert et al. 2015) and for genome editing like CRISPR/Cas (Nakanishi et al. 2014) and TALEN (Naitou et al. 2015; Nakanishi et al. 2015) have been developed and adapted.

Transcription activator-like effectors (TALEs) can be engineered to bind to specific sequences of DNA, combined with a nuclease like FokI DNA can be cut at specific locations (Miller et al. 2011; Pan et al. 2013). This artificial nuclease can generate double strand breaks at its target site that can be repaired by error-prone non-homologous end-joining (NHEJ), resulting in gene disruptions through the introduction of small insertions or deletions (in-dels) (Carroll 2014; Pan et al. 2013). TALENs have recently been utilized in *D. magna* to integrate a 4.5 kb long donor DNA plasmid into its target locus with only small in-dels at the junction sites (Nakanishi et al. 2016). The TALENs used in this study were developed previously (Naitou et al. 2015), they target a sequence within the red fluorescence protein DsRed2's ORF which the minos-red line of *D. magna* carries on one allele. Therefore induction of a double strand break within the DsRed2 ORF will cause a loss of red fluorescence.

The minos-red line used in this study contains a hemizygous EF1α-1:DsRed2, it shows ubiquitous dsred2 fluorescence. This line was constructed previously (Naitou et al. 2015) and was kept stably for over 50 generations. As shown in Figure 13, induction of a double strand break within the *dsred2* ORF causes a loss of fluorescence.

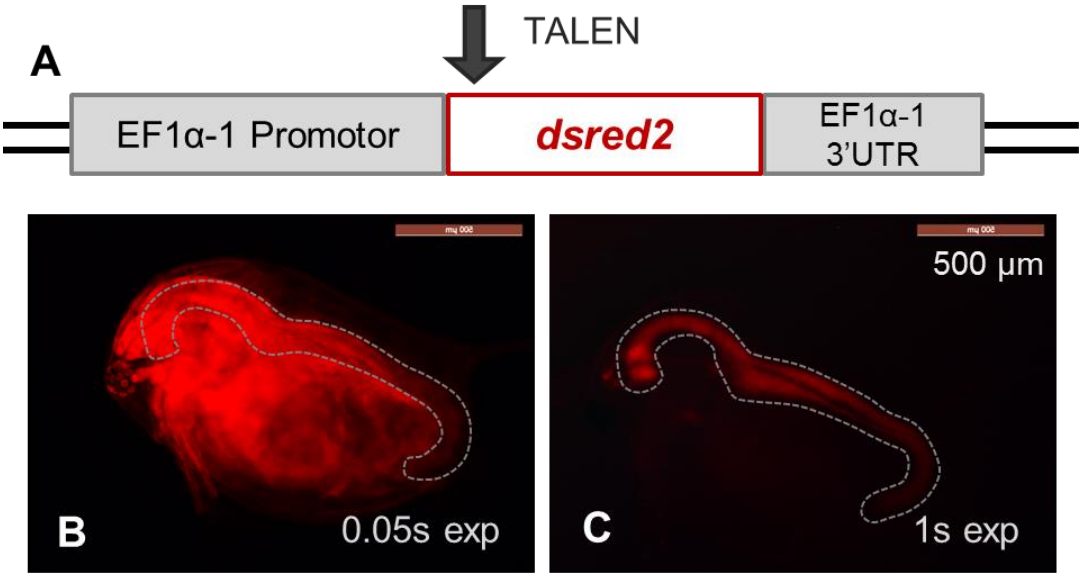

Figure 13. Target for genomic integration in the minos-red line

A Schematic representation of hemizygous dsred2 cassette in the minos-red line. The target site for TALEN induced double strand break is marked by a grey arrow.

B Minos-red juvenile, four days old, exposure time 50 ms

C ES line juvenile, four days old, exposure time 1 s

Bar = 500 µm. Dotted lines mark the gut (autofluorescence from fed algae).

Daphnia contain no estrogen receptor (Clubbs and Brooks 2007) so to make an estrogen biosensor *daphnia* it is most useful to employ a human estrogen receptor and to introduce a full reporter cassette, since *daphnia* do not contain known ERE sequences either (Colbourne et al. 2011).

They do however have a system of Ecdyson Receptor and Ecdyson Response Elements (EcRE) interacting, so those sequences can be used as positive control since they have been proven functional in a biosensor assay previously (Asada et al. 2014).

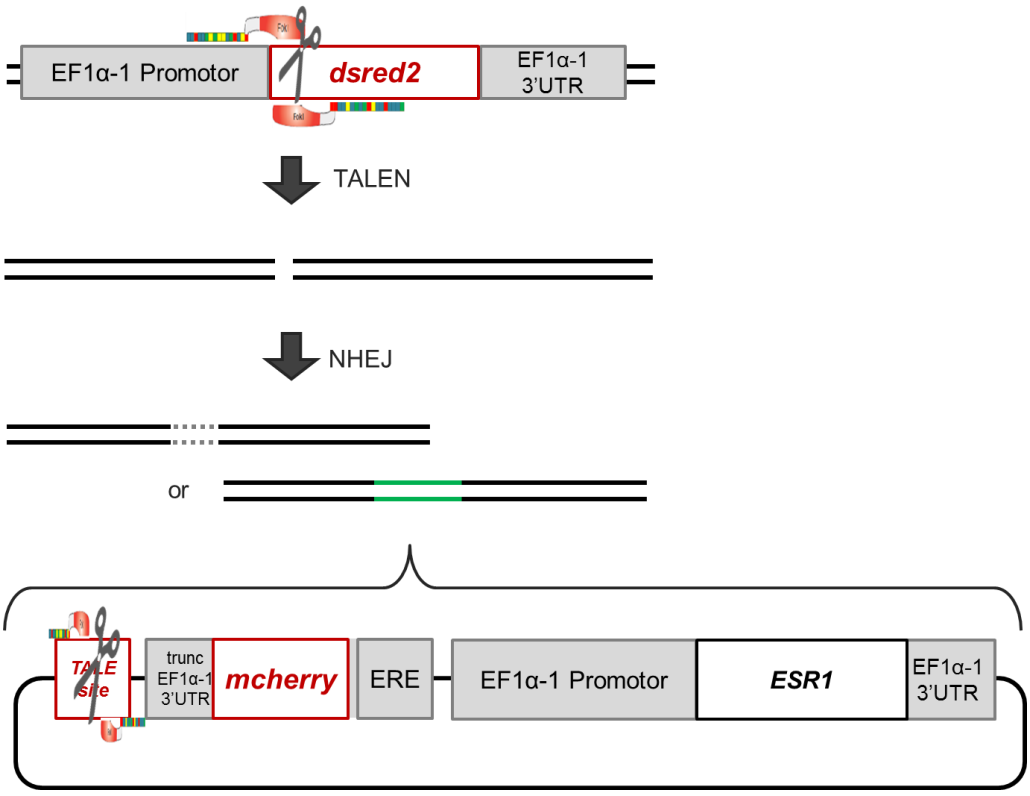

Figure 14. Schematic process of the knock-in of the biosensor plasmid via TALEN and NHEJ

TALEN induce a double strand break within the DsRed2 ORF and at the TALE site of the ES plasmid, linearizing the latter. Via NHEJ, the double strand break is repaired either with or without insertion of the linearized plasmid.

In Figure 14 the process of knock-in via TALEN is shown schematically. TALENs induce a double strand break on one allele of the minos-red genomic DNA, as well as linearizing the donor DNA plasmid by inducing a double strand break at its TALE site. The genomic double strand

break is then repaired via NHEJ with in-dels, either with or without insertion of the linearized plasmid.

In this chapter, my goal was to knock-in an estrogen biosensor plasmid into *D. magna*. This was accomplished by testing the separate biosensor components by microinjection into *D. magna* embryos, and once functional, proceed to knock-in via NHEJ using TALEN.

3.2 Methods

Daphnia strain

A transgenic line of *D. magna* containing a hemizygous *DsRed2* gene under the control of the *D. magna EF1a-1* promoter was generated previously (Naitou et al. 2015) from a *D. magna* NIES clone (obtained from the National Institute for Environmental Studies, NIES; Tsukuba, Japan). This Minos-red line has been maintained for more than 50 generations. It exhibits ubiquitous *DsRed2* expression.

Daphnia culture conditions

80 neonates of NIES *daphnia* (under 24 h old) were cultured in 5 L AdaM (Klüttgen et al. 1994) at 22–24 °C under a light/dark cycle of 16/8 h. The culture medium was renewed once a week. *Daphniids* were fed every day with 5 mg of *chlorella vulgaris* (Nikkai Center, Tokyo, Japan) during the first week. After maturation, offspring was removed daily and adults were fed with 10 mg *chlorella* per day.

After establishment of the estrogen biosensor line (ES line), ES *daphnia* were cultured under the same conditions with the exception of feeding and juvenile removal. ES *daphniids* were fed every other day with 6 mg of *chlorella vulgaris* (Nikkai Center, Tokyo, Japan) until maturation. Then, offspring was removed twice a week and adults were fed with 10 mg *chlorella* every other day.

All methods regarding animal use were carried out in accordance with the relevant guidelines and regulations.

