Testing different approaches for generating transgenic zebra finches towards optogenetic manipulation of interneurons for functional studies

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Erklärung über die eigenständige Verfassung der vorgelegten Dissertation

Hierdurch versichere ich, dass ich meine Dissertation selbstständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet habe.
Grundsätzlich wurden die Experimente und Auswertungen eigenständig durchgeführt. Bei den Slot Blots, der Genotypisierung, der Kloakenmassage und der Elektroporation wurde ich von Ulla Kobalz, Janett Meinecke und Dr. Ezequiel Mendoza unterstützt.
Die Dissertation ist weder in dieser noch in ähnlicher Form in einem früheren Promotionsverfahren angenommen oder abgelehnt worden.

Berlin, 11.10.2022 Sina Denise Girra

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Abstract

Transgenesis is a very effective tool to study the function of genes *in vivo* as it affords the best experimental control over particular cell types from the start of development. For popular animal models, such as mice or fruit flies, a variety of methods to generate transgenic animals are established and many transgenic strains are commercially available. In avian species, most research has been conducted on chickens and quails, but for songbirds, a pipeline to reliably produce transgenic individuals is still lacking. Songbirds offer a unique possibility to unravel questions concerning the neural basis of behavior, as many species are easy to keep and breed, exhibit a diversity of social behaviors including a rich repertoire of vocalizations used for communication. The use and production of many songbird vocalizations need to be learned during infancy, akin to speech learning in humans. Most research on the topic has been conducted on zebra finches (*Taeniopygia guttata*). A few publications describing the generation of transgenic zebra finches exist (Agate et al., 2009; Liu et al., 2015; Abe et al., 2015; Gessara et al., 2021), but have not enabled the scientific community of songbird researchers to create transgenic finches as a routine technique. It thus remains of great interest to investigate alternative methods or to improve on already published ones.

In this thesis, two different methods for the generation of transgenic songbirds were tested. First, a method described in mice was tried to transfer to zebra finches involving microinjection and electroporation of testes. The difficulty was to overcome the different location of testes in birds and mice, e.g. inside versus outside of the body cavity respectively. Second, a previously published approach using progenitor cells of the germ line (PGC) was chosen (Gessara et al., 2021). This method was reported to lead to transgenic founders and offspring in a more efficient way than the first publication of transgenic finches, in which lentivirus was injected into the earliest stage of embryogenesis' (Agate et al., 2009).

For the first approach, access to zebra finch gonads was achieved via laparotomy, and the manipulation of testes was conducted successfully without affecting male’s fertility. Different parameters were tested, and best conditions were determined by amount of reporter fluorescence in tissue sections of treated testes. Stable expression of the fluorescent reporter was achieved through stable integration of it by HypBase; suggesting that manipulation had long-term effect through integration of the transgene by transposition. The transgene could be detected in testes by PCR and slot blot genotyping as well as histology. Nevertheless, no transgenic offspring was identified yet.

For the second part of the study PGC extraction, culturing and transduction by lentivirus was implemented successfully. Similar levels in development of manipulated embryos as in Gessara et al., 2021 (45.5% vs. 56.3%) were generated. Hatching rates could not be compared as all tests were performed before 2/3 of embryonic development. Unfortunately, genotyping did not exhibit any transgenic founder or offspring among embryonic samples, which was the case for every hatchling in the previous study. In both approaches, difficulties with PCR genotyping (apparently due to contamination) were noted and consequently the method was switched to Slot blot, which lead to more reliable results.

Here, a new method for transgenesis in zebra finches is presented that led to genetically modified testes of treated male zebra finches (through '*In vivo* microinjection and electroporation of testes'). Unfortunately, this study was neither able to generate transgenic offspring by '*In vivo* microinjection and electroporation of testes', used for the first time in a songbird, nor to repeat the outcome of Gessara's PGC based approach. Still, both methods might be interesting for future studies as the Gessara publication had convincing more founder individuals than the classical 'Lentiviral injection into stage x embryos' approach. However, PGC heterogeneity (Jung et al., 2022) might be considered in future projects, that are PGCs based. Testes manipulation seems promising for songbird transgenesis after some refinements (e.g. optimized parameters, hemi-castration).

Zusammenfassung

Transgenese ist ein sehr effektiver Prozess, um die Funktion bestimmter Gene *in vivo* zu studieren, da hierbei keine natürlich auftretenden Interaktionen vernachlässigt werden und im Gegensatz zu anderen Ansätzen (z.B. *in vitro* Experimente) auch frühe Entwicklungsstadien betrachtet werden können. Für bekannte Tiermodelle, wie Mäuse oder Fruchtfliegen, sind bereits viele veränderte Linien kommerziell erhältlich sowie diverse Strategien zur Generierung transgener Tiere erfolgreich etabliert. Im Vogelreich wurde vor allem Forschung and Hühnern und Wachteln betrieben. Jedoch fehlt noch eine Pipeline für Singvögel. Singvögel bieten zu einen die Möglichkeit Fragestellungen bezüglich ihres Verhaltens zu erforschen, da es sich um sehr soziale Tiere handelt. Zum anderen sind Wissenschaftler besonders in Gesangserwerb, Gedächtnisbildung und vokale Lautäußerungen wegen der Parallelen zur menschlichen Sprache und der Relevanz für unsere Gesellschaft, interessiert. Es existieren bislang wenig Publikationen zu transgenen Zebrafinken (*Taeniopygia guttata*). Die meisten von ihnen weisen eine geringe Effizienz bei der Generierung transgener Gründertieren auf (Agate et al., 2009; Liu et al., 2015; Abe et al., 2015; Gessara et al., 2021), daher sollten weitere Bemühungen unternommen werden neue oder bereits veröffentlichte Methoden zu entwickeln bzw. zu verbessern.

In dieser Studie wurden zwei Methoden zur Generierung transgener Singvögel angewendet: Zunächst wurde versucht die Methode '*In vivo* Mikroinjektion und Elektroporation von Hoden', angewandt bisher bei Mäusen, auf Zebrafinken zu übertragen. Die Schwierigkeit bestand darin die Artunterschiede zu überwinden. Das ist z.B. die Lage der Hoden, welche sich bei Vögeln im Inneren des Körpers befinden und somit schwerer zugänglich sind als bei Mäusen (Hoden liegen außen am Körper.). Darüber hinaus wurde eine früher publizierte Methode gewählt. Diese basiert auf den Vorläuferzellen der Keimbahn (primordial germ cells, PGCs), führte zu transgenen Gründertieren und Nachwuchs (Gessara et al., 2021) und war effizienter als der klassische Ansatz ('Lentivirus Injektion in stage x Embryonen'; Agate et al., 2009), der zur ersten transgenen Zebrafinklinie führte.

Über eine Laparotomie konnte Zugang zu den Gonaden gewährt werden. Die Hoden wurden erfolgreich manipuliert, ohne die Fertitilät der Männchen zu beeinträchtigen. Verschiedene Parameter wurden getestet und die besten Bedingungen (5µl Injektionsvolumen & 30 Volt) konnten über die Detektion von Fluoreszenz (eines eingebrachten Reporters) in Gewebeschnitten behandelter Hoden bestimmt werden. Die Langzeit Expression des Fluoreszenz-reporters konnte erreicht werden, in dem er stabil über Transposition durch das Enzym HypBase im Wirtsgenom integriert werden konnte. Obwohl das eingebrachte Transgen

in Hoden über PCR, SlotBlot und histologische Untersuchungen nachgewiesen wurde, konnten keine transgenen Nachkommen identifiziert werden.

Im zweiten Teil der Studie wurde PGC Extraktion. Kultivierung und Transduktion über einen Lentivirus erfolgreich implementiert. Bei der Entwicklung manipulierter Embryonen wurden ähnliche Werte wie in Gessara et al., 2021 erreicht (45.5% vs. 56.3%). Daten zum Schlupf manipulierter Eier konnten jedoch nicht verglichen werden, da hier alle Tests vor 2/3 der Embryonalentwicklung durchgeführt wurden. Leider konnten weder transgene Gründer noch transgene Nachkommen über Genotypisierung festgestellt werden. Wohingegen das Transgen für jedes geschlüpfte Tier in der vorangegangenen Studie nachgewiesen werden konnte (Gessara et al., 2021). In beiden Methoden wurden Schwierigkeiten bei der Genotypisierung mittels PCR verzeichnet (offensichtlich wegen Kontamination) und es wurde daraufhin zu Genotypisierung mittels Slot Blot, wobei verlässliche Ergebnisse erzielt wurden, übergegangen.

Es wurde eine neu etablierte Methode für Transgenese bei Zebrafinken (über '*In vivo* Mikroinjektion und Elektroporation von Hoden'), welche zu genetisch veränderten Hoden bei Versuchstieren führte, gezeigt. Im Rahmen dieser Studie konnte leider kein transgener Nachwuchs erzeugt werden mit der Methode '*In vivo* Mikroinjektion und Elektroporation von Hoden', welche erstmals in Zebrafinken Anwendung fand, oder die Ergebnisse von Gessara et al. wiederholt werden.

Nichtsdestotrotz können beide Methoden von Interesse für zukünftige Projekte sein, da in Gessaras Publikation überzeugendere Ergebnisse (bezüglich der Generierung von Gründertieren) hervorbrachte als der klassische Ansatz 'Lentivirus Injektion in stage x Embryonen'. Jedoch sollte bei PGC basierten Methoden die von Jung et al. (2022) entdeckte Heterogenität der PGCs nicht unberücksichtigt bleiben.

Hodenmanipulation scheint vielversprechend für die Transgenese von Singvögeln zu sein, auch wenn es ggf. noch einiger Veränderungen bedarf (z.B. optimierte Parameter, Hemikastration).

1 Introduction

1.1 Zebra finches as animal models for social behavior and song learning

Zebra finches (*Taeniopygia guttata*) are gregarious birds living in flocks of different size in Australia and Indonesia. They are opportunistic breeders and may produce offspring at any suitable time, assuming sufficient amount of resources (i.e. food, water, nesting opportunity) to rear their brood. Usually, one egg per day is laid, resulting in a clutch of 3 to 6 eggs. Consequently, hatching of the chicks happens daily after a mean of approx. 14 days of embryonic development. Whereas, the male is responsible for nest building and impressing the female by courtship behavior, consisting of stereotyped song and special dance, brood care tasks are shared among/between partners of a couple (Zann, 1996). The general assumption is that zebra finches form monogamous, lifelong partnerships, although in captivity extra pair copulations (EPC) were described (Forstmeier et al., 2011; Houtman, 1992) supposedly resulting in better genetic fitness than having offspring only from their own mate and so improve their reproductive success, while male birds profit from greater number of descendants. EPCs are less common in the wild (Griffith et al., 2010).

The neural structures relevant for song acquisition and song production are well described in various songbird species (Prather et al., 2017). Birdsong is learned by imitation of a tutor's song. Unlike in the majority of songbird species, in the zebra finch only the males sing (Immelmann, 1969) and accordingly the neural substrates subserving song production and song acquisition are vestigial in females in this species (Odom et al., 2014).

Females were tested in past studies for song preferences revealing that complex songs, delivered in a stereotyped manner are preferred (Woolley & Doupe, 2008). Females do influence male song development by providing gestural feedback (Carouso-Peck & Goldstein, 2019). Calls are produced by both sexes and most seem to be innate, having basic functions such as begging for food early in life or warning in dangerous situations (Elie & Theunissen, 2018).

As zebra finches are very social animals and can be bred and kept under laboratory conditions, this species has been used extensively to study social behavior/interactions and to investigate the evolution of vocalizations, the neuroethology and timing of vocal learning by imitation, the neural machinery behind sensorimotor learning as well as the impact of acoustic early life experiences on female song preferences and the impact of the tutor's song on female song preferences (Hauber et al., 2021).

These research fields could profit immensely from the establishment of a general pipeline for the generation of transgenic songbirds as it would provide new possibilities to study the genetic contributions to behavioral traits and their neurobiological substrates.