Plasmid construction

To generate the hERα expression plasmid, full length *D. magna EF1a-1* promoter (Kato et al. 2012) (fwd 5'-GCGGTCCTATTGACGAAGCTTCAGCGACCTTGGGGAAAA T-3', rev 5'-ACCCGGGCATTGTGATTGGAGTTTA-3'), full length human *ESR1* (Zhang et al. 2017) (fwd 5'- TCACAATGCCCGGGTATGACCATGACCCTCCA CACCAAAGCAT-3', rev 5'-TCAGACCGTGGCAGGGAAACCCTCT-3'), and a pRC21 backbone with full length *EF1a-1* 3'UTR (fwd 5'-TTCCCTGCCACGGTCTGA ATGGAGGCTACTATTCCATCCAACCG-3', rev 5'- CGTCAATAGGACCGCCCA TATGAC-3'), were amplified via polymerase chain reaction (PCR), introducing overlapping sequences for InFusion cloning with the fwd primers. The fragments were purified by gel extraction and column purification using the MinElute Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany) and following the manufacturer's instructions. The purified fragments were joined via InFusion (TAKARA, Kusatsu, Shiga, Japan) following the manufacturer's instructions; the resulting construct was termed pRC21-hERa.

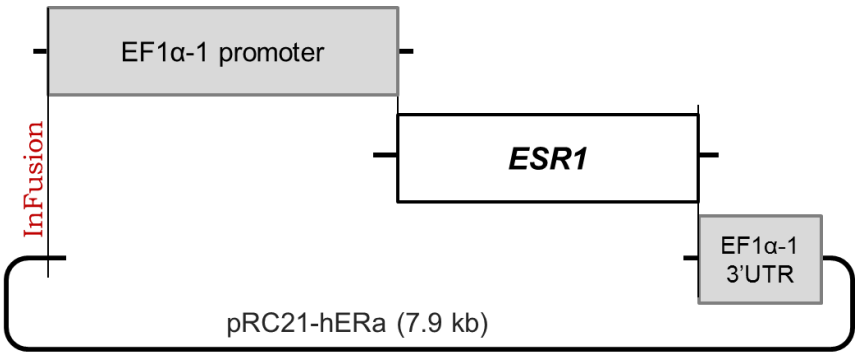

Figure 15. Construction of pRC21-hERa

To generate the EcRE reporter plasmid, full length *mCherry* and the first 60 bp of *EF1a-1* 3'UTR (Törner et al. 2014) was amplified via PCR (fwd 5'- CCACCGGTCGCCACCATGGTGAGCAAGGGCGAG-3', rev 5'-

TTACTTGTACA GCTCGTC-3‘) and joined into a pRC21 backbone containing the last 13bp of *EF1a-1* 3’UTR and therefore providing a poly(A) signal via InFusion (TAKARA). A 4xEcRE promoter (Asada et al. 2014) was amplified via PCR with primers introducing a restriction site for MscI (fwd 5’- CCTGACGGGCCCCGCGTCCCATTCGCCATTCA-3’, rev 5’-tgatgatggccatgttatcctcctcgcccttgctcaccatGGTGGCGACCGGTGGAATG-3‘). Both the PCR fragment and the *mCherry* plasmid were digested with MscI and EcoO109I (NewEngland BioLabs, Ipswich, MA, USA) for 1h and joined via MightyMix ligation (TAKARA); the resulting construct was termed pRC21-EcRE:*mCherry*.

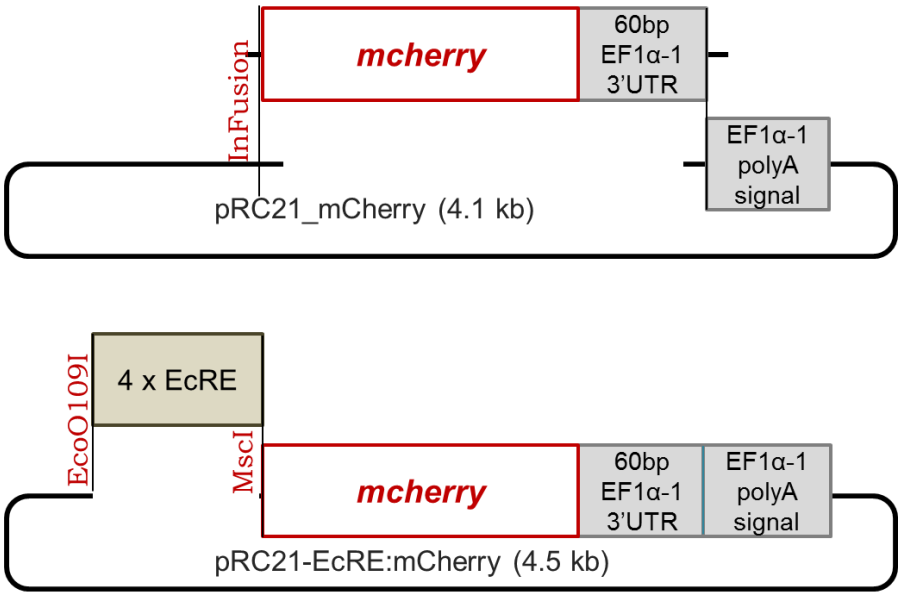

Figure 16. Construction of pRC21-EcRE:*mCherry*

To generate the ERE reporter plasmid, the EcRE repeats were excised out of pRC21-EcRE:*mCherry* with EcoO109I and XmaI (NewEngland BioLabs). A 4xERE sequence (Yoshioka et al. 2012) was amplified with primers introducing a restriction site for XmaI (fwd 5’-GACGTCATATGGGCGGTCC-3’, rev 5’-CTCGAGCCCGGGCTAG AGGATGAATTCGATCTTTGATCAGGTC-3‘), it already contains an

EcoO109I site. This PCR fragment was also digested with EcoO109I and XmaI (NewEngland BioLabs) and joined into the backbone plasmid via MightyMix ligation (TAKARA); the resulting construct was termed pRC21-ERE:*mCherry*.

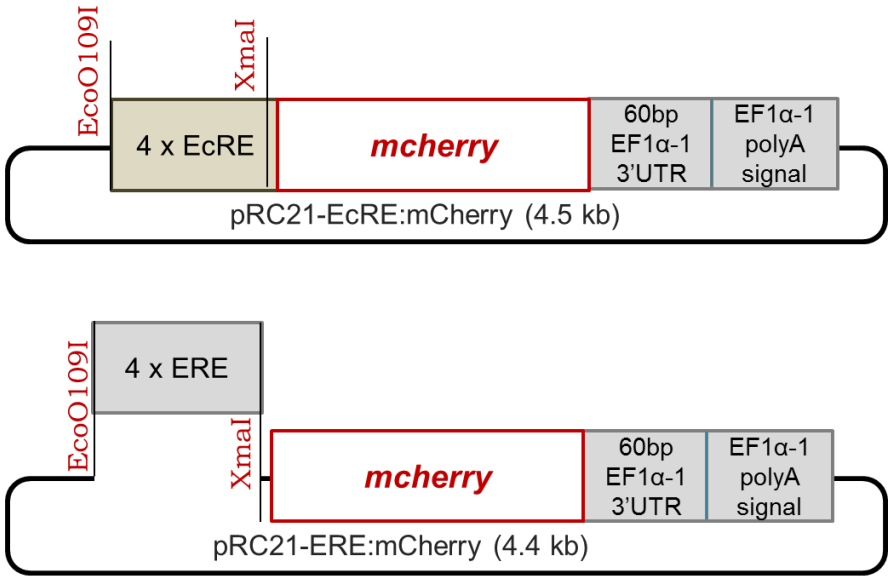

Figure 17. Construction of pRC21-ERE:*mCherry*

For genomic integration, pRC21-hERa as the backbone was digested with SalI and NdeI (NewEngland BioLabs). pRC21-ERE:*mCherry* was digested with BssHII and NdeI (NewEngland BioLabs). The *dsred2* TALE site was amplified from genomic DNA with PCR primers introducing digestion sites for SalI and BssHII. After digestion, all three fragments were joined via MightyMix ligation (TAKARA); in the resulting construct the ERE reporter and the ER sensor halves face opposite directions, it was termed pRC21-estrogensensor (see Results, Figure 21).

All plasmids were transformed into XL10-GOLD *E. coli* after ligation, harvested with a PureYield Miniprep kit (PROMEGA, Fitchburg, WI,

USA), purified by phenol/chloroform extraction followed by ethanol precipitation and their sequence was confirmed by sequence analysis.

In vitro synthesis of TALEN mRNAs

For TALEN mRNA synthesis, left and right TALEN expression plasmids (Naitou et al. 2015) were linearized with Acc65I (NewEngland BioLabs), and purified using the QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany). Linearized DNA fragments were used for *in vitro* transcription with the mMessage mMachine kit (Life Technologies, CA, USA). Poly(A) tails were attached to TALEN RNAs using a Poly(A) Tailing Kit (Life Technologies), following the manufacturer's instructions. The synthesized RNAs were column purified using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany), followed by phenol/chloroform extraction, ethanol precipitation, and resuspension in DNase/RNase-free ultra-pure water (Life Technologies).

Microinjection

Microinjection was conducted according to established procedures (Kato et al. 2011b). In short, adult *D. magna* with empty brood chambers were selected and observed until ovulation started. Then *D. magna* were transferred to ice-chilled M4 medium (Elendt and Bias 1990) containing 80 mM sucrose (M4-suc) and dissected to collect the eggs within the brood chamber. The eggs were stored in ice-chilled M4-suc medium until injection to slow the hardening of the egg membrane. Microinjection was performed within 1 hour post ovulation (hpi) for the same reason. Successfully manipulated eggs were transferred to fresh

medium and were cultured in a 96-well plate at 23 ± 1 °C. From each clutch of eggs, 2 – 3 eggs that were not injected served as control for development.