1.2 Parallels and differences in humans and zebra finches

1.2.1 Human speech and birdsong

Only few species share the capacity to communicate by expressing learned vocalizations, among them elephants, bats, dolphins, parrots, hummingbirds and songbirds (Bolhuis & Gahr, 2006; Bolhuis et al., 2010; Doupe & Kuhl, 1999; Fitch, 2000; Hauser et al., 2002). Speech and song learning in human infants and young songbird hatchlings share remarkably many parallels on a behavioral, genetic and neuronal level (Condro & White, 2014; Griffith & Buchanan, 2010; Pfenning et al., 2014; Sakata & Birdsong, 2022). For both, humans and birds, there exists a critical period when vocal production is learned best. Although this time window differs in duration for humans and songbirds, it is for both divided into several phases: In the sensory phase, song/speech is heard and memorized. A variety of sounds but not all might be learned during this time period, since there are innate predispositions towards which sounds are species specific (Doupe & Kuhl, 1999). Juvenile songbirds start to practice their song by comparing their own copy to the stored template and to gradually adjust further refinements, as is the case for human babies who start to babble (Fig. 1). In the beginning, the zebra finch vocalizations are akin to babbling, few not very accurate syllables are produced, but at the end of the sensorimotor phase they match the stored adult song and song the stays quite stable during the rest of the zebra finches life (Eales, 1985). In contrast, so called ‘open-ended learners’, like the canary, are able to change their song even in adulthood (Nottebohm et al., 1986).

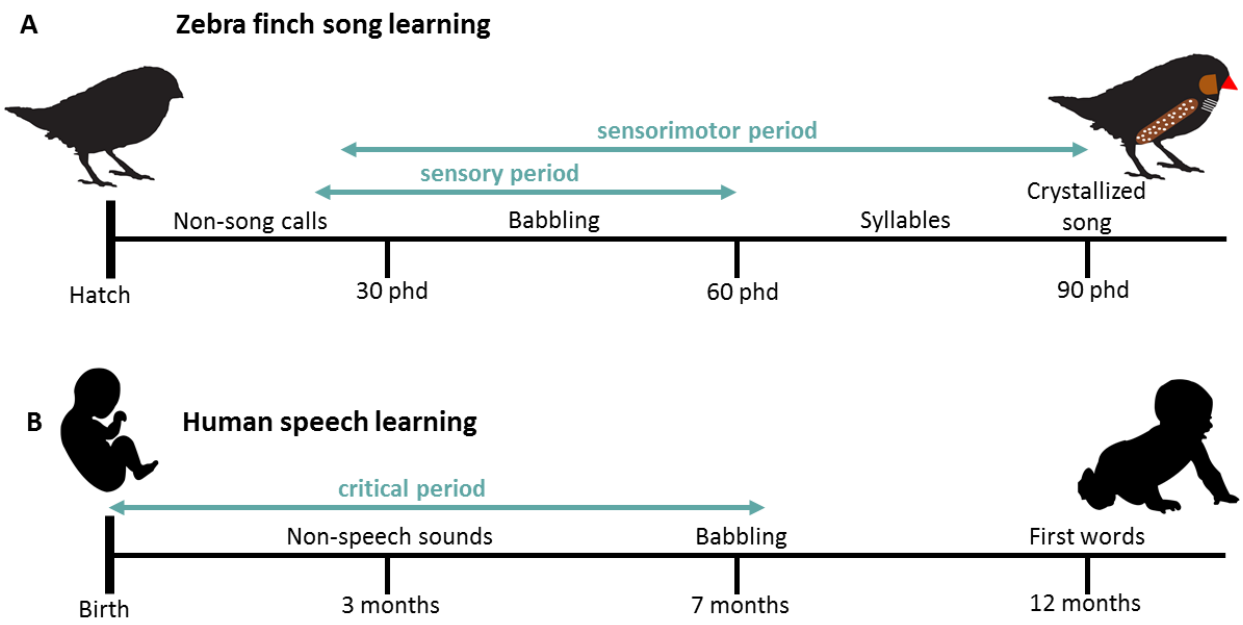

Figure 1 Timeline for song learning in zebra finches and speech acquisition in humans.
Zebra finches (A) and humans (B) share a similar structure in the phases during song learning and speech acquisition. In both, vocal learning is achieved by imitation of conspecifics. In the sensory period, young birds and infants store the auditory input from a tutor in their memory. It is overlapping with the sensorimotor phase. There, young male zebra finches start babbling and later expressing syllables until the tutor's song is completely copied and crystallized. During practicing, the produced vocals are compared to the memorized template in order to refine the song repeatedly, increasing the similarity to the template. Of course, the duration of the developmental phases differs in both species due different lifespans. (Brainard & Doupe, 2002; Doupe & Kuhl, 1999; Hyland Bruno et al., 2021)

Human and bird brains look superficially very different, but extensive genetic, developmental and neuroanatomical studies have revealed many similarities (Jarvis, 2019; Petkov & Jarvis, 2012; Prather et al., 2017); see Fig. 2.

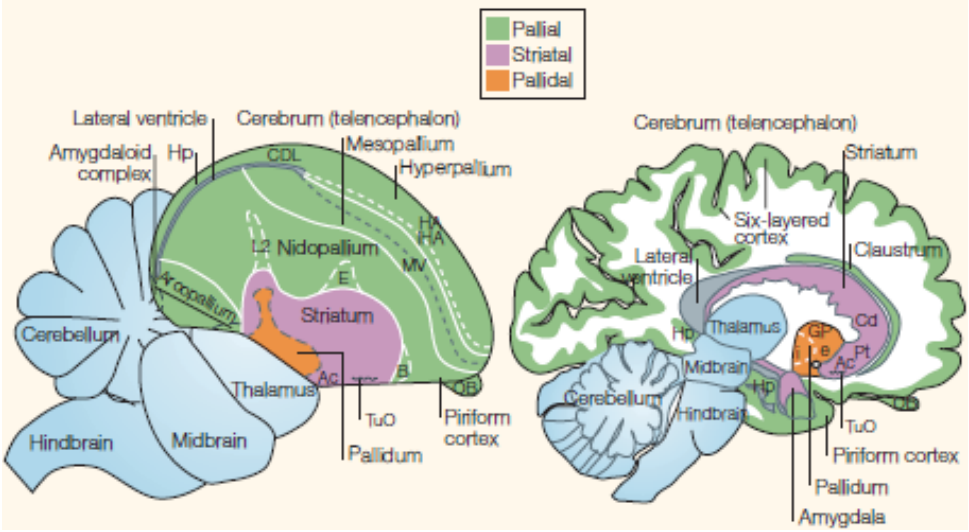

Figure 2 Comparison of brains from mammals and birds according to the conclusions of the Avian Brain Nomenclature Forum.
Sagittal cuts through an avian (L.), to which songbirds and therefore zebra finches belong to, and a mammalian (R.), including humans, brain are shown. Same colored regions code for equivalent brain areas in both. from (Jarvis et al., 2005)

Two main pathways (Fig. 3) characterize the organization of the song system (Wood et al., 2013): First, the motor pathway, that is required for song production. It projects from the forebrain nucleus HVC (used as a proper name) to the robust nucleus of the arcopallium (RA), whereas RA projects to brainstem motor nuclei, where respiration and muscles of the vocal organ (syrinx) are controlled. For song learning and adult song plasticity, a second pathway is required. This so called anterior-forebrain-pathway (AFP) is homologous to the cortico-basal gangliathalamocortical loop in mammals and is involved in sequenced motor behaviors (Bolhuis et al., 2010). Area X, DLM (medial portion of the dorsolateral nucleus of the thalamus) and LMAN (lateral subdivision of the magnocellular nucleus of the anterior nidopallium) belong to this pathway. The AFP connects to the motor pathway at the level of RA.

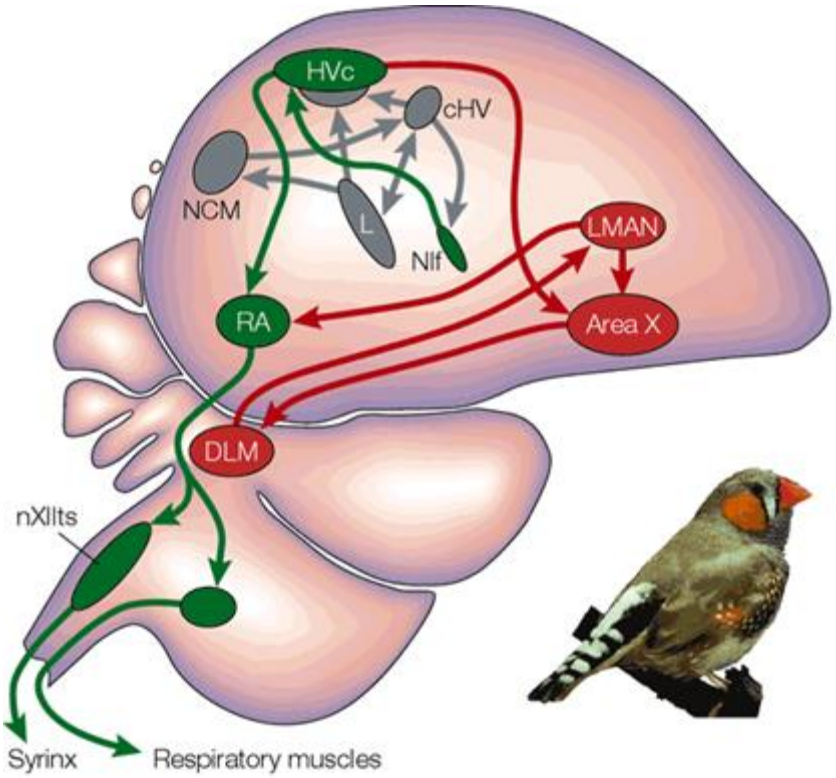

Figure 3 Neuronal pathways of the avian song system.
Scheme of sagittal cut through the songbird's brain. Shown are the projections of the key nuclei in the avian song system. The descending motor pathway is displayed by green arrows and is relevant for song production, whereas red connections are demonstrating the anterior forebrain pathway including the Area X (striatum) which is important for song learning and song plasticity. Figure from (Brainard & Doupe, 2000)

Regarding the genetic level, FoxP2 has a high impact for human speech acquisition as well as for avian song learning. This transcription factor belongs to the family of forkhead proteins and regulates many genes. Mutations in human FOXP2 lead to many different abnormalities including impaired speech production, speech perception and motor deficits, together known as Developmental Verbal Dyspraxia (DVD) (Bacon & Rappold, 2012; Hamdan et al., 2010; Horn et al., 2010; Lai et al., 2001; Morgan et al., 2018). The best-known and most studied family, affected by speech and language disorders based on a FOXP2 mutation, is the KE family (Lai et al., 2001).

In zebra finches, FoxP2 mRNA and protein has been detected in similar areas of the brain as in humans, e.g. the striatum and other subcortical structures (Haesler et al., 2007; Mendoza & Scharff, 2017; Teramitsu et al., 2004).
Experimental up- or downregulation of the FoxP2 expression level in the song nuclei Area X of zebra finches results in impaired song learning. Incomplete and inaccurate song imitation, impaired spine formation of striatal medium spiny neurons and lack of social context induced neural plasticity are characteristic for treated animals (Haesler et al., 2007; Heston & White, 2015; Norton et al., 2019). Most functional studies are based on the principles of gain or loss

of function mutations. Mutations of genes may lead to expression at lower levels (reduced or lacking functionality) on one site or on the other site at higher expression levels (enhanced or new function). In experiments, one could achieve this by knockdown/out (KD/KO) or overexpression, i.e. in cell culture (*in vitro*) or in living animals (*in vivo*) (Haesler et al., 2007; Norton et al., 2019).

For *in vitro* experiments species specific cell lines are often necessary. There were only few cell culture lines published for zebra finches and an immortalized cell line was not available until recently. Embryonic fibroblasts were immortalized by applying a lentivirus providing SV40 T small and large antigen (with oncoprotein function: inducing immortalization) resulting in a stable cell line capable of density-independent proliferation and clonal propagation. The CRISPR/Cas9 method has already been successfully applied to them (Biegler et al., 2021). Two other cell lines exist from naturally occurring tumors, one derived from males, which is diploid and one from a female, which is tetraploid. Both are assumed to be fibroblast like (Itoh & Arnold, 2011). A protocol for establishment of primary cell culture was also recently published (Kulak et al., 2021), but there might be genetic differences due to the diverse donor individuals endangering reproducibility of results and duration of cultivation is temporally limited. Although *in vitro* experiments are a useful tool for many experiments, they cannot replace transgenic individuals to study the effects of gene manipulations on the brain and behavior, which cannot be simulated by *in vitro* methods.

1.2.2 Reproduction in mammals and birds

Birds are oviparous in contrast to mammals, which are mostly viviparous (Blackbum, 1999). This means after ovulation, fertilization and oviposition, the avian embryo develops inside the incubated egg (Fig. 7), which raises difficulties when aiming to manipulate avian oocytes or single cell embryos (Cooper et al., 2017; Han & Park, 2018). In the process of generating transgenic birds, primordial germ cells (PGCs), progenitor of gametes (oocyte and sperm cell), are target cells as they offer the opportunity to deliver genetic information to the next generation, which is crucial to gain a transgenic line (Van de Lavoir et al., 2006). Once settled, PGCs differentiate, according to the sex of the individual, into testes or the ovary respectively (Meyer, 1964).

As precursor of the germ lineage PGCs give rise to oogonia and spermatogonia. Different types of spermatogonia either undergo a homonymous or heteronymous division to maintain the stem cell population (self-renewal) or to enter the process of spermatogenesis (S. Gilbert, 2000). Consequently, PGCs play a crucial role in the formation of spermatozoa, as there would be no loss of the manipulation once introduced into PGCs. Thus, PGCs offer a great possibility

to generate genetically modified birds by reproduction after the successful establishment of a germ line-transmitting founder individual.

PGCs express germ cell-specific proteins, CVH (chicken vasa homologue) and CDH (chicken DEAD end homologue), as well as some pluripotency markers and a stem cell marker, c-Myc, cKlf4, cPouV, cSox2, cNanog and SSEA-1 (Macdonald et al., 2010), pointing towards their potential to still undergo differentiation. Moreover, this cell type is represented by a specific morphology: PGCs differ in size (diameter: >10-20µm vs. <10µm) from other cells (red blood cells, hematopoietic stem cells, hemangioblasts) circulating in the vascular system of chicken (*Gallus gallus*) embryos at early developmental stages (Anstrom & Tucker, 1996; Fujimoto et al., 1976). These characteristics are useful when aiming to identify PGCs or to separate them from other cell types. A variation of methods has been applied to detect PGCs; among them: periodic-acid-Schiff (PAS) staining (Meyer, 1960), immunohistochemical staining (Karagenc et al., 1996), MACS or FACS (Mozdziak et al., 2005; Ono & Machida, 1999) and density gradient centrifugation (Yasuda et al., 1992).

PGCs are found in the embryo's center of freshly laid eggs (stage x) in small number, but at later developmental stage become more numerous in the germinal crescent before they migrate from there through the vascular system to the gonadal anlagen (Meyer, 1964) (Fig. 4). PGC migration via blood vessel was uniquely discovered for birds and offers the chance to get access to manipulation experiments intending the generation of transgenic birds.

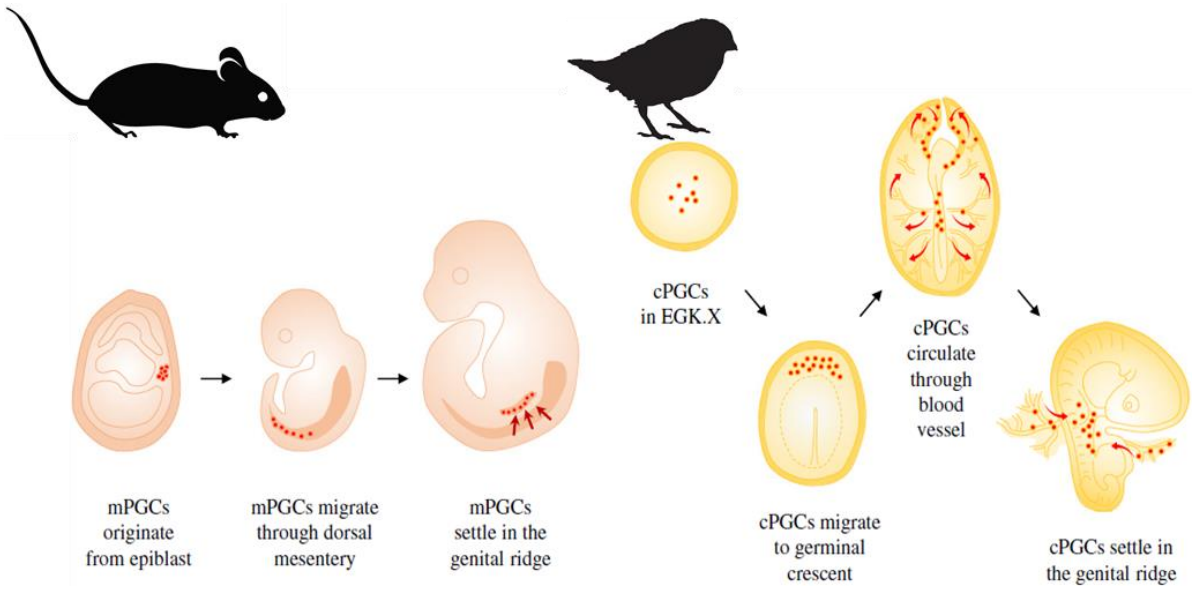

Figure 4 Comparison of the development & migration of PGCs during embryonic development in mice & chicken.
Red dots represent PGCs, whereas red arrows show the direction of migration. 'm' short for mouse and 'c' for chicken. A: In mice, the origin of the PGCs is the epiblast. Before settlement at the genital ridge, they migrate through the dorsal mesentery. B: For birds, few PGCs are located the center of the area pellucida after oviposition. After duplication, PGCs migrate to the germinal crescent during the first day of embryonic development. From there, they then migrate through the vascular system around 2/3 to settle in the genital ridge at day 5-6 to form the gonads. Figure modified from (Han & Park, 2018)

Avian spermatogenesis

During spermatogenesis PGCs differentiate into spermatogonia, which self-renew by mitosis. After the last mitotic division, a primary spermatocyte is generated. This primary spermatocyte goes through the first meiotic division and leads to secondary spermatocytes. A second meiotic division follows and results in haploid spermatids. Before their release into the lumen of the seminiferous tubules as spermatozoa or simply sperm cells, these spermatids become elongated and get their characteristic shape. While maturating, the germ cells move gradually to the lumen of the seminiferous tubules (see cross sections of the seminiferous tubules in Fig. 5).

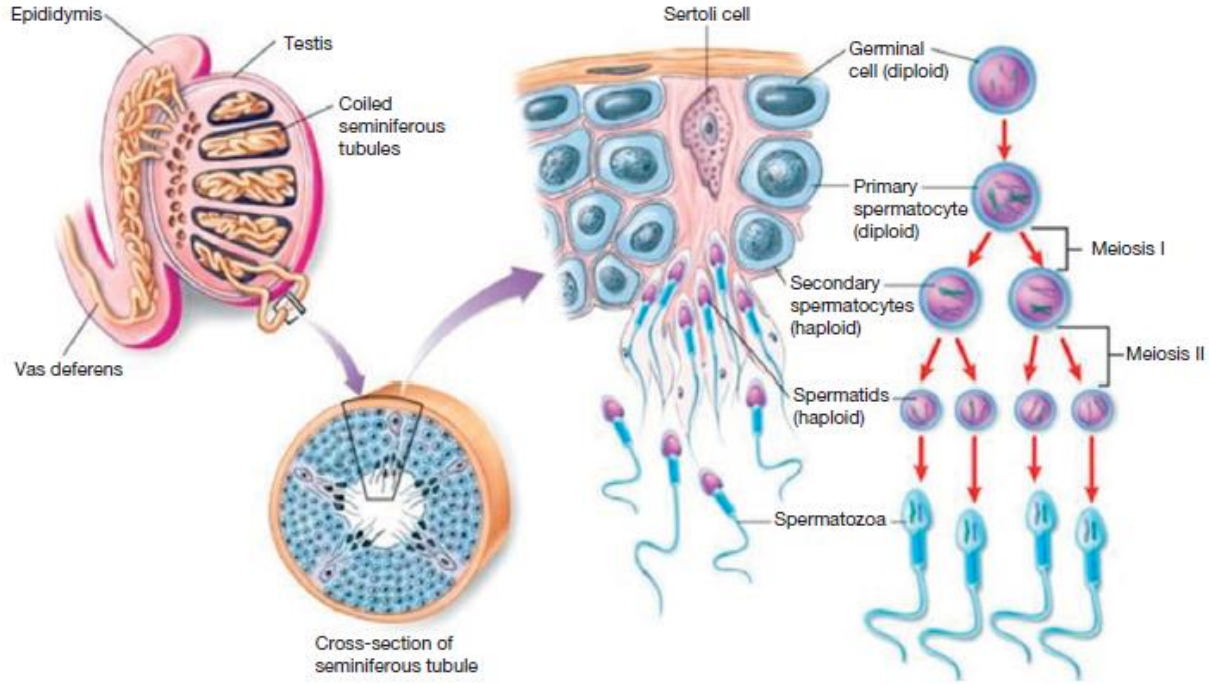

Figure 5 Cross section of seminiferous tubules & spermatogenesis (Lin & Troyer, 2014).
Illustration of seminiferous tubules (inside testes); zoomed in to follow differentiation process to form mature spermatozoa (spermatogenesis).

Sertoli cells have an essential supportive function as they release several important substances needed to maintain spermatogonial stem cells in adult testis or to stimulate spermatogenesis for instance (Chen & Liu, 2015). The time required for the entire differentiation process into sperm cells is not exactly examined for zebra finches, but there exist estimated durations for species belonging to the order Galliformes: 12.77 days for Japanese quails (*Coturnix japonica*) (Lin & Jones, 1992), 11-12 days for the domestic fowl and the Barbary drake (Jones & Lin, 1993) (all non-passerine birds). Although a study for passerine birds was not conducted or published yet, it was assumed that the process of spermatogenesis in passerines is accelerated compared to non-passerines (Bhat & Maiti, 1988).

It is known that individuals of many avian species exhibit an asymmetry of testes. Mostly, left testes are bigger in size, hypothesizing only one to be functional while the other one is retained

as a compensatory alternative. In zebra finches the natural occurrence of this phenomenon has been confirmed for both testes (Calhim & Birkhead, 2009), so any manipulation of testes could induce the increased functionality of the other one. This has to be kept in mind, when aiming to manipulate male germ cells and for later reproduction.

Sperm and Sperm storage tubules

Across species a huge variation concerning sperm morphology has been identified, despite the shared function it occupies in the reproduction process (Birkhead et al., 2005; Calhim et al., 2007). In birds, one reason for this fact was found to be the level of sperm competition (Lüpold et al., 2009). Sperm traits, e.g. sperm morphology and velocity, of zebra finches may vary between different individuals, but are strongly comparable within and across ejaculates from the males (Birkhead et al., 2005; Birkhead & Fletcher, 1995). Sperm length is strongly hereditary (Birkhead et al., 2005), so offspring of a male with long sperm are expected to produce sperm of similar length. A positive correlation between sperm velocity and fertilization success was discovered (Birkhead et al., 1999), particularly for species with sperm competition like the zebra finch (Parker, 1998). Later studies could positively correlate sperm morphology and velocity, too. This genetic link of both characteristics explains the coevolution of faster and longer sperm (Mossman et al., 2009).

Many internally fertilizing species lose sperm after insemination; thus only the minority of the ejaculate stays in the urogenital tract and has the chance to fertilize the ovum (Holt & Van Look, 2004). Several avian species share the ability to store some sperm cells of an ejaculate inside epithelial invaginations at the utero-vaginal junction, named sperm storage tubules (SST) (Birkhead, 1998). 1200 to 1700 of these cellular structures can be detected per bird and storage duration was observed for about 10 days in zebra finches and even more (up to 13 days) in bengalese finches (Birkhead & Hunter, 1990). It is assumed that sperm cells compete about limited storage capacities via their velocity (Froman et al., 2002). There is evidence that females might to some degree have influence over the sperm storage via SST and thereby having impact on which sperm fertilizes the egg (Birkhead & Møller, 1992). Longer sperm, more precisely sperm with longer midpiece, is more likely kept in SST and also more likely fertilizing the ovum (Bennison et al., 2015; Hemmings & Birkhead, 2017).

Avian oogenesis

Before oogenesis begins, germ cells differentiate into oogonia. Oogonia undergo a mitotic division to multiply and then differentiate into primary oocytes. Oogenesis describes the oocyte maturation inside the ovarian follicles. The first meiotic division of the primary oocyte leads to

the secondary oocyte and a polar body. Meiosis is an unequal cell division and only one functional cell is formed, here the oocyte, so the polar body is non-functional. The second division by meiosis is completed after the fertilization of the ovum by a sperm cell. A polar body is again built during meiosis II (S. F. Gilbert, 2000; Raven, 2013).