3.3 Results

Design of an estrogen biosensor for function in the crustacean *D. magna*

I implemented different lengths of the endogenous *daphnia* EF1α-1 3'UTR to adjust expression levels, in accordance with the findings in Chapter 2. I suggested there that the first 60 bp of the EF1α-1 3'UTR might be essential for ubiquitous and stable expression and implemented that length in the ERE:*mCherry* reporter, adding the last 19 bp of the UTR which contain its poly(A) motif to a total length of 79 bp ("trunc. EF1α-1 3'UTR"). For the EF1α-1:*ESR1* sensor on the other hand, I used the full length EF1α-1 3'UTR, 522 bp, for low translation rates of ERα (Figure 18).

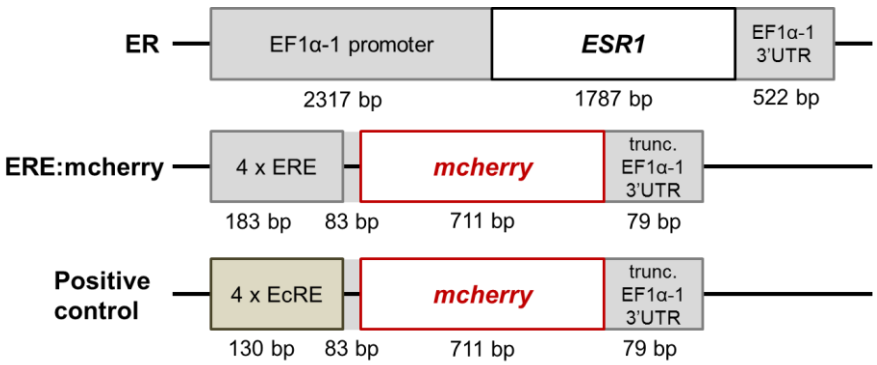

Figure 18. Structure of the different plasmids

ER: pRC21-hERa. Human estrogen receptor α (*ESR1*) with EF1α-1 promoter and full length EF1α-1 3'UTR

ERE:*mCherry*: pRC21-ERE:*mCherry*. *MCherry* with 4 x ERE repeats and a truncated version of the EF1α-1 3'UTR

Positive control: pRC21-EcRE:*mCherry*. *MCherry* with 4 x EcRE repeats and a truncated version of the EF1α-1 3'UTR.

The genetic sequence constructing an estrogen biosensor was then prepared on two separate DNA plasmids. One expresses the human estrogen receptor α (hERα) (Figure 18 A) ubiquitously. The other plasmid (Figure 18 B) contains 4x repeats of the Estrogen Response Element (ERE), *mCherry* as visible reporter, and the truncated EF1α-1 3'UTR. The sequence between the ERE repeats and the *mCherry* start codon is the same as that between endogenous EcRE (Ecdysteroid response element) repeats and its reporter start codon in a previously established ecdysteroid reporter in this species (Asada et al. 2014). That 4xEcRE reporter was used as a positive control in this experiment (Figure 18 C).

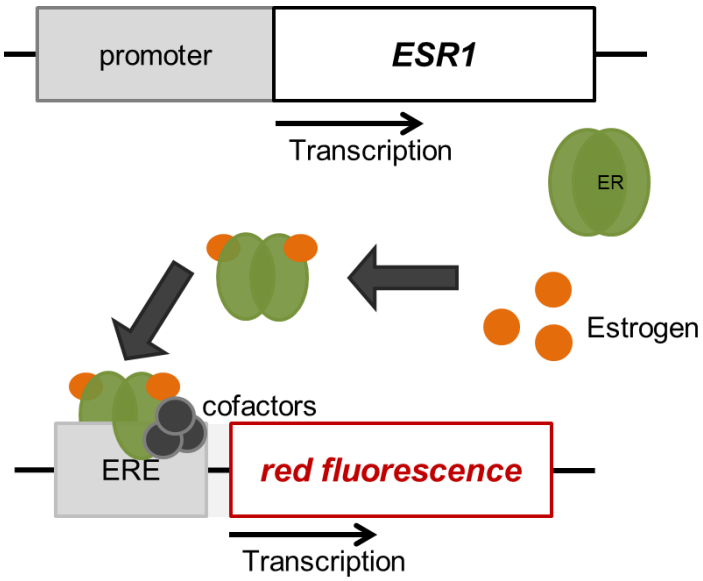

Figure 19. Schematic functions of the different plasmids and response to estrogen exposure

ESR1 transcription and translation result in the presence of ERα in the cells. In the presence of estrogen, ER is activated and binds to EREs, inducing downstream transcription of *mCherry*, resulting in red fluorescence.

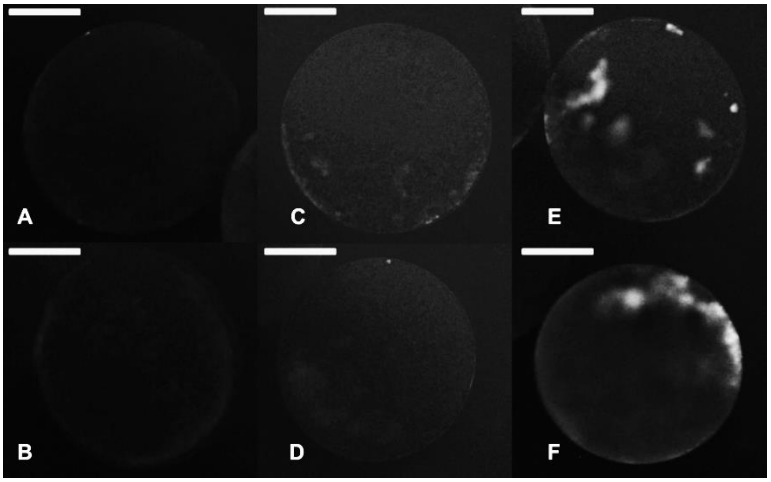

Figure 20. Wild type *daphnia* embryos injected with DNA plasmids

A Uninjected embryo

B Injection of pRC21-hERa and pRC21-ERE:*mCherry*

C Injection of pRC21-ERE:*mCherry* and DES

D Injection of pRC21-hERa and DES

E Injection of pRC21-hERa, pRC21-ERE:*mCherry* and DES

F Injection of positive control plasmid pRC21-EcRE:*mCherry*.

A – D are negative controls. Concentration of plasmids was 50 ng/µL each, 26.8 ng/µL (100 µM) for DES. Pictures taken at 16 hpi. Bar = 100 µm

To test the functionality of these plasmids (see Figure 19) in the presence of an estrogenic compound, DES, microinjection was conducted with wild-type (NIES) *daphnia* eggs. 50 ng/µL pRC21-hERa plasmid, 50 ng/µL pRC21-ERE:*mCherry* plasmid and 26.8 ng/µL (100 µM) DES were injected as single solution, in combinations of two or all three together. When only 1/3rd (data not shown) or 2/3rd of the components were injected (Figure 20 B-D), no red fluorescence could be detected after 18 h, similar to uninjected control eggs (Figure 20 A). When injecting the two plasmids together with an estrogenic compound, DES, on the other hand, fluorescence could be detected (Figure 20 E), as well as after injection of the pRC21-EcRE:*mCherry* control plasmid that responds to endogenous ecdysteroids (Figure 20 F). Thus the

biosensor, including hERα, is functional in an estrogen-dependent manner in *D. magna* embryos. I also concluded that this estrogen sensor is not activated by endogenous compounds in *D. magna* eggs of this stage.

Biosensor plasmid for genomic insertion

For genomic integration using transcription activator-like effector nucleases (TALEN), both parts of the biosensor need to be joined into one plasmid together with a TALE site so the plasmid will be linearized inside the cells. As target, the DsRed2 ORF (EF1α-1:dsred2) of the transgenic minos-red was chosen since a successful knock in will be marked by a loss of red fluorescence in the absence of estrogen exposure. The necessary TALENs for this have been developed previously, they were shown to be highly effective (Naitou et al. 2015). Since the EF1α-1 promoter is fairly strong, particular attention was paid regarding the arrangement of the sensor and reporter cassettes to avoid especially cross-activation of *mCherry* transcription and therefore background fluorescence. In the biosensor plasmid I constructed, the sensor and reporter cassette face in opposite directions (Figure 21). Also, when the linearized plasmid is integrated into the DsRed2 locus, in either direction the upstream cassette will face in the opposite direction from DsRed2's EF1α-1 promoter.

To test the functionality of this estrogen sensor plasmid (ES plasmid), microinjection was conducted with wild-type (NIES) *daphnia* eggs. The plasmid was injected alone at a concentration of 50 ng/µL (not shown) or together with 26.8 ng/µL (100 µM) DES (Figure 22 C). Similarly to previous injections of the positive control plasmid pRC21-EcRE:*mCherry* (Figure 22 B) and to previous injections of two separate biosensor

plasmids with DES (Figure 22 D); presence of both the ES plasmid and DES resulted in significant fluorescence increase.

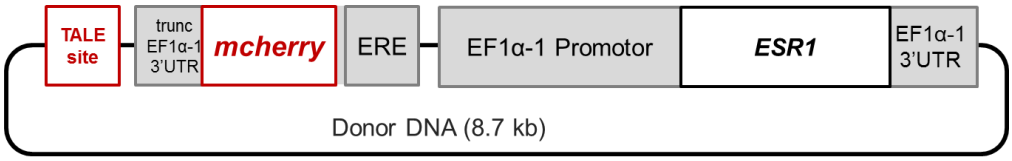

Figure 21. Structure of the estrogen biosensor plasmid

The estrogen biosensor plasmid consists of a TALE site targeting the DsRed2 ORF, a ERE:*mCherry* reporter cassette and an EF1α-1:*ESR1* sensor cassette in opposite directions. Lengths of the elements not to scale.