From egg development to fertilization and oviposition

The reproductive tract of birds is divided into several parts: the ovary, the infundibulum, the magnum, the isthmus, the uterus the vagina and the cloaca. The ovary is the place where folliculogenesis takes place. During folliculogenesis, follicle matures into the oocyte and yolk, the latter consisting of nutrients like proteins, lipids, carbohydrates and further components. During ovulation, the secondary oocyte enters the oviduct at the infundibulum. It is here that fertilization occurs; if sperm cells are present. Several sperm cells are required to fertilize the ovum due to polyspermy in birds. Sperm gets access through the urogenital tract via the cloaca and the vagina. A successful copulation encompasses ejaculate (that contains enough intact sperm cells) that reaches the ovum by swimming, so that fertilization happens. The ovum then passes the magnum and isthmus, where the shell membrane is added, on its way to the uterus. There the shell of the egg becomes harder to protect the inner components of the egg from external influences (Bakst, 1998; Johnson, 2015). The complete egg is finally everted by the cloaca, a process referred to as oviposition. An overview about the egg development is illustrated in Fig. 6.

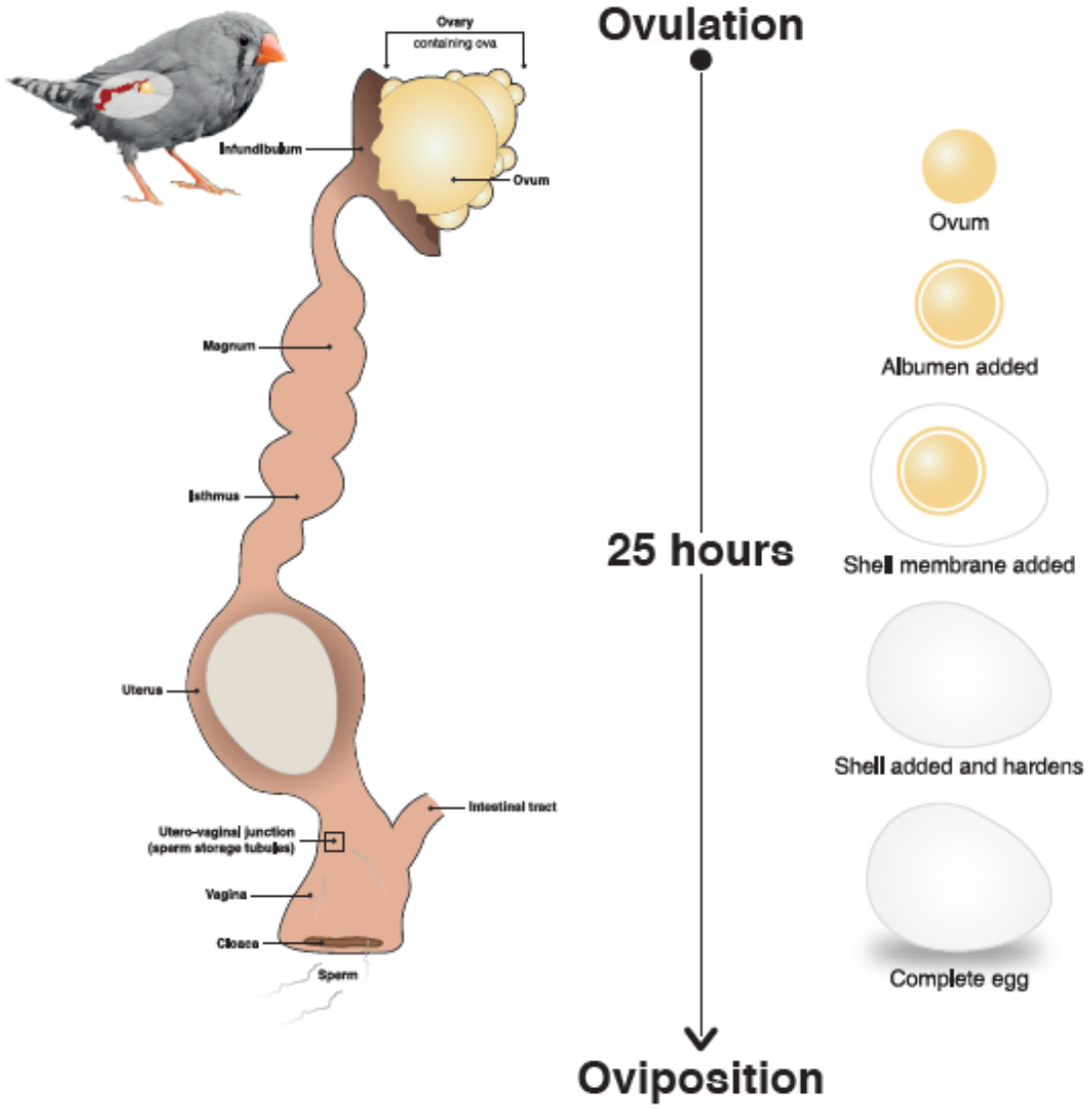

Figure 6 Avian female reproductive system & egg development.
L.: Scheme of reproductive tract; R.: developmental process from ovum to egg.
The ovary carries developing follicles and releases the mature follicle into the oviduct, when it comes to ovulation. Here, sperm fertilizes the ovum, if copulation occurred and the spermatids reached the infundibulum by swimming movements. While passing the oviduct albumen (magnum), shell membrane (isthmus) and shell (uterus) are added. Finally, the complete egg is laid (oviposition) through the vagina and cloaca. At the urogenital junction, spermatids might be stored in sperm storage tubules and released at a suitable time point.

Breeding

Zebra finches are opportunistic breeders, meaning they do not follow any season, but start breeding, if environmental conditions are suitable. Especially in captivity, breeding might begin at any point in time. The male shows courtship behavior: song and dance directed towards the females (Fig. 7) as well as nest building.

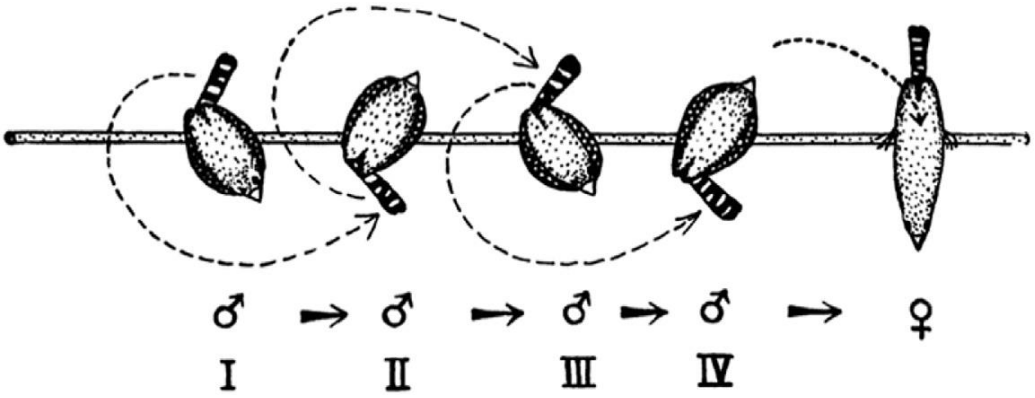

Figure 7 Illustration of a zebra finch male's courtship dance directed towards a female zebra finch. From (Morris, 1954)

In the reproductive period, copulations happen every other morning after the lights are turned on and the female lays each day one egg until the clutch is completed. Usually, clutch size ranges from 2 to 8 eggs (Zann, 1996). Embryonic development inside the eggs needs about 12 to 16 days before chicks start to hatch one after the other. Both partners share brood care, which not only includes breeding to ensure adequate incubation temperature, but also feeding hatchlings until they are weaned (around post hatching day, PHD, 35).

1.3 Transgenesis

Transgenesis describes the process of the integration of foreign DNA into the genome of a host individual and is often used in animals to study gene function *in vivo.* Many different approaches were applied to vertebrates and invertebrates for the generation of transgenic animals. Mostly, mouse (*Mus musculus*), rats (*Rattus norvegicus*), zebra fish (*Danio rerio*) as vertebrate species, and the fruit fly (*Drosophila melanogaster*), nematodes (*Caenorhabditis elegans*), as invertebrate species, are chosen for the production of transgenic animals (Apfeld & Alper, 2018; Do Carmo & Cuello, 2013; Houdebine et al., 2007; Leung et al., 2008; Rinkwitz et al., 2011). The poultry industry and the potential of the chicken egg as bioreactor for protein production had a high impact on the investigations concerning avian transgenics (Ivarie, 2003; Lillico et al., 2005). An overview about methods for the generation of transgenic songbirds is illustrated in Fig. 8. It contains methods already used in the past, but also a new approach applied in the recent study.

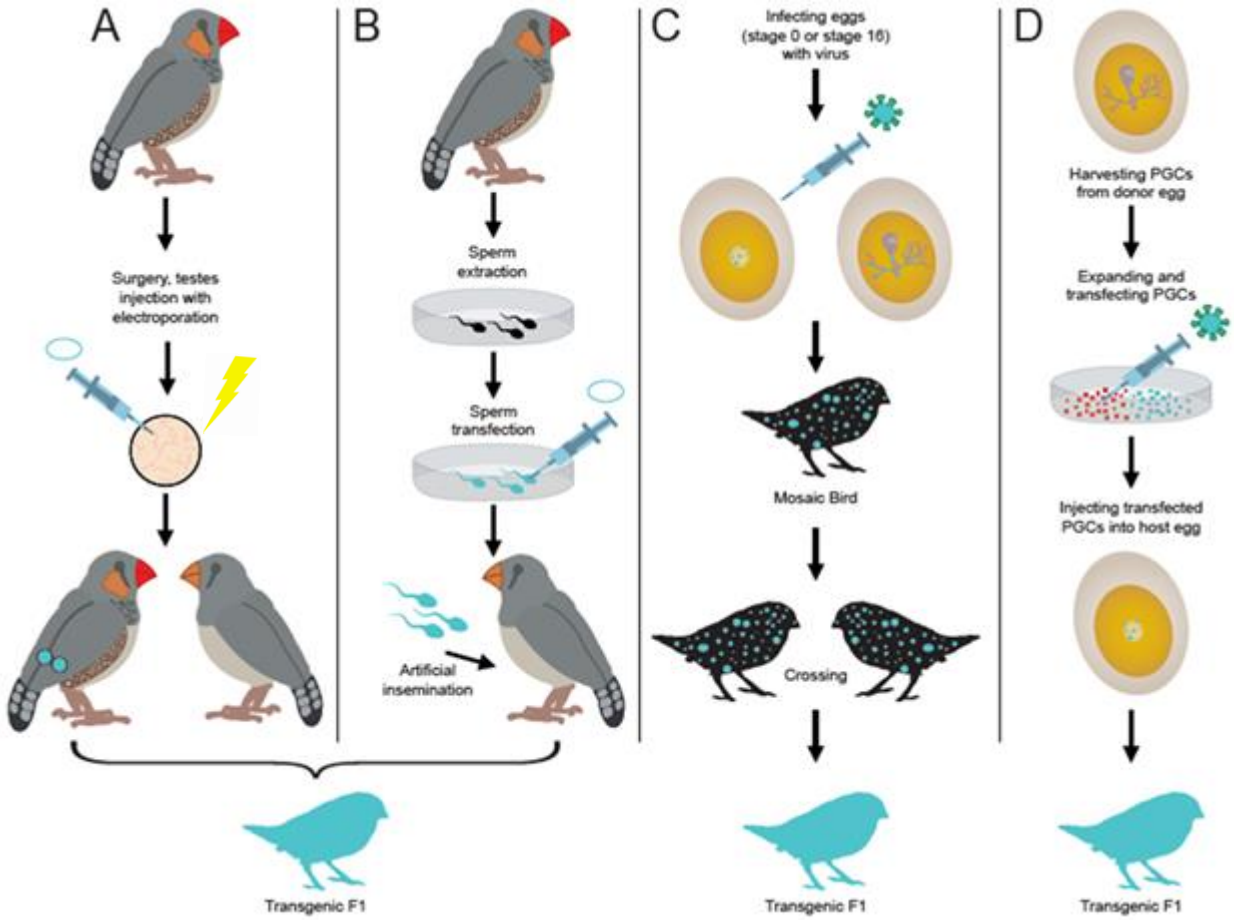

Figure 8 Different ways for the generation of transgenic animals.
There are several possibilities to access and manipulate the germline of animals in order to establish transgenic lines. Not all methods have been successfully applied for every species. In general, there exist three main approaches to get transgenic animals: the manipulation of the germ cells from males or females and the manipulation of early embryos. Some of the mentioned methods lead first to chimeric or mosaic animals (expressing the transgene not all over the body) and require subsequently an additional crossing step to produce transgenic offspring and therefore need more time. A: **in vivo** ***microinjection and electroporation of testes (done in mice; i.e. in Usmani et al., 2013); B: sperm modification by STAGE (done in chicken; i.e. in Cooper et al., 2017); C: different manipulation attempts on embryos of different developmental stage (done in chicken, quail and zebra finches; i.e. in Han and Park, 2018; Poynter et al. 2009 and Agate et al., 2009); D: injection of*** **in vitro** ***transduced PGCs into stage x embryo of zebra finches (done in zebra finches; Gessara et al., 2021). The only approaches, which were also published for zebra finches, were C & D.***

1.3.1 Avian transgenics

Inaccessibility of the ovum and of single cell embryos in birds hinder scientists to transmit many of the currently available methods for the generation of transgenic mammals, to avian species. To create avian transgenic lines, manipulation of the ovum, the sperm or any precursor cells (like PGCs) have to be achieved to ensure the germline transmission of the modification (Han & Park, 2018) as these cell types are able to transmit the genetic information to the next generation (Van de Lavoir et al., 2006). Different methods have been described in lots of publications over the last years, especially for chicken due to its role in the poultry industry

(Dimitrov et al., 2016; Macdonald et al., 2012; Oishi et al., 2016; Park & Han, 2012a; Park et al., 2014; Schusser et al., 2013). More details to them can be found in next paragraphs (1.2.1, 1.2.2).