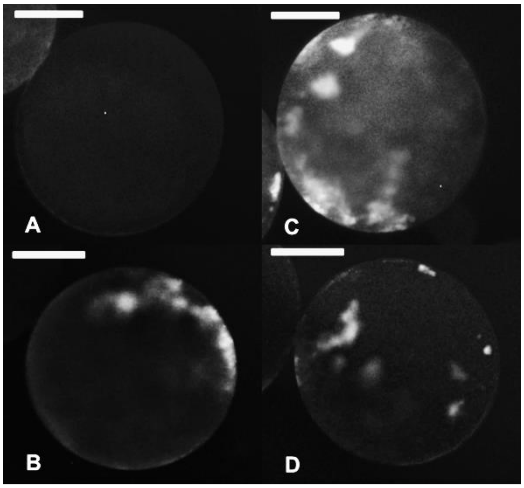

Figure 22. Wild type *daphnia* embryos injected with the biosensor plasmid

A Uninjected embryo

B Injection of positive control plasmid pRC21-EcRE:*mCherry*.

C Injection of ES plasmid and DES

D Injection of pRC21-hERa, pRC21-ERE:*mCherry* and DES

Concentration of plasmids was 50 ng/µL each, 26.8 ng/µL (100 µM) for DES. Pictures taken at 16 hpi. Bar = 100 µm

I concluded that this ES plasmid was functional and suitable for genomic integration to make an estrogen biosensor *daphnia* line.

Genomic insertion of biosensor plasmid

I injected the donor plasmid designed above (**Figure 21**) (25 – 50 ng/µL) together with in vitro synthesized mRNAs that code for DsRed2-targeting TALENs (250 – 500 ng/µL each) into minos-red line *daphnia* embryos.

Table 3. Injected solutions

Injected Solution	Injected eggs at 1 hpi	Hatched eggs	Adults
50 ng/µL plasmid, 500 ng/µL TALEN mRNAs	22	0	0
25 ng/µL plasmid, 250 ng/µL TALEN mRNAs	69	0	0
50 ng/µL plasmid, 500 ng/µL TALEN mRNAs, ½ injection volume	15	**1**	**1**

As shown in Table 3, I first tried microinjection with 50 ng/µL donor ES plasmid and 500 ng/µL each of TALEN mRNAs. Due to high lethality of the injected embryos, this was then adjusted to 25 ng/µL donor ES plasmid and 250 ng/µL each of TALEN mRNAs. Embryos injected with this lower concentrated mixture survived longer than previously but still could not develop past 16 hpi (data not shown).

I further adjusted the injection solution to the first, higher concentration (50 ng/µL donor ES plasmid and 500 ng/µL each of TALEN mRNAs), but injected only half of the usual injection volume of 0.3 nL (Kato et al. 2011b), with the goal to minimize physical stress from the microinjection on the embryos.

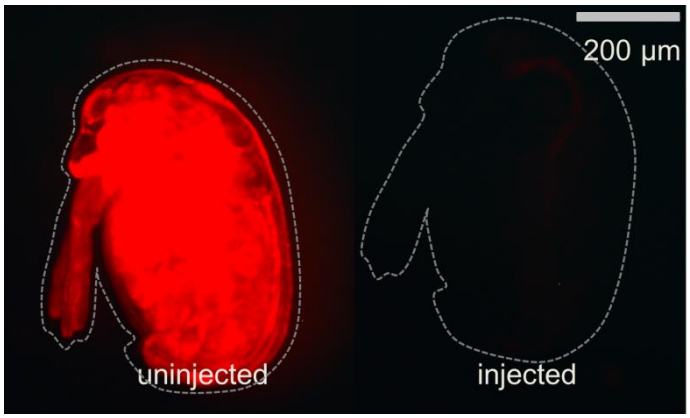

Figure 23. DsRed2 knock-out phenotype

Left: uninjected minos-red juvenile. Right: Single minos-red juvenile surviving microinjection of donor ES plasmid and TALEN mRNAs. 48 hpi, bar = 200 µm.

This approach proved successful, one of the 15 embryos surviving at 1 hpi in this condition survived into adulthood, it showed a loss of DsRed2 fluorescence (Figure 23).

In conclusion, of the 106 embryos total surviving at 1 hpi, one (0.94%) that showed a loss of red fluorescence survived into adulthood. This G0 founded the ES line of *D. magna*.

3.4 Discussion

Daphnia contain no estrogen receptor (Clubbs and Brooks 2007) so to make an estrogen biosensor *daphnia* it is most useful to employ a human estrogen receptor. No human gene has been expressed in *daphnia* before, but comparison of the results from a complete sequence of the mitochondrial genome of *daphnia pulex* (Crease 1999) with human genetic code shows high similarity (see Table 4).

Table 4. Genetic code in *Daphnia* compared to human

	T	C	A	G
T	**TTT Phe F** TTC **TTA Leu L** TTG	**TCT Ser S** TCC TCA TCG	**TAT Tyr Y** TAC **TAA STOP** **TAG STOP**	**TGT Cys C** TGC TGA Trp W TGG
C	**CTT Leu L** CTC CTA CTG	**CCT Pro P** CCC CCA CCG	**CAT His H** CAC **CAA Gln Q** CAG	**CGT Arg R** CGC CGA CGG
A	**ATT Ile I** ATC ATA Met M ATG	**ACT Thr T** ACC ACA ACG	**AAT Asn N** AAC **AAA Lys K** AAG	**AGT Ser S** AGC AGA AGG
G	**GTT Val V** GTC GTA GTG	**GCT Ala A** GCC GCA GCG	**GAT Asp D** GAC **GAA Glu E** GAG	**GGT Gly G** GGC GGA GGG

Table 4 is based on the genetic code in humans. Shown in **broad** the code is the same in human and in *D. pulex*, in light grey there is no information in *D. pulex*, and in dark red the code is different in *D. pulex*. Namely, ATA codes for Met instead of ATG, TGA codes for Trp instead of TGG, and AGA does not encode Arg.

Only a few amino acids are encoded differently in *D. pulex* compared to human, and those don't feature prominently in the ORF of *ESR1*, the

human estrogen receptor α, if they are present at all. Therefore this human gene *ESR1* should in theory be possible to be expressed in *daphnia* based on its genetic code without necessarily requiring codon optimization. Estrogen receptors do however require different cofactors for transcription activation. The estrogen-dependent fluorescence response after microinjection showed that *D. magna* cofactors are sufficient for some transcription activation, though it remains to be seen how effective they are. In Chapter 4 I will evaluate the ES line's response to different concentrations of synthetic and natural estrogens, this should clarify if *daphnia* cofactors are sufficient or if future designs should consider including human cofactors.

A lack of estrogen receptors implies also the possibility of a lack of estrogen response elements, and indeed *daphnia* do not contain known ERE sequences (Colbourne et al. 2011). They do however have a system of Ecdyson Receptor and Ecdyson Response Elements (EcRE) interacting, so those sequences can be used as positive control since they have been proven functional in a biosensor assay previously (Asada et al. 2014).

Functional ERα requires a variety of post-translational modifications, including among others phosphorylation, acetylation, ubiquitination, and palmitoylation (Le Romancer et al. 2011; Vrtačnik et al. 2014). As many crustaceans conduct such post-translational modifications (Hecker et al. 2003; Ollivaux et al. 2009) it is consider likely that *daphnia* may be able to do the same, and indeed, the estrogen-dependent fluorescence response after microinjection (Figure 20, Figure 22) suggests that hERα is functional in *D. magna* embryos. Since this could be shown by a selective fluorescence response, i.e. only in presence of hERα, ERE:*mCherry* and DES, not if any of the elements was missing, I did not quantify expressed ER in injected embryos. Now that an ES line is established it would be interesting to quantify ER expression in different tissues and at different life-stages.

Using minos-red line *daphnia* had advantages as well as disadvantages. This line carries DsRed2 only on one allele, so a single cut was sufficient as a visible marker for genome editing. However, since integration will occur just downstream of the strong EF1α-1 promoter, this needs to be factored in when designing donor DNA for genomic integration. One can implement it in the design and therefore realize shorter, promoter-less donor plasmid sizes for example by implementation of the viral 2A peptide (Kumagai et al. 2017a), avoid it by utilizing long spacer DNA in between the genomic EF1α-1 promoter and the newly integrated cassette, or avoid it by facing the new DNA cassette in the opposite direction. I opted for the latter option (see Figure 24 for possible integration sequences) since it showed the most promise in terms of keeping the plasmid for insertion small enough without using additional, at the time of designing in *daphnia* unproven, sequences like 2A peptide.

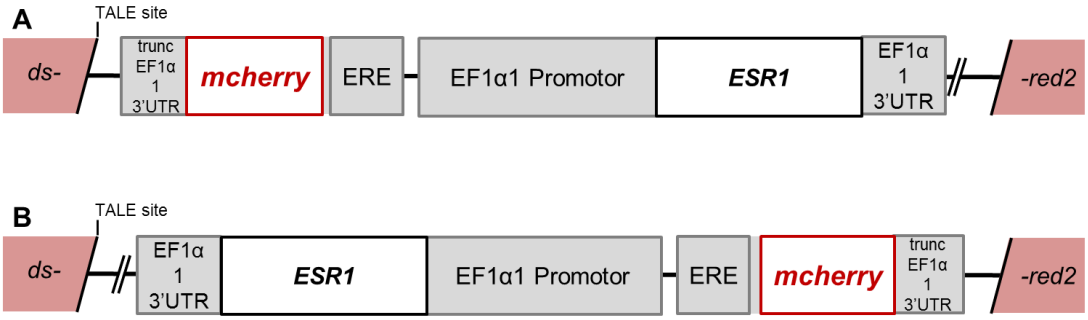

Figure 24. Possible structure of plasmid inserted in DsRed2 locus

A Structure of "forwards direction" integrated biosensor plasmid

B Structure of "reverse direction" integrated biosensor plasmid

Injection of this plasmid together with TALEN mRNAs proved challenging due to a survival rate of zero in the first two tested conditions, 50 ng/µL plasmid and 500 ng/µL TALEN mRNAs (each) compared to 25 ng/µL plasmid and 250 ng/µL TALEN mRNAs (each). Under these conditions, none of the 22, respective 69, injected embryos

could develop past 20 h. I then used the higher concentration (50 ng/µL plasmid and 500 ng/µL TALEN mRNAs (each)), but only half of the usual injection volume. Out of 15 injected embryos, one could develop into adulthood and founded the ES line. Because of the low sample number, I cannot make any substantial conclusions about the positive effect of a lower injection volume, particularly since other experiments found higher rates of genomic insertion from higher injection volumes (data not shown). It is conceivable that in general, a higher volume may be advantageous since the probability of the donor plasmid being present at the genomic DNA double strand break site increases, but that in this specific case, the negative effects of high concentrations of donor DNA required lower concentrations to allow for survival.

ERα should be expressed ubiquitously but in relatively low concentration to avoid possible adverse effects on the organism. Once ERα is activated by estrogens and induces transcription of the marker gene, maximum possible yield was desirable to make a sensitive biosensor. To ensure this, I employed different lengths of EF1α-1 3' UTR which were shown to have the desired effect in Chapter 2. In the next chapter about characteristics of the resulting transgenic *daphnia* line I will explore how successful this fine-tuning of expression was.

4 Characteristics of the ES *Daphnia magna*

4.1 Introduction

In this study I focused on two compounds with estrogenic activity. 17β-Estradiol, E2, the main natural estrogen in humans, and Diethylstilbestrol, DES, a strong synthetic estrogen formerly prescribed to pregnant women to avoid miscarriage. DES has fallen out of most medical use since its strong correlation with uterine cancers in the offspring from mothers who took DES during pregnancy was discovered in the 1970s (Martino et al. 2002; Veurink et al. 2005); it is nowadays mostly limited to treatments of prostate cancers in men and some breast cancers in women who have passed the menopause. Structures of both E2 (molecular weight 272.38 g/mol) and DES (molecular weight 268.35 g/mol) are shown in Figure 25.

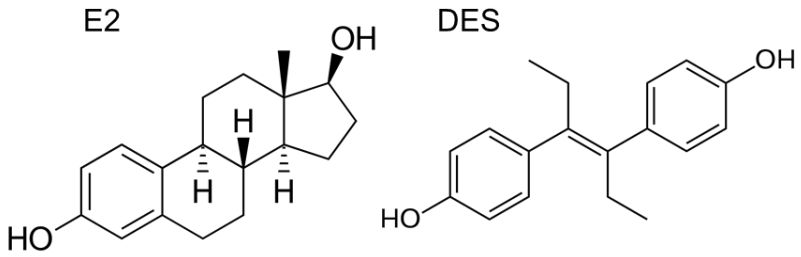

Figure 25. Structures of E2 and DES

Chemical structure of E2 (17β-Estradiol) and DES (Diethylstilbestrol)

Neither E2 nor DES is readily solvable in water, so N,N-dimethylformamide (DMF) was chosen as solvent instead. DMF has an LC_{50} (48 h) with *D. magna* (Straus) of about 13 mg/L, with

recommendations for chronic tests to stay under 1.2 – 2.5 mg/L (LeBlanc and Surprenant 1983).

For exposure of EDCs to biosensor organisms, a wide variety of life stages and exposure durations is used (see studies quoted in Table 1), ranging from shorter exposures to embryonic and larvae stages to longer adult exposures. In this study I focus on the juvenile stages since they are generally still more sensitive than adult stages but less laborious to use than embryonic stages.

How *daphnia* take up and accumulate or metabolize chemicals depends on their physical and biochemical properties. Larger nanoplastic particles appear to be taken up by filter feeding and then pass the gut (Mendonça et al. 2011) while smaller sized nanoplastics can pass the gut epithelial membrane (Mattsson et al. 2016; Santo et al. 2014) and accumulate in or on lipophilic cells like yolk granules. That way they can also be transferred maternally (Brun et al. 2017). Food ingestion aside, water filtration and dermal adsorption are uptake mechanisms for various chemicals (Dai et al. 2013; Jiang et al. 2018), though a common method to differentiate between them include exposure of dead *daphnia*. In the case of ES line *daphnia*, the reporter results from a genetic cascade resulting in fluorescence, so unfortunately exposure of dead *daphnia* is not very useful. For this reason I will not differentiate between different possible uptake mechanisms of E2 in this study.

In this chapter, my goal was to characterize ES line *D. magna* and determine their practical feasibility. This was accomplished by a reproduction assay and exposure to DES and E2 for comparison of sensitivity and tissue specific responses.

4.2 Methods

Reproduction assay

One neonate *daphnia* (under 24 h old) was put in 2 mL AdaM (Klüttgen et al. 1994) per well in a 24 well plate. *Daphniids* were cultured at 22–24 °C under a light/dark cycle of 16/8 h. The culture medium was renewed once a week. *Daphniids* were fed every day with 0.06 mg of *Chlorella vulgaris* (Nikkai Center, Tokyo, Japan) during the first week. After maturation, offspring was counted and removed every second day and adults were fed with 0.12 mg *Chlorella* per day until day 28.

Exposure

Diethylstilbestrol (DES) (Sigma Aldrich, St. Louis, MO USA) and 17β-Estradiol (E2) (Sigma Aldrich) were dissolved in 100 % *N,N*-Dimethylformamide (DMF) (Nacalai tesque, Kyoto, Japan) to final concentrations of 10 mg/mL as stock solutions. For exposure, the stock was diluted with AdaM (Klüttgen et al. 1994) to final solvent concentrations of under 0.2%. For exposure, neonate *daphniids* (under 24 h old) were kept in 24 well plates (Thermo Fisher Scientific, Waltham, MA USA), one individual in 2 mL medium per well, at 23 ± 1 °C under a light/dark cycle of 16/8 h, with medium renewal every day.

Fluorescence microscopy

Daphnia were partially immobilized in minimal amounts of medium on micro slide glasses (Matsunami, Osaka, Japan). Red fluorescence intensity was recorded with a colour digital camera (Leica DC500) mounted on a Leica M165C fluorescence microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) equipped with a 545 nm excitation and a 620 nm barrier filter. The pictures were taken under 63 × magnification with 100 % aperture, 1 s exposure, 3.0 gain, 1.5 saturation, and 1.0 gamma. The red fluorescence intensity of neonates was recorded every 24 h in the first exposure experiment, at day 4 of exposure henceforward.

Fluorescence quantification

Fluorescence intensity (fluo) was quantified using ImageJ software. Previously reported methods (Gavet and Pines 2010; Potapova et al. 2011; Törner et al. 2014) were adapted to reduce background interference. In this study, only the area of thoracic appendages (thorap) was used for calculations with the following formula.

Fluo (thorap) = total fluorescence of thoracic appendages − (number of pixels of the selected area × mean of three background fluorescence measurements)

This value was then normalized for exposed *daphnia* by defining the value of unexposed *daphnia* of the same age as 1.

4.3 Results

Reproduction

Testing the maturation and reproduction rate of ES line *D. magna* compared to the minos-red line and the NIES wildtype showed that the first clutch was both released later (day 12 – 15 instead of day 10) and clutch size was smaller in the ES line, resulting in a much lower total number of juveniles from one *daphnia* at 28 days of age, 28 ± 5 for ES line, 50 ± 6 for minos-red line, and 50 ± 2 for NIES wildtype (Figure 26).

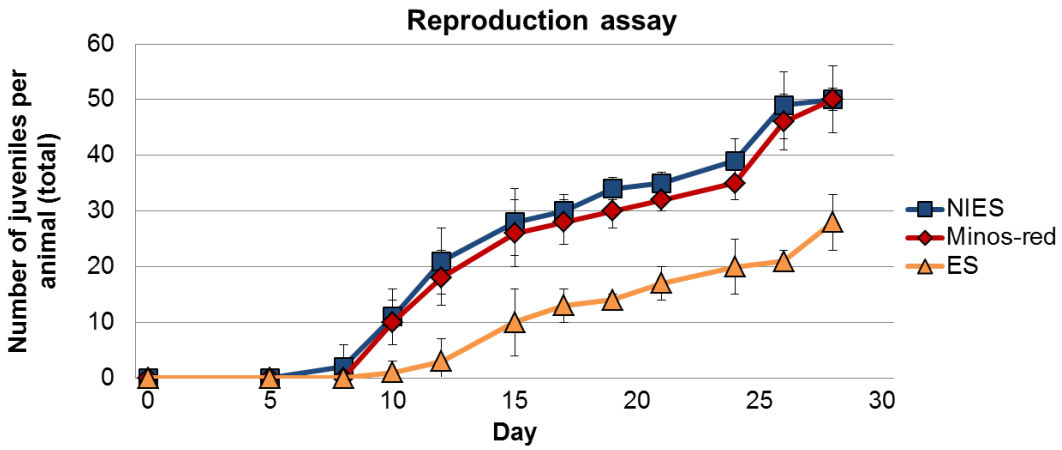

Figure 26. Reproduction assay

Total number of juveniles from one adult *daphnia* over the course of four weeks. Wildtype NIES in blue, minos-red line in red, and ES line in yellow. Error bars show standard deviation, n = 6.