1.3.2 Transgenic chicken and quail

Chickens are often used as bioreactor to produce proteins to serve humans e.g. as medicine in case of diseases. The need for suitable systems accelerated the development of different methods to generate transgenic animals (Han, 2009). In birds, the focus for genetic manipulations lies on PGCs. These target cells might be isolated at different time points of embryonic development: from the germinal crescent at stage x (day 1), from the vascular system at stage 13-15 (day 2-3) or after settlement at the gonadal ridge Hamburger Hamilton (HH) stage 28 (day 5-6) (Fig. 9).

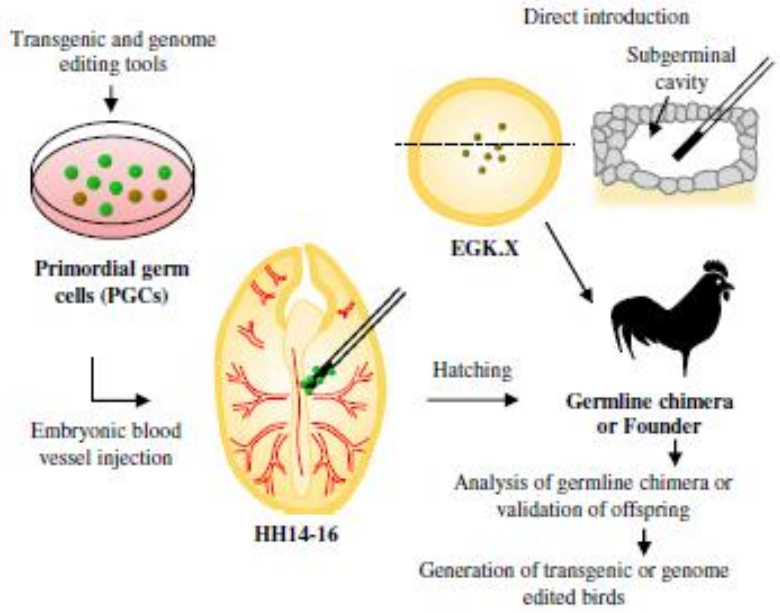

Figure 9 Methods for generating transgenic chicken.
Transgenic avian offspring resulting after injection of genetic manipulated PGCs into the vascular system of stage HH14-16 recipient embryo or after lentiviral injection into the blastoderm of stage x embryo. (modified from (Han & Park, 2018)

Chicken PGC culturing has already been feasible for a long time (Van de Lavoir et al., 2006) and methods for cryo-conservation are available as well (Naito et al., 1994). This facilitates the genetic manipulation, selection and reintroduction of PGCs into recipient embryos.
Early in the history of transgenic chickens, retrovirus approaches were chosen. First injecting them into stage x embryo to target PGCs present in the very center of the blastoderm (Salter et al., 1987; Salter et al., 1986); later also trials were successful in quails at stage HH14-16,

where PGCs are distributed in the vascular system before settling at the gonadal ridge (Sun et al., 2012). Another retroviral-based attempt was the injection into the germinal crescent, which led to infected PGCs and so resulted in transgenic chicken too (Vick et al., 1993).

A new direction was taken when PGC cultivation was established. Cell numbers could be multiplied, PGCs were then manipulated *in vitro* to harvest and reintroduce them afterwards in recipient embryos. Different methods were tested for genetic modification of these target cells: a) Piggyback transposase was used to insert GFP (green fluorescent protein) in the genome of PGCs (gonads derived) and these cells were introduced to embryos, which hatched and carried the transgene (Park & Han, 2012b). b) Application of Tol2 transposase to insert GFP under the control of CAGG promoter in PGCs (blood derived), that were injected into stage HH16 embryos (Macdonald et al., 2012). c) Homologous recombination (HR) of the joining (J) gene segment of the chicken Ig heavy chain gene by injecting PGCs (blood derived) into stage HH13-15 embryos (Schusser et al., 2013). d) TALEN mediated knockout of the ovalbumin (OV) gene via PGCs (gonads derived) (Park et al., 2014). e) Modifications by CRISPR/Cas9 system concerning chicken proteins, OV and ovomucoid (Oishi et al., 2016), as well as the JH gene of the immunoglobulin heavy chain locus of chicken (Dimitrov et al., 2016). d) and e) were both achieved by injecting manipulated PGCs into stage HH14-16 recipient embryos. Moreover, transgenic quails were successfully generated within one generation by injecting sperm into oocytes (Hrabia et al., 2003; Mizushima et al., 2014; Mizushima et al., 2017; Mizushima et al., 2010). However, this method is very time consuming, difficult, and requires a large number of donor animals.

Although these examples have proven their feasibility of transgenesis, the afore mentioned methods require a huge number of manipulated individuals yielding just a few transgenic animals (not before the second generation).

This means for the manipulation of zebra finch embryos inside the egg, hatchlings might be chimeric for the transgene. Only in the case of manipulated cells in the germ line, there exists a realistic chance of a founder individual to start a transgenic line. After reaching sexual maturity these potential founder animals are given the opportunity to breed and reproduce. Some hatchlings of a clutch might carry the transgene, which is identified by genotyping the offspring.

In contrast to such methods, there are possibilities to achieve the generation of transgenics within one generation. In fact, this would not only be faster, but at the same time reduce the number of experimental animals in a project. Producing a transgenic animal in the first generation could result in accelerated offspring production and therefore produce many transgenic individuals. Until now, these one-generation approaches were not applied to zebra finches. In chicken, some of these one-generation approaches were used. Usually, the aim is

to modify sperm cells either by manipulating testis or the sperm itself: *In vivo* transfection of testis successfully expressed a foreign gene in sperm, but the generation of transgenic chickens was not assessed (Liu et al., 2011). Manipulated PGCs were transplanted into the testes of infertile roosters, and resulted in transgenic offspring (Trefil et al., 2017). Female chickens were inseminated by *in vitro* manipulated sperm and so generated transgenic chickens (Cooper et al., 2017).

1.3.3 Transgenic zebra finches

Zebra finches are used as an animal model to investigate the genetic and neural mechanisms of song learning and production because they might have bearing on the equivalent mechanisms in human speech. Although a huge amount of studies has been published on songbirds on these topics, an efficient way for the generation of transgenics is still unavailable. Until now, approaches for the generation of transgenic chicken and quails were difficult to transfer to zebra finches.

So far, a total of only fivetransgenic zebra finch lines were generated. Lentiviral injection into stage x embryos was first reported in 2009 (Agate et al., 2009) and only three other studies, each by a different lab, successfully employed this method during the next 10 years.

Agate et al. used a lentivirus carrying a ubiquitin promoter to ensure the ubiquitous expression of the reporter gene GFP. For lentiviral microinjection, freshly laid eggs were collected and opened to get access to the embryos at stage x. Eggs were closed again and incubated by foster parents represented in Fig. 10 (Velho & Lois, 2014). Hatched birds were potentially positive for the transgene and consequently crossed with wildtypes (WT). Some of their offspring were successfully screened for GFP by polymerase chain reaction (PCR) and thus proved that this approach results in chimeric founders, which may produce transgenic offspring.

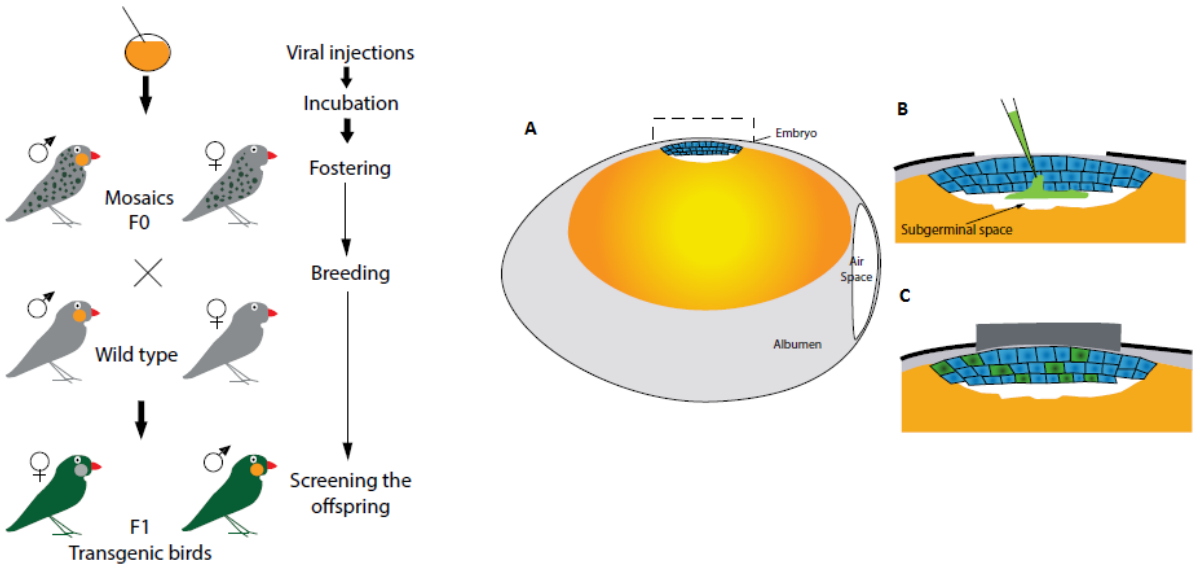

Figure 10 Lentiviral mediated transgenesis in zebra finches.
L.: Schematic workflow; R.: Steps of lentiviral injection into avian embryo (scheme): (A) cross section of the egg displaying the embryo attached on top of the yolk (orange) at early developmental stage, (B) viral injection via opening in the shell into the embryo (accumulation of cells in blue) using glass capillaries, (C) post injection state: some of the embryonic cells were infected by the virus (green) and the transgene is integrated in to the genome. (from (Velho & Lois, 2014))

In the study of Agate et al., a total of 265 eggs were treated with this protocol and 35 (13%) birds hatched. 9 birds (25.7%) died shortly after hatching. Of the remaining 26 birds three (11.54%) were founders showing germline transmission by raising transgenic hatchlings at a rate of 6 to 12%. Regarding all manipulated eggs the rate of successful manipulation is low (1.13%). Crossing of F1 generation resulted in GFP positive offspring as well.

Two other studies based on this method were published in 2015. In Liu et al. (2015), the first functional zebra finch mutant was obtained. A mutant huntingtin protein, which is responsible for severe neurodegenerative symptoms that arise in patients suffering from Huntington's disease, was expressed in zebra finches. Song learning of affected birds was poor and indicated several differences to the template regarding imitation goodness, syntax and others. Of approx. 350 manipulated eggs 83 hatched (23.7%) and 8 were identified as transgenic founders (2.29%).

In the same year zebra finch lines carrying two different variants (enhanced or suppressed) of the transcription factor CREB (cAMP response element-binding protein; CREB) were published (Abe et al., 2015). Transgenics were done to elucidate CREBs function in song learning of young zebra finches. Depending on the used constructs, hatching rate was about 10-25% of the manipulated eggs. 20 birds were defined as transgenic founders after genotyping (20/1473: 1.36%).

All above mentioned mutant zebra finches were generated by making use of the same method named 'Lentiviral injection into stage x embryo', which resulted in all studies having low efficiencies.