Estrogenic exposure of neonates

To find the optimal time point and method to quantify fluorescence response to estrogen exposure, I first exposed neonates (under 24 h old) to 2 mg/L DES over seven days.

Then I exposed ES *daphnia* to different concentrations of DES and E2 to determine sensitivity.

Diethylstilbestrol

For fluorescence quantification, the area of thoracic appendages (marked by dashes and dots in Figure 27) was considered optimal since they showed a significant fluorescence response to exposure. This also avoids distortion of quantification values due to varying autofluorescence in the gut from algae feeding (marked by square dots in Figure 27). Second antennae also show some fluorescence response (marked by smaller round dots in Figure 27), but not as strong as that of thoracic appendages.

I then exposed neonate ES *daphnia* (under 24 h old) to 2 mg/L DES for seven days and calculated the total fluorescence of the thoracic appendages, normalized by the same value in unexposed control *daphnia* of the same age (Figure 28).

Day four and day seven or later show the highest difference between control and exposed individuals so all later neonate exposures were conducted for four days.

When testing this line for sensitivity regarding DES, I exposed neonates to 0.1 mg/L – 2.0 mg/L DES for four days (Figure 29). In these experiments, the detection threshold for DES was 0.5 mg/L (Figure 30).

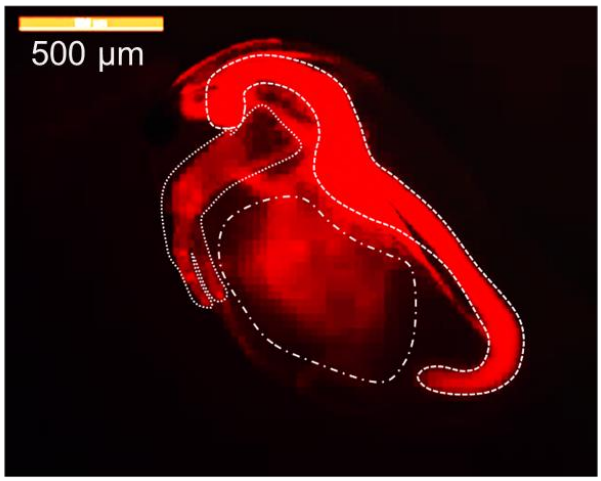

Figure 27. ES line *D. magna* exposed to 1mg/L DES for four days

Area of thoracic appendages used for fluorescence calculation is marked by dashes and dots. Second antennae are marked by round dots. Square dots mark the gut (autofluorescence from algae food). Bar = 500 µm.

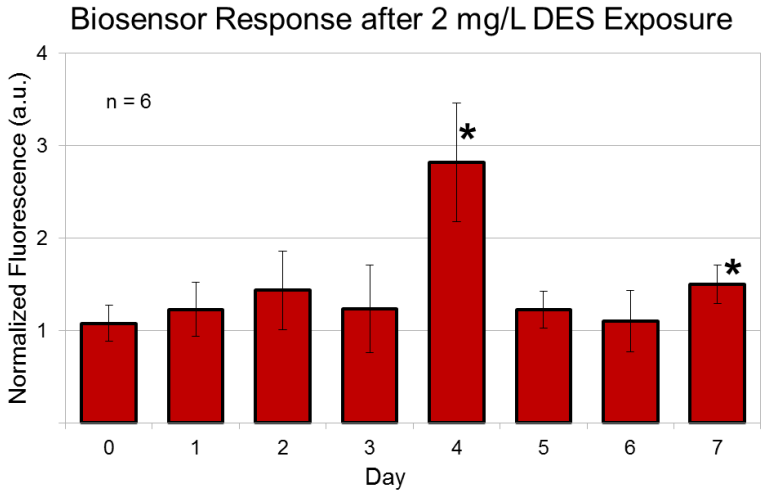

Figure 28. Fluorescence of thoracic appendages of ES daphnia exposed to 2 mg/L DES

Time course of fluorescence change over seven days, pictures taken every 24h. Normalized to fluorescence of control *daphnia* of the same age, units are arbitrary (a.u.). Significant differences (*) compared to control ($p<0.002$) at day 4 and from day 7 onward; n =6.

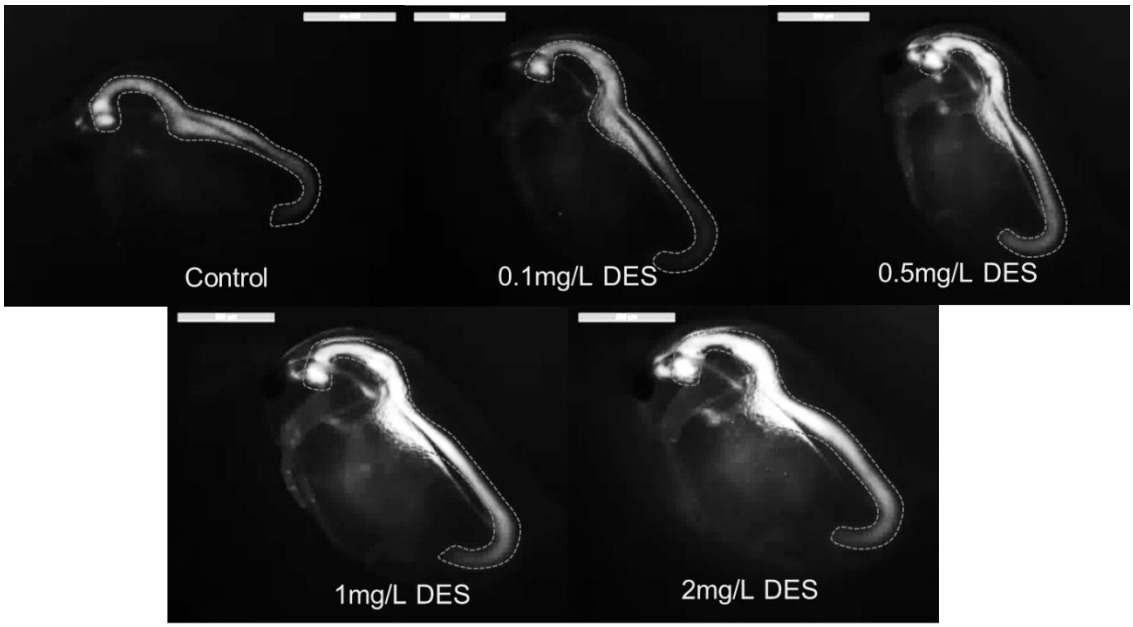

Figure 29. Biosensor responses to different concentrations of DES

ES *daphnia* juveniles exposed to different concentrations of DES, pictures taken at day 4, bar = 100 µm. Dotted lines marking the gut (autofluorescence from fed algae).

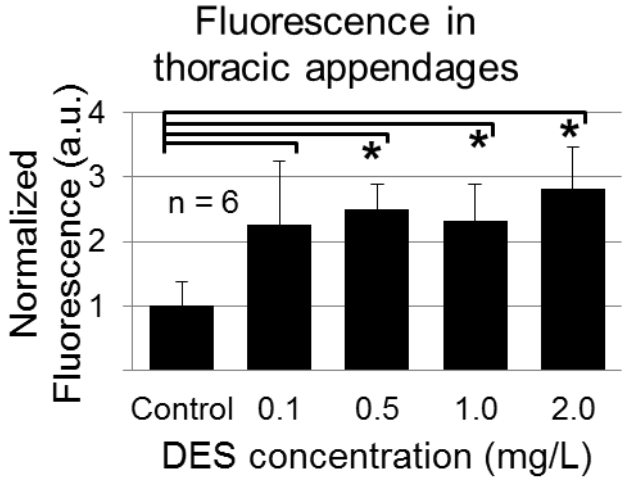

Figure 30. Quantified biosensor response to different concentrations of DES

Fluorescence calculated from thoracic appendages, normalized to control *daphnia* of the same age. Asterisks (*) indicating $p<0.002$ compared to control; n =6.

Estradiol

When testing the ES line for sensitivity regarding E2, I exposed neonates to 0.1 mg/L – 4.0 mg/L E2 for four days (Figure 31). In these experiments, the detection threshold for E2 was 4 mg/L (Figure 32).

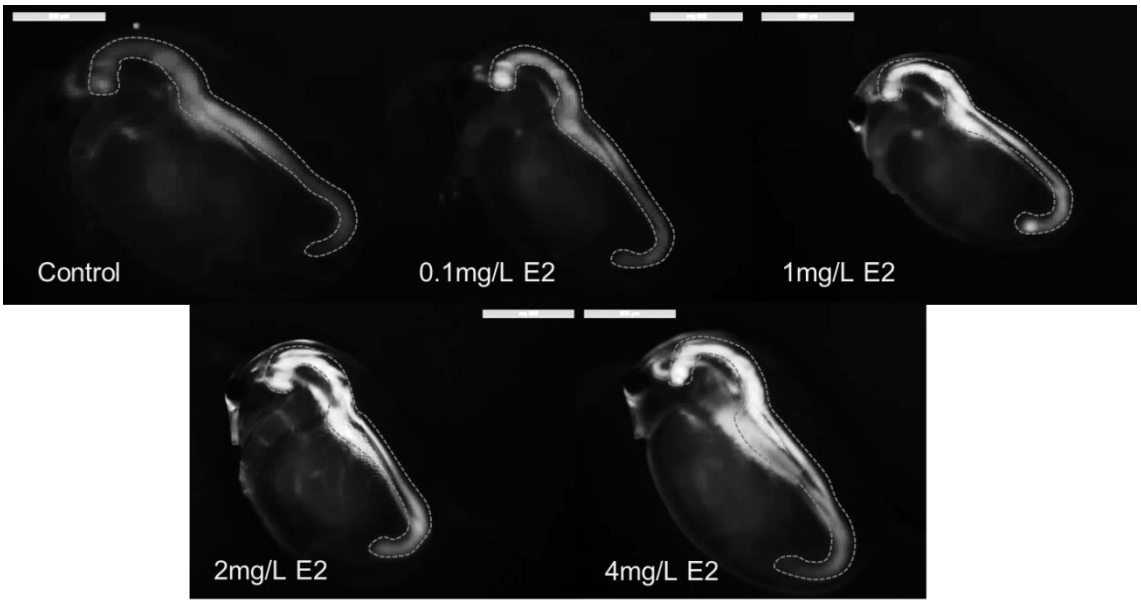

Figure 31. Biosensor responses to different concentrations of E2

ES *daphnia* juveniles exposed to different concentrations of E2, pictures taken at day 4, bar = 100 µm. Dotted lines marking the gut (autofluorescence from fed algae).