Recent studies that addressed the same aim concentrated on approaches with culturing PGCs. Jung et al. published in 2019 a method to culture PGCs, which were extracted from dissected gonads (embryo stage HH28) and cultured on a feeder layer. PGC culturing up to 30 days could be achieved by applying this method. *In vitro* lipofection of PGCs was done to express ubiquitous GFP. Finally, they succeeded in detecting GFP expressing PGCs, that were incorporated in the gonads of a recipient embryo (HH stage 13-16), but no transgenic animal had been published by applying this approach. Recently, another study about *in vitro* manipulation of PGCs was published concluding that PGC characteristics are maintained after *in vitro* manipulation and hence offer a great opportunity for the generation of transgenic songbirds (Jung, Kim, et al., 2021), but the author did not report any generated transgenic bird. Another possibility to obtain PGCs for cultivation has been described (Gessara et al., 2021). Extraction of PGCs happened at a very different embryonic stage (here HH13-15). In this case, PGCs still circulate through the vascular system (before they migrate through the vascular system to settle at genital ridge and form the gonads) and could be extracted from the blood vessel via a needle/glass capillary. Blood samples were cultured in a special culture medium for about one week before infection with lentivirus. 48h after infection cells were harvested, washed, treated with papain to convert cell clumps into singles cells and re-suspended in a small volume of medium to inject them into a recipient embryo (stage x). This resulted in transgenic zebra finches. 22 eggs (embryos at stage x) were injected with successfully infected PGCs resulting in a 45.4% hatching rate, which is a remarkably higher value than for all previously published transgenic zebra finch studies (i.e. 13.2% in Agate et al., 2009).

1.3.4 Promising approaches to generate ‘Transgenics in one generation’

So far, methods for the generation of transgenics in particular based on two-generation approaches were mentioned. First, a G0 generation, which might be chimeric, is produced and has to be screened for insertions. After confirming the genetic modification, the potential founder is mated to raise the F1 generation of which some should be transgenic birds.

There are also several studies, which focus on the manipulation of sperm to generate transgenics. Different methodologies were tried to achieve this aim: either manipulations that affected testes/spermatogonial cells, i.e. via *in vivo* injection of lentiviral constructs into testis (Sehgal et al., 2011), transplantation of manipulated PGC into testes (Trefil et al., 2017), *in vivo* transfection (Huang et al., 2000; Liu et al., 2011)/electroporation (Michaelis et al., 2014; Usmani et al., 2013) and resulted thereby in modified sperm or collected sperm itself was directly manipulated, i.e. by transfection/lipofection (Cooper et al., 2017; Rottmann et al., 1992) and electroporation (Rieth et al., 2000; Sin et al., 1993). Some of these technologies might be

promising for zebra finches or any other birds as well, since entering the ovum or single cell embryos is quite difficult due to oviparity. Furthermore, these approaches using sperm do not require two generations to guarantee transgenic offspring like many other methods, which first generate chimeric individuals.

One of them is the approach named Sperm Transfection Assisted Gene Editing (STAGE) (Cooper et al., 2017), and another one is about the microinjection and a following electroporation of testes in anesthetized animals (Michaelis et al., 2014; Usmani et al., 2013). STAGE was first published for chickens and intended to help obtaining a huge variety of different transgenic avian species. Sperm from roosters was collected in a special semen extender and washed before manipulation was conducted. Afterwards the sperm samples were transfected with corresponding Cas9 mRNA and guide RNA using Lipofectamine 2000 (Invitrogen, USA) as transfection reagent. Subsequently, the modified sperm could be transferred to the hen's cloaca. Consecutive artificial inseminations were carried out for an interval of 3 up to 7 days and were achieved by using a syringe. All laid eggs were collected, incubated and at later stage screened for embryonic development and expression of the reporter, here GFP. Applying this method in combination with the CRISPR/Cas9 system the authors succeeded in generating GFP KO and DMRT1 mutants (Cooper et al., 2017).

In vivo microinjection and electroporation of testis to generate transgenics was reported for mice (Dhup & Majumdar, 2008; Huang et al., 2000; Majumdar et al., 2009; Michaelis et al., 2014; Usmani et al., 2013; Yomogida et al., 2003). First, the animals were anesthetized and the construct of interest (plasmids: ds circular DNA) could be microinjected into both testes, so the genetic material was located in the extra cellular space, near the actual target cells (spermatogonia). Temporal enhancement of the cell membrane permeability by creating an electric field allows the uptake of the constructs into the cells (electroporation). Then integration into the host genome is possible via the process of transposition (i.e. hyperactive Piggybac transposase; see 1.4). In both studies, which made use of this method, animals were still fertile after the treatment and reproduction lead to transgenic offspring. The efficiency in the F1 generation was, depending on the construct, 57-62%, which is noticeably higher than the efficiency of the commonly used method 'Pronuclear DNA microinjection' (approx. 10-20%) (Wall, 2001) to generate transgenic mice and the same is true for the comparison with zebra finch transgenics having efficiencies between 1-10% (Abe et al., 2015; Agate et al., 2009; Liu et al., 2015). Here, the authors also achieved a specific tissue expression. Another advantage of this method is the small number of manipulated animals, which is needed. Thus, one successfully manipulated mouse is able to produce many litters, of which some individuals would be transgenic, after mating. In the scheme of Fig. 11 the classical approach 'Lentivirus injection into stage x embryos' and the new approach '*In vivo* microinjection & electroporation

of testes' to generate transgenic zebra finches are compared with respect to time required to achieve a transgenic F1.

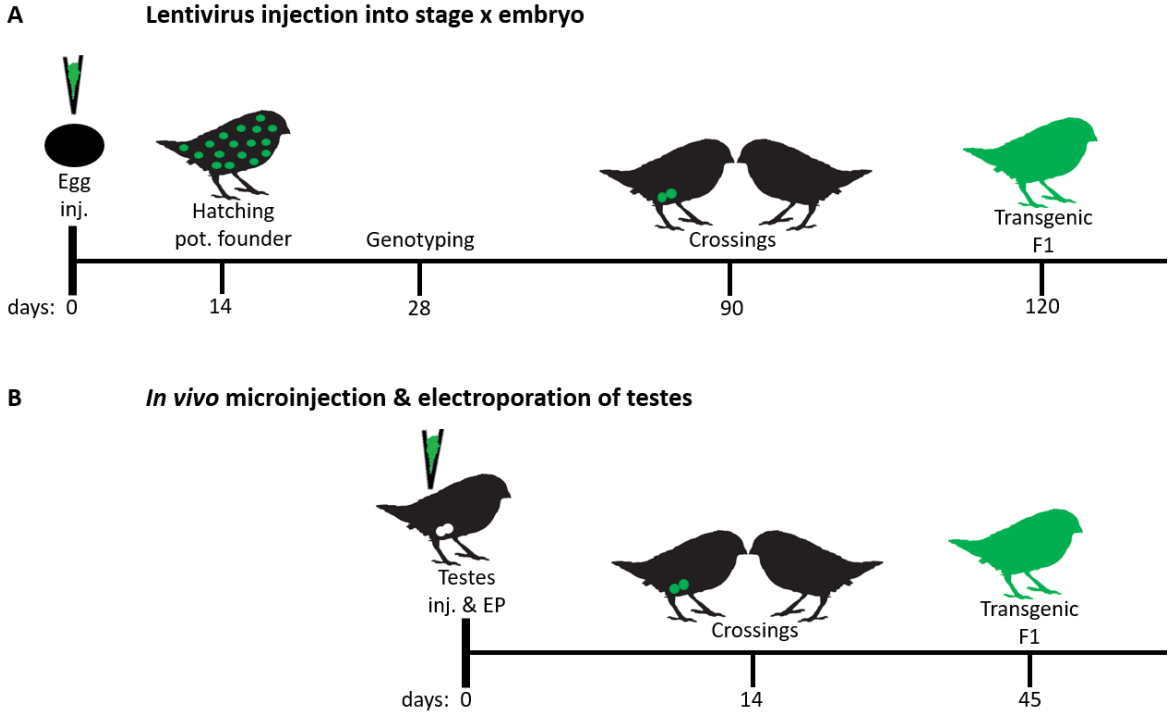

Figure 11 Timeline for the generation of transgenic songbirds applying different approaches.
Classic 'Lentiviral injection into stage x zebra finch embryos' (A) leads first to chimeric hatchlings, which need to be genotyped to confirm the presence of the transgene before it can reproduce. Only transgenic offspring is produced, if the germline was manipulated. It takes about 120 days to obtain transgenic F1 generation. Novel 'In vivo microinjection & electroporation of testes' method (B) targets the germline of adult males, that could be crossed to a female within two weeks and therefore transgenic offspring is generated faster (approx. after 45 days) and without a chimeric animal in between. 'inj.': injection, 'pot.': potential, 'EP': electroporation.

In the above mentioned method for the generation of transgenic zebra finches by injecting *in vitro* transduced PGCs described in Gessara et al. (2021) 100% of all generated founder individuals produced transgenic offspring ranging from 4-22%, so transmission of the inserted transgene via the germline to the F1 was achieved with similar results as reported for Lentivirus injection into stage x embryo (6-22% transmission via germline to F1), but Gessara's approach was much more efficient in producing founder animals (100% vs. 1.13-2.29%) than all projects based on Lentivirus injection into stage x embryos (Abe et al., 2015; Agate et al., 2009; Liu et al., 2015). Consequently, this new strategy seems the better choice to obtain founder individuals in an appropriate time interval compared to direct lentivirus injection into same stage embryos.

These approaches are most promising due to the reduced time for generating transgenics and their increased efficiency in contrast to other avian embryonic manipulations. In addition, harvesting and manipulating sperm is also less time consuming than PGC culturing and manipulation. This alternative approach from mice research for which testes are manipulated

and the recently published PGC culturing based approach already successfully applied to zebra finches open new exciting possibilities to manipulate the genome of songbirds and perhaps other avian species (Usmani et al., 2013; Michaelis et al., 2014, Gessara et al., 2021).

1.4 Specific targeting of interneurons

To specifically target a single cell type requires that only the cell type of interest is manipulated or that the expression of the introduced constructs only occurs in these target cells. To do this, we used a murine enhancer, which specifically addresses GABAergic interneurons of the forebrain. This enhancer lies between the genes DLX (Drosophila distal-less) 5 and 6. The similarity was high enough to also drive expression in zebra finch (Dimidschstein et al., 2016). Optogenetic manipulations were already done in adult zebra finches (Hisey et al., 2018; Xiao et al., 2018; Zhao et al., 2019). Experimental animals received an injection of an AAV carrying a channelrhodopsin into the song nucleus HVC. Specific targeting was achieved by the just mentioned mDLX enhancer. The more target cells were hit, the more likely an effect in their (singing) behavior could be observed (unpublished data from Fabian Heim, MPI for ornithology Seewiesen).

1.5 Stable integration via Transposon

For long-term expression of genes in certain cells, it is not enough to introduce the genetic information into the target cell, but necessary to integrate into the genome. This allows stable expression of the gene of interest. Transposons offer the opportunity to bring in genetic material based on ‘copy and paste’ or ‘cut and paste’ mechanism. Despite the existence of several recombination systems as Cre/loxP (The Cre/loxP system requires two lines, which need to be crossed. This is a disadvantage, if generating even one line is a difficult as in a songbird species so far.) and others, the Hyperactive PiggyBac transposase was selected for this project. The precision of the excision, the possibility to transpose large DNA sequences and the efficiency for germline transmission are advantageous for the generation of transgenic animals (Wang et al., 2008). Moreover, this transposon has already been successfully used to generate avian transgenics (Park & Han, 2012b).
Hyperactive PiggyBac transposase (Fig. 12) belongs to the second type of transposon systems and was chosen in this project for insertion of required sequences.