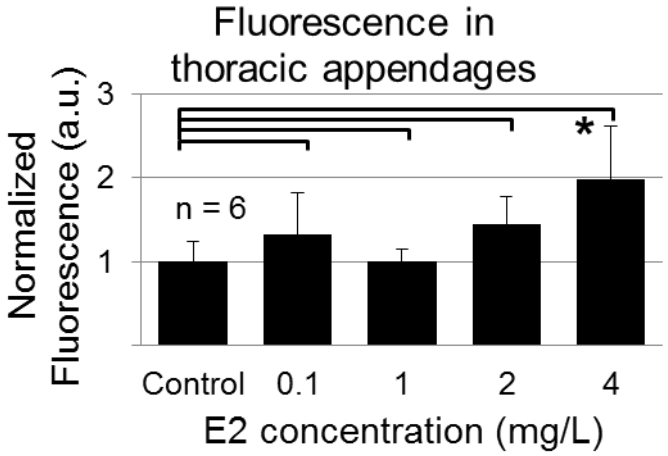

Figure 32. Quantified biosensor response to different concentrations of E2

Fluorescence calculated from thoracic appendages, normalized to control *daphnia* of the same age. Asterisks (*) indicating $p<0.002$ compared to control; n =6.

4.4 Discussion

My ES line *daphnia* reproduce considerably slower compared to the minos-red line and the NIES wildtype, as shown by both a later first clutch release and smaller clutch sizes (see Figure 26). However, *daphnia* are typically kept in tanks of 80 individuals per 5 L AdaM medium, so we can still harvest over 2 000 juveniles after 28 days from one single tank. Therefore for high throughput testing a simple increase of tanks should be sufficient.

For exposure evaluation, day four of exposure was chosen since the fluorescence response was the strongest at this instar 2 point-in-time (see Figure 28), only rivalled by response after day eight (instar 4, data not shown). While the exact reasons for the high sensitivity at this one early day remain unclear, the juvenile period around instar 1-2 is generally known to be sensitive to kairomones from fish for predator-induced polyphenisms. In the *daphnia* species that can form neck-teeth (*Daphnia pulex*) or helmets (*Daphnia cucullata*), kairomones are thought to act directly during these early larval stages to promote such growths (Harris et al. 2012). However, for helmet formation, embryonic maternal exposure is required for such polyphenisms' induction, while exposure during instars is required to maintain it (Agrawal et al. 1999). This suggests a possibility to increase ES line *daphnia* sensitivity by exposing not only the juvenile to estrogenic chemicals but the mother animal as well.

The detection thresholds for DES and E2 in the presented experiments were 0.5 mg/L and 4mg/L respectively. This is well below what is common in other E2 biosensor organisms, ng/L to µg/L range in human cells, fish and yeast, undermining this line's practical application until increased sensitivity is achieved. Sensitivity could

potentially be improved by further changing the method for fluorescence quantification, by exposing embryos instead of neonates, by changing the genetic cassette or its genomic integration locus. Since estrogen receptors do require cofactors for transcription activation it might be beneficial to consider including human cofactors in future biosensor designs in *daphnia*.

The response to EDCs was constant for over 15 generations, tested with DES exposure of G2, G10 and G15 juveniles. Resulting fluorescence was not significantly different (data not shown), so the ES line can be considered stable. Individual differences exist even within offspring of the same mother who should all be genetically identical (see standard deviations in Figure 30 and Figure 32), which was a factor in the low sensitivity of this line.

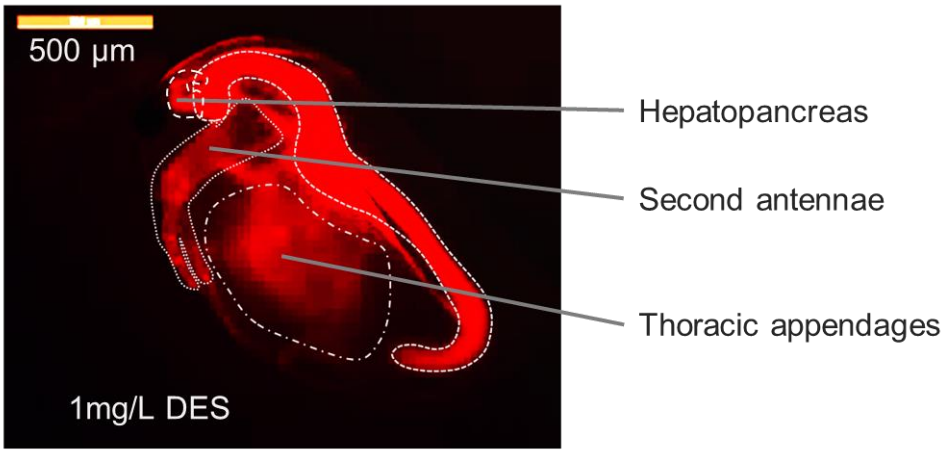

Figure 33. Fluorescence localization

ES line *D. magna* exposed to 1mg/L DES for four days. Fluorescence response is marked in the hepatopancreas, the second antennae and the thoracic appendages. Bar = 500 µm.

For fluorescence quantification, I exclusively focused on the area of thoracic appendages in this study. This area showed the highest

difference between exposed and unexposed *daphnia*, as well as little background fluorescence in general. Using only this particular area for quantification also has the advantage of cutting out gut autofluorescence from algal feeding, which could not be reduced to zero even on a minimal diet of yeast (data not shown). In the end, quantification of thoracic appendage fluorescence failed to show a linear correlation between exposed concentration of ECDs and fluorescence intensity, while visually, such a dose-dependent effect seemed to be present. The exact reasons for this remain yet to be elucidated, but focusing on other organelles might be an option. Somatic cells in the second antennae also showed fluorescence response after estrogen exposure, but thoracic appendages are vastly more convenient to photograph and show a higher response to exposure, if only for their much higher cell number from their bigger size. Both of them move through a considerable volume of water so it is not unlikely that their response is not singularly from estrogenic compounds taken up by feeding but also directly from the medium via dermal adsorption. The hepatopancreas is involved in the sequestering of hormones taken up by feeding, so quantification of that small area should give us the line's sensitivity for ECDs from feeding (bioaccumulation).

Focusing on thoracic appendages and using four days of exposure, I could show that ES line *daphnia* can detect both DES and E2, though the sensitivity for both needs improvement for practical applications of the line.

5 Discussion

Protein overexpression is commonly used to understand the role certain proteins play, in addition to classic loss-of-function experiments. Overexpression screens are readily available in many organisms, as of recently including *daphnia*. Commonly promoters might be exchanged to achieve higher expression, though if expression is too high there will be growth defects from resource overload, promiscuous interactions, or negative pathway modulations (Reviewed in (Moriya and Cohen-Fix 2015; Prelich 2012). Expression of fluoresce tagged proteins is employed in reporter assays both in vitro and in vivo to gain insight into cell type-specific spatio-temporal dynamics of gene expression and signal transduction. In *D. magna*, a transgenic line expressing a histone H2B-EGFP fusion protein was utilized to visualize the dynamics of specific cell populations, especially during development (see Figure 6) (Kato et al. 2012). These transgenic lines are laborious to make, so an efficient system for transient overexpression of proteins in *daphnia* had to be developed. I was successful focusing on the 3' UTR's effect on RNA stability and therefore translation rate of protein, full length EF1α-1 3' UTR was identified as a negative regulator of protein expression in *D. magna* while shortening the 3' UTR increased the reporter expression. Following this, shortened versions of the EF1α-1 3' UTR have been implemented both by me and other researches especially when using fluorescence reporters to achieve good signal-to-noise ratios (Kumagai et al. 2017b, 2017a). Further exploration of different promoters and 5' UTRs will hopefully allow us to fine-tune expression levels and cellular location of protein even more in the future. Potential applications are manifold and range from more detailed gain-of-function experiments to the production of protein and their secretion into fat droplets or eggs for easier harvest and purification.

The new estrogen biosensor line of transgenic ES *D. magna* could successfully detect both DES and E2 at different concentrations and with different sensitivity, in accordance with their different binding affinities to hERα (Folmar et al. 2002). The general background fluorescence was relatively low so it is sensitive enough to detect EDCs four days after exposure (Figure 28). The biosensor was not functional without any exposure of estrogenic compounds even though the control EcRE-reporter (Asada et al. 2014), different in only the promoter sequence, could detect endogenous ecdysteroids. This result is consistent with previous finding that *Daphnia* do not have any endogenous estrogens nor any ERs (Baker 2008). However, sensitivity was too low for practical applications with 0.5 mg/L for DES and 4mg/L for E2.