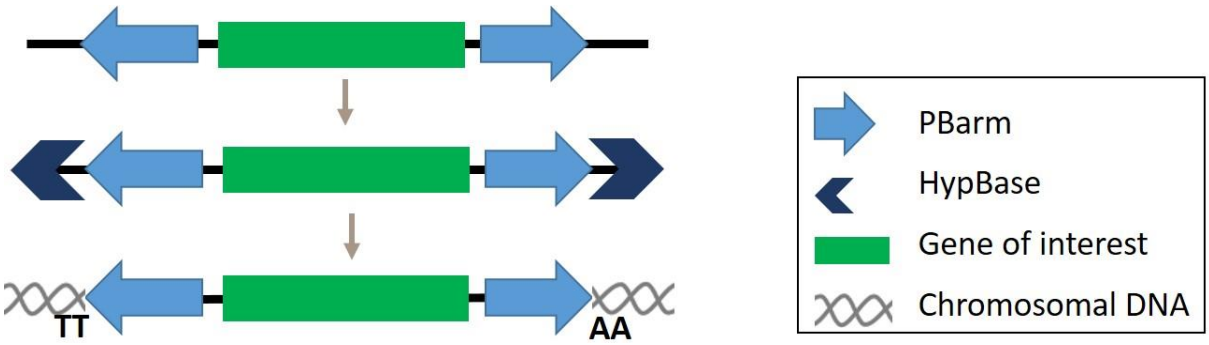

Figure 12 Hyperactive PiggyBac transposase system.
Integration of the gene of interest into chromosomal DNA (random between TTAA sites) by cut & paste mechanism of the hyperactive PiggyBac transposase. The gene of interest is flanked by PBarms, which are recognized by the transposase. By the cut and paste mechanism the gene is then integrated between TT^AA sites of the host genome.

This approach makes use of transposition to integrate channelrhodopsins in interneurons to ensure expression over the whole lifetime of the generated transgenic animals and has already been used for manipulation experiments resulting in transgenic chickens (Macdonald et al., 2012).

1.6 Aim of the study

In this project, the main aim was to create a pipeline for the generation of transgenic songbirds. Although the generation of the first transgenic songbird (Agate et al., 2009) was published more than a decade ago, this method has not become routine. As mentioned in 1.3.1 and 1.3.4 previously published methods to produce avian transgenic lines were mostly successfully applied in chicken and quails, whereas for the zebra finch, as an animal model for vocal communication, few approaches were adapted so far. The injection of lentivirus injection into stage x embryo resulted in low transgenic founder outcome (1.13%; Agate et al., 2009) and could not be much improved by later studies (1.36% in Abe et al., 2015 and 2.29% in Liu et al., 2015). Since then no more transgenic zebra finch lines based on lentivirus injection into stage x embryo were reported. In 2021, Gessara et al. presented an alternative approach based on the *in vitro* transduction of previously extracted PGCs. There, extremely high efficiency concerning founder generation was achieved (100%). However, the ratio of transgenic offspring resembled the one of Agate et al. (Agate: 6-22%; Gessara: 4-22%).
For this study, different ways to generate transgenic zebra finches were tested with the purpose to improve the efficiency of the first approach ‘Lentiviral injection into stage x embryos’ and furthermore offer an alternative way to generate transgenic songbirds. Especially focusing on one generation approaches as they lead to a faster generation of transgenic animals and at the same time reduce the number of required animals in contrast to two generation

approaches. '*In vivo* microinjection and electroporation of testes' and different *in vitro* modifications of sperm followed by artificial insemination belong to possible methods aiming to achieve transgenics in one generation. Both have been successfully used for the generation of transgenic animals: Several studies were published for mice applying '*In vivo* microinjection and electroporation of testes' (Usmani et al., 2013; Michaelis et al., 2014) and one time 'sperm transfection assisted gene editing' in chicken (Cooper et al., 2017).

The following two approaches were selected for this project: '*In vivo* microinjection and electroporation of testes', because of the advantages of one generation approaches and the repeated success in mice studies and '*In vitro* manipulation of PGCs and re-injection of them into recipient stage x embryo' (Gessara et al., 2021), as it yielded always in a transgenic founder, if hatching succeeded.

At the beginning the feasibility of the new method '*In vivo* microinjection and electroporation of testes' was checked (first part). Therefore, the following requirements and questions were investigated: 1) the accessibility of gonads; 2) adjustments of the parameters for microinjection and electroporation (due to species differences: body size, location of testes, kinetics); 3) detection of introduced constructs (by fluorescent reporter, co-localization of two constructs possible?), 4) the examination of long-term manipulation (stable integration into the host genome by transposition) and 5) the transmission of the manipulation to the next generation (via natural reproduction).

For the second part, the promising way to generate transgenics by '*In vitro* manipulation of PGCs and re-injection of them into recipient stage x embryo' was tried to reproduce and results could finally be compared to the previous study. The main steps to obtain transgenic songbirds by this method were the following: 1) virus production, 2) blood extraction of embryos for 3) PGC cultivation, 4) transduction of PGCs, 5) re-injection of modified PGCs into stage x embryos, 6) genotyping of manipulated embryos and 7) the transmission of the transgene to their progeny. Because step 6 was not successful, step 7 was not taken, as mandated by the governmental rules for the protection of animals in research.

In general, both approaches were expected to lead to transgenic founders and a transgenic F1-generation as they were successfully used in the past. The results show that it remains questionable, whether the '*In vivo* microinjection and electroporation of testes' can be transferred successfully to zebra finches and it remains to be seen whether the results gained by '*In vitro* manipulation of PGCs and re-injection of them into recipient stage x embryo' from Gessara can be replicated by other laboratories.

2 Material & Methods

2.1 Nomenclature

The nomenclature for avian brain regions determined by (Reiner et al., 2004), recommended by The Avian Brain Nomenclature Forum (http://avianbrain.org/), has been applied.

2.2 Animal husbandry

2.2.1 Animal housing

All birds, which were part of any described experiment in this dissertation, were raised in their own breeding colony (Permit Number: ZH147, ZH144) of the department for animal behavior at Free University Berlin, Takustr. 6, 14195 Berlin.
Breeding cages (dimensions: 180 cm x 34 cm x 41 cm) were provided for couples to reproduce. Additionally, some zebra & Bengalese finches coexisted in aviaries (dimensions: 2m x 1m x 2m, max. 20 animals). All birds were housed under a 12:12h light:dark-cycle. Young zebra finches were not separated from their parents before post hatching day 90 (PHD 90). At any time, seeds (Deli Nature 41-Exoten Zucht), fresh water, sepia and grit *ad libitum*, as well as crumbled egg with eggshell and sprouted seeds/grass on a weekly basis were offered. Moreover, we offered nesting material (coconut fibers) and nesting boxes for breeding couples. For egg production pairs of wild type zebra finches were selected, and each couple was housed in a breeding cage as already described. Couples of zebra & bengalese finches were housed together in an aviary with nesting opportunities to eventually replace their eggs by manipulated eggs, so they would take care of/rear the potentially transgenic hatchlings as foster parents.

2.2.2 Marking and Sex determination of zebra finch hatchlings

To ascertain the order of hatchlings in one clutch, every day all nesting boxes of the breeding couples were checked. The birthday of each chick was recorded by a unique pattern of cutting the down feathers (Adam et al., 2014). At around PHD10 the young zebra finch's body size allows placing numbered leg ring to distinguish individuals.

The bird's sex chromosomes are named 'Z' and 'W'. Males are homogametic with a ZZ karyotype, whereas female birds are heterogametic and possess a ZW karyotype. The molecular sexing procedure makes use of the chromo box helicase DNA binding gene (CDH), that is located on both avian sex chromosomes, but contains a sex specific polymorphism, which can be detected by polymerase chain reaction (PCR) (Griffiths et al., 1998). Here, a modified approach, using saliva from nestlings as starting material, has been applied (Adam et al., 2014). After the DNA extraction of the samples, a PCR was performed (from about 18µL genomic DNA). With specific primers differently sized fragments of the Z- and W-linked genes, CHDZ and CHDW, were amplified and thus showed whether the samples derived from a male or female bird. Two signals on an agarose gel (after electrophoresis of the PCR product) indicate a female zebra finch due to its heterogamy, in contrast to the amplification of a single fragment, which indicates that the bird is male (homogamy).

2.2.3 Crossing of manipulated birds

Birds with manipulated testis were usually crossed with WT female birds (age of 6 months to 1.5 years, to ensure reproductivity) after approx. two weeks to assess transmission via the germ line. To prevent inbreeding, we did not cross birds from the same or related families.
Additionally, sperm samples of manipulated and crossed male birds were collected during their reproductive phase (as described in 2.5.1).

2.2.4 Egg incubation

Eggs were collected on a daily basis for either manipulation experiments of embryos (stage x) or in order to collect blood from embryos of stage HH13-15 for cultivation of PGCs.
Incubation of zebra finch eggs was carried out in commercial incubators (ProCon CTD7 and EHRET BSS 300). The following parameters were programmed:

- Humidity: approx. 40%
- Temperature: 38°C
- Turn rate: 6x repetitions per day (each turning for 15min)

After oviposition, all eggs chosen to serve as a source of blood samples for PGC culturing were directly moved to an incubator until they reached after approx. 2-3 days the appropriate embryonic stage (HH13-15). To determine the correct stage, characteristics were classified according to an embryonic staging atlas for zebra finches (Murray et al., 2013).

After manipulation of embryos, eggs were incubated for three to five days, until embryonic development was visible by shining a light through the eggshell and then put into foster nests for further development.

All eggs that were part of this study were checked regularly for embryonic development. For this purpose, a hoop lamp and/or a digital egg monitor (‘Buddy’, Avitronics, Cornwall, UK) were applied. The hoop lamp allows to shine through the eggs and to detect an embryo and a developing vascular system, whereas the egg monitor is able to detect the heartbeat of the embryo from a certain stage on (approx. day 3 of incubation, when heartbeat is as well visible via hoop lamp).

2.3 Laboratory materials

2.3.1 Solutions and Buffers

A list of all solutions and buffers required for any experiment of this project is shown in Tab. 1.

Table 1 Solutions and buffers

10x PBS -NaCl 80g (1370mM end concentration) -KCl 2g (27mM end concentration) -Na_2HPO_4 14.2g (100mM end concentration) -KH_2PO_4 2.4g (200mM end concentration) -Adjust to pH7.4 and add ddH_2O to 1L	**PBST 0.1%** -1mL Tween20 in 1L 1x PBS
	PBS-Tx 0.3% -15mL 10% Triton X in 1L 1x PBS
	Ethidium bromide stock solution -10mg/mL in ddH_2O
DEPC water -Add 0.5mL DEPC to 500mL H2O -Shake vigorously -Leave open under hood overnight -Autoclave	**DAPI solution** -1:10,000 in water
	formalin-ethanol fixative solution -5mL 37% formaldehyde -45mL 95% EtOH
50x TAE -Tris Base 242g (2M end concentration) -acetic acid 100% 57.1mL (5.71%) -0.5M EDTA pH 8.0 100mL (0.05M end concentration) -Add ddH_2O to 1L	**0.5M PB** -7.10g Na2HPO4 in H_20
	1x TAE -50x TAE 40mL (1x end concentration) -Add ddH_2O to 2L
8% PFA -500mL ddH_20 (heated to 60°C) -40g PFA -add 10N NaOH dropwise to dissolve PFA -filter solution -store at 4°C or freeze at -20°C	**10xPCR buffer** -Tris Base (670mM) -Ammoniumsulfate (166mM) -Tween20 (1%)
4% PFA -500mL 8%PFA -200mL 0.5M PB -add ddH_2O to 1L	**Mowiol** -6g Glycerin -2.4g Mowiol -6mL ddH_2O -Stir at RT -Add 12mL 0.2M TRIS (pH 8.5) -Stir at 53°C overnight -Centrifuge at 500rpm for 20min -Aliquot and store at -20°C
DNA extraction buffer -2.5mL 2M Tris (pH 8.5) (100 mM end concentration) -0.5mL 0.5M EDTA (0.2% end concentration) -1.0mL 10%SDS (100 mM end concentration) -2mL 5M NaCl (200 mM end concentration) -ad 50mL ddH_2O	**Analgesic** -Meloxicam For zebra finches: 0.4mg/kg body weight -Carprofen For zebra finches: 4mg/kg body weight
Reconstitution buffers for growth factors a) 10mM Citric acid pH3.0 + 0.1% BSA b) 1x PBS + 0.1%BSA c) 4mM HCl + 0.1%BSA	**TDMH** -1069.65µL 10xPCR buffer -76µL 25mM dNTP's -854.35µL 25mM $MgCl_2$
RVP -5mL DEPC -3mL 20x SSC -2mL Formaldehyde	**Semen extender Lake & Ravie 1984** -L-Glutamic acid monosodium salt hydrate 1.92g (0.1M end concentration) -D(+) Glucose 0.8g (0.04M end concentration) -Magnesiumacetate Tetrahydrate 0.08g (0.004M end concentration) -Kaliumacetate 0.5g (0.05M end concentration) -Polyvinylpyrrodion 0.3g (0.0003M end concentration) -Add ddH_2O to 100mL
1x TE buffer (pH7.6-8.0) -0.2mL 0.5M EDTA (pH 8.0; 1mM end concentration) -1.0mL 1M Tris HCl (pH 7.6; 10mM end concentration) -ad 100mL ddH_2O	

Cryoprotectant -30% ethylene glycol and 30% sucrose in PB -Recipe adapted from Nordeen laboratory -Dilute 100mL 0.5M PB in 150mL ddH_2O -Dissolve 150g sucrose in diluted -Add 150mL ethylene glycol to PB/sucrose solution -ddH_2O to 500mL -store at 4°C	

2.3.2 Cell culture media

Different cell culture media were used for virus production and PGC cultivation. They can be found in Tab. 2.