Table 5. E2 sensitivity of various biosensors

Receptor	Reporter	Organism	Source	Detection threshold for E2
hERα	ERE:*Luc*	Human cell lines	(Legler et al. 1999)	**0.13 ng/L**
hERα	ERE:*GFP*	Drosophila	(Thackray et al. 2000)	**0.3 ng/L**
hERα	ERE:*Luc*	Yeast cell lines	(Leskinen et al. 2005)	**0.13 µg/L**
zER	ERE-zvtg1:*GFP*	Zebrafish	(Chen et al. 2010)	**0.1 µg/L**
zER	ERE:*GFP*	Zebrafish	(Gorelick and Halpern 2011)	**0.5 µg/L**
hERα	ERE:*mCherry*	Waterflea	(presented here)	**4 mg/L**

The sensitivity of the yeast assays is generally approximately an order of magnitude lower compared to in vitro bioassays based on mammalian cells, though comparable with zebrafish sensors. The main disadvantage of the yeast tests as well as the zebrafish sensors is that the activity of the anti-estrogens and estrogenic compounds seems to be different in yeast cells, zebrafish cells and mammalian cells, which is why I used a human ER in my research.

For the mRNA microinjections in Chapter 2 I used DsRed2. This red fluorescence protein had already been shown functional and non-toxic in *daphnia* at the time. It matures slowly but was considered reasonably bright. For the estrogen biosensor construct in Chapter 3, I switched from the tetramer DsRed2 for the monomer mCherry, which matures very rapidly, with the goal of achieving a fast fluorescence response to EDC exposure. Given the present leisurely response of ES *D. magna* to exposure, maturing pace is not an important factor, though it might be in future iterations of the *daphnia* biosensor.

Sensitivity for example could be improved by testing different iterations of the sequence between ERE and the *mCherry* start codon (Nardulli et al. 1996). From the known timeframe for kairomone induced polyphenisms (Agrawal et al. 1999; Harris et al. 2012), exposing not only the juveniles to estrogenic chemicals but the mother animal as well might improve sensitivity too. Since growth and reproduction in ES line *daphnia* was already slowed compared to wild-type, increasing expression of hERα is likely to have adverse effects, so that is unlikely to be a solution. Replacement of *mCherry* with a brighter fluorescent protein is another option, like tdTomato to stay in the red spectrum, or mNeonGreen (Heppert et al. 2016; Hostettler et al. 2017), as is codon optimization (see Table 4) or the introduction of human coactivators like SRC-1 and -3, and LRP16 (Han et al. 2007; Yi et al. 2015). To test environmental estrogens from environmental samples it would be advantageous to first improve sensitivity to dissolved, pure DES and E2,

but if the water sample is concentrated appropriately ES line *daphnia* should be able to detect estrogenic activity after exposure.

To express hERα we used the promoter of *EF1α-1* which produces highly abundant mRNA in this species (Kato et al. 2012), which could easily lead to over-expression of hERα. And indeed reproduction was slowed down in the ES line compared to both a wild-type strain of *D. magna* (NIES) and the minos-red line (Figure 26). Therefore particularly the EF1α-1:*ESR1* sequence might need to be adjusted to lower expression levels by truncating the promoter sequence or by using promoters of other ubiquitously expressed genes such as ribosomal protein L32 and β-actin.

From the genetic make-up of the biosensor, fluorescence was expected and detected all over the body, with stronger fluorescence in digestive tissues presumably from feeding and the hepatopancreas which is involved in sequestering hormones taken up by feeding (Mykles 2011). Stronger fluorescence was also detected in the second antennae which are responsible for locomotion. It was shown previously that negative effects from E2 exposure to *D. magna* are caused by its bioconcentration in the medium, not by bioaccumulation from feeding exposed algae (Beecher 2013), so dermal adsorption in second antennae and thoracic appendages, both of which move through a considerable volume of medium, likely plays a role in E2 uptake and therefore localization of fluorescence response.

In conclusion, both hERα and ERE could be shown to be functional in water fleas for the first time, suggesting a huge potential for use of *daphnia* to study the interaction of human genes with environmental factors like the effect of EDCs in this study. This also indicates that *daphnia* cells can conduct the post-translational modifications necessary for formation of active hERα (Leader et al. 2006) and that their cofactors are sufficient though not optimal for ER to initiate transcription.

It would be interesting to cross this line with other transgenic lines of Daphnia and visualize more than one stressor at a time. To increase ease of use especially in the field, it would be beneficial to implement a reporter that is not based in fluorescence but that can be detected by the naked eye or a simple light microscope. Over-expression of haemoglobin and the subsequent redder colour of *D. magna* (Gorr et al. 2004; Ha and Choi 2009) is a promising approach, as is increased black colour from expressing melanin or other darker pigments which are usually only found in *daphnia* species exposed to higher UV radiation like *daphnia melanica* (Rhode et al. 2001; Schumpert et al. 2015).

Summary

- I developed a method for protein overexpression in *D. magna* using mRNA microinjection.
- I characterized the effect of different lengths of EF1α-1 3' UTRs on translation modulation, finding several RNA instability motifs.
- I designed, tested and integrated an estrogen biosensor construct into the *D. magna* genome.
- ES *D. magna* express functional hERα and exhibit fluorescence responses to exposure to both natural and synthetic estrogens as tested with E2 and DES, but sensitivity must be improved for practical application.
- Functional hERα was expressed in *daphnia* for the first time.

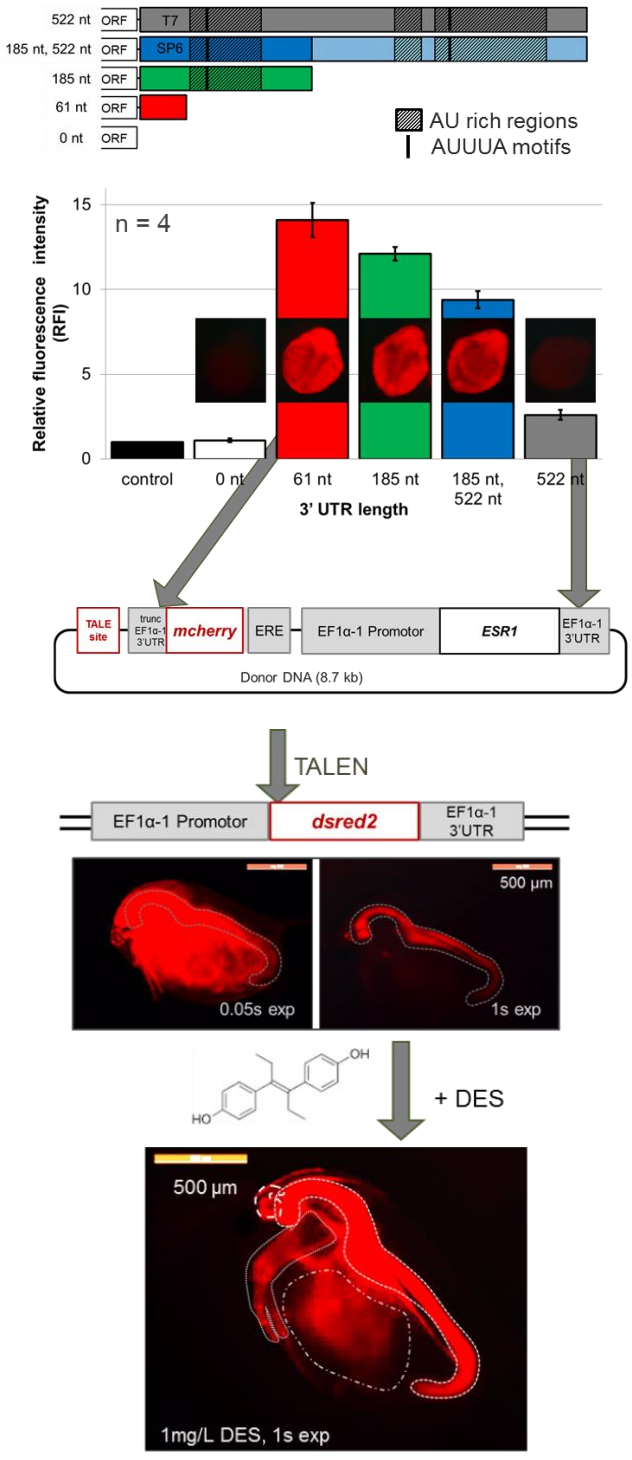

Figure 34. Schematic thesis summary

Conclusion

For the first time, a human gene (*ESR1*) was successfully expressed in the crustacean *D. magna* as part of a biosensor for estrogen. The new transgenic line of *D. magna* expresses *ESR1* at a low concentration due to implementation of full length EF1a-1 3'UTR, and *mCherry* after estrogen-dependent transcription activation. This transgenic *daphnia* can indicate presence of both DES and E2 after exposure.

Publications

Paper

Törner K, Nakanishi T, Matsuura T, Kato Y, Watanabe H. (2018) Genomic integration and ligand-dependent activation of the human estrogen receptor α in the crustacean *Daphnia magna*. PLoS ONE 13:e0198023; doi:10.1371/journal.pone.0198023.

Törner K, Nakanishi T, Matsuura T, Kato Y, Watanabe H. (2014) Optimization of mRNA design for protein expression in the crustacean *Daphnia magna*. Mol Genet Genomics 289:707–715; doi:10.1007/s00438-014-0830-8.

Poster

Törner K, Kato Y, Watanabe H. (2017) TALEN-mediated knock-in of an estrogen biosensor into *Daphnia magna* genome via non-homologous end joining. 2nd Annual Meeting of the Japanese Society for Genome Editing, Osaka, Japan.

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