Table 2 Cell culture media

HEK293-T / Hela cell culture medium	**Medium for freezing cells**
500mL DMEM (Invitrogen, Carlsbad, USA) 55mL Foetal Calf Serum (this corresponds to ~ 10 %) 6mL L-Glutamine (200mM; Invitrogen) 7mL ready-to-use Penicillin/Streptomycin-Mix (Penicillin 10.000 U/mL, Streptomycin 10.000µg/mL) (Invitrogen)	35mL culture medium 10mL FCS 5mL DMSO
PGC Medium (Whyte et al., 2015) 1mL (1x end concentration) 50x B27 supplement, Gibco (Thermo Fisher Scientific) 0.5mL (2mM end concentration) 100x Glutamax, Gibco (Thermo Fisher Scientific) 0.5mL (1x end concentration) 100x MEM-NEAA, Gibco (Thermo Fisher Scientific) 0.3546µL 14.1M (0.1mM end concentration) 2-Mercaptoethanol (Sigma, Japan) 2.5mL (0.2% end concentration) 4% Ovalbumin, Sigma 600µL (1.2mM end concentration) 100x Sodium Pyruvate (Thermo Fisher Scientific) 2mL (0.2% end concentration) 5% Sodium Heparin, Sigma 0.5mL (1x end concentration) EmbryoMax® 100x Nucleosides, Millipore 12.5µL (end concentration 25ng/mL) BMP4 Recombinant human Protein (0.1µg/µL in 0.1%BSA/4mM HCl), Gibco (Thermo Fisher Scientific) 12.5µL (end concentration 25ng/mL) Recombinant Human TGF-β1 (HEK293 derived) (0,1µg/µL in 0.1%BSA/10mM Citric Acid pH3.0), PeproTech 2µL (end concentration 4ng/mL) Recombinant Human FGF basic/FGF2/bFGF (146 aa) Protein (0.1µg/µL in 0.1% BSA/1xPBS), R&Dsystems 6.25µL (end concentration 25ng/mL) Recombinant human IGF-I/IGF-1 Protein, CF (0.2µg/µL in 1xPBS), R&Dsystems 42.3289mL KnockoutD-MEM 4.5g/L Glucose + Sodium Pyruvate, Gibco (Thermo Fisher Scientific)	
Virus production media 1) DMEM (1g/L glucose) with Glutamax 10% FCS without antibiotics 2) DMEM (1g/L glucose) with Glutamax & Pyruvate 10% FCS without antibiotics 3) DMEM (1g/L glucose) with Glutamax & Pyruvate 2% FCS 1% penicillin/streptomycin (100x stock solution), Gibco	

2.3.3 Antibodies for IHC

All antibodies needed for immunohistochemistry and Slot blot are listed in Tab. 3.

Table 3 List of antibodies utilized for IHC or Slot Blot

Primary antiboby	**animal (host)**	**company**	**AB type**	**dilution**
anti SSEA1	mouse	abcam, ab16285	monoclonal	1:200
anti GFP	rabbit	abcam, ab290	antiserum	1:1,000
anti DsRed (Living Colours)	rabbit	Clontech, 632496	polyclonal	1:1,000
anti Dig-AP	mouse	Roche 11093274910	monoclonal	1:2,000
Secondary antibody	**company**	**conjugated**	**dilution**	
Donkey anti Mouse	Invitrogen	Alexa 488	1:200	
Donkey anti Mouse	Invitrogen	Alexa 568	1:200	
Donkey anti Rabbit	Invitrogen	Alexa 568	1:200	
Streptavidin	Invitrogen	Alexa 488	1:200	
Streptavidin	Invitrogen	Alexa 568	1:200	

2.3.4 DNA probe for Slot Blot

A specific DNA probe for eYFP and mcherry was produced for the detection of the transgene in genomic DNA by Slot Blot. Therefore, plasmid #462 (lab intern numbers, see 2.3.5) was cut by the restriction enzymes KpnI and PmeI resulting in an 803bp long fragment (eYFP probe for later use in eYFP genoytping by Slot Blot). Plasmid #217 was restricted by AgeI and EcoRI to generate a 723bp long mcherry probe (for later mcherry genoytping by Slot Blot) (Fig. 13). Both were subsequently DIG labelled utilizing the DIG High Prime Labeling and Detection Starter Kit 11585614910 (Roche, Switzerland).

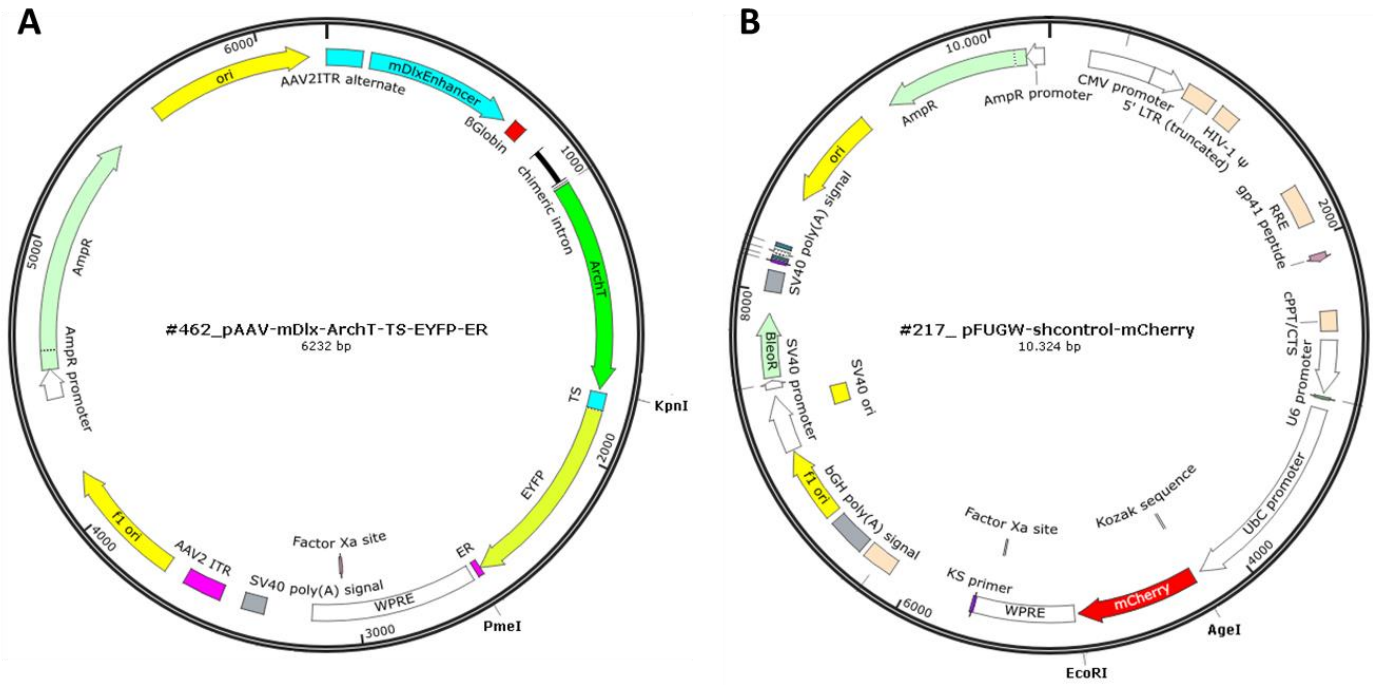

Figure 13 Plasmid maps of plasmid #462 and #217 for DNA probe design.
Plasmids were digested with displayed restriction enzymes to generate DNA probes for Slot Blot genotyping (A: for EYP, B: for mcherry).

2.3.5 Plasmids

Plasmids were either used for cloning, transfection of cells or virus production in cells (full list of all plasmids see App. 1). Several plasmids were kindly gifted by Prof. Avihu Klar from the Hadassah Medical School (The Hebrew University of Jerusalem) and by the Hegemann laboratory (Humboldt University Berlin).

Plasmids #14 to 16 were required for virus production.

Some plasmids were created by laboratory intern cloning.

Plasmid #62: This plasmid served as backbone for #137.

Plasmid #86: This plasmid was needed to build #207. FoxP2 promoter was amplified from #50 by primers 9 and 10. The PCR product and plasmid #30 were restricted by EcoRV and HindIII and finally ligated.

Plasmid #132: It was used for mcherry expression and to detect its fluorescence after injection into and electroporation of zebra finch testis. #146 served as backbone, was cut by EcoRI & NheI to remove RAGE sequence and restriction sites were added applying primers 455 and 456.

Plasmid #135: This plasmid delivered ß Globin for #137 cloning. Primer 85 and 86 amplified the FoxP2 enhancer sequence from zebra finch DNA. The resulting PCR product (1791bp)

and the plasmid #34 were digested by the enzymes BamHI and NheI. Afterwards both were ligated.

Plasmid #137: It was used for virus production and to detect EGFP in embryos, which were injected by the virus. This plasmid made out of three plasmids. The backbone of #62 was used, but ubiquitin promoter was removed by BamHI and Bsp119I. FoxP2 enhancer and ß-Globin was amplified from plasmids #135 and #141 with the primers 89 & 90 and 88 & 91. Afterwards, a Megaprime PCR was run on both products with the primers 88 & 89. The backbone and the Megaprime PCR product were finally ligated.

Plasmid #148: This plasmid was required to express the transposase (HypBase) to achieve stable integration from desired construct co-injected and electroporated into testis.

Plasmid #177: This plasmid was an intermediate product needed to build #179 and later #457. PCR war performed on template plasmid #129 with the primers 141 and 174. The resulting PCR product and the plasmid #127 were cut by AgeI and EcoRI and digested fragments were finally ligated.

Plasmid #179: This plasmid was an intermediate product needed to build #457. PCR war performed on template plasmid #129 with the primers 145 and 175. The resulting PCR product and the plasmid #177 were cut by NheI and EcoRI and digested fragments were finally ligated.

Plasmid #207: This plasmid was an intermediate product needed to build #457. Two PCRs were performed: First on the template plasmid #86 with the primers 193 and 194; second on the template plasmid #179 with the primers 195 and 197. The two resulting PCR products served as template for a Megaprime PCR with the primers 193 and 197. The Megaprime PCR product and the plasmid #146 were cut with EcoRI and SpeI and digested fragments were finally ligated.

Plasmid #316: It was used for EGFP expression and to detect its fluorescence after injection into and electroporation of zebra finch testis. #132 served as backbone and was cut with NotI and NheI. The eGFP sequence has its origin in #140 and was cut out by NotI and NheI. The backbone and the eGFP sequence were finally ligated.

Plasmid #389: This plasmid was an intermediate product for cloning #442 (Chrimson eGFP). Plasmid #218 served as template for the generation of a 4667bp long fragment (amplified by the primers 669 and 670). Plasmid #387 served as template for the generation of a 2819bp long fragment (amplified by the primers 671 and 672). Both PCR products were restricted by the enzymes NheI and PmeI and finally ligated.

Plasmid #436: This plasmid was used to construct plasmid #442 in order to get Chrimson eGFP sequence for later cloning of #460.The plasmid #432 was cut by BamHI and HindII. A PCR was performed with the 1773bp fragment of plasmid #432 as template and the primers

760 and 761. The plasmid #389 and the PCR product were digested with NheI and PmeI. The 4641bp fragment of plasmid #436 and the cut PCR product were ligated.

Plasmid #441: This plasmid served for cloning the CoChR eGFP sequence into plasmid #459. A PCR was performed with plasmid #434 as template and the primers 770 and 771. The plasmid #436 and the PCR product were digested with KpnI and SpeI. The 5450bp fragment of plasmid #436 and the cut PCR product were ligated.

Plasmid #442: This plasmid served for cloning the Chrimson eGFP sequence into plasmid #460. A PCR was performed with the plasmid #435 as template and the primers 772 and 773. The plasmid #436 and the PCR product were digested with KpnI and SpeI. The 5457bp fragment of plasmid #436 and the cut PCR product were ligated.

Plasmid #457: This plasmid served as backbone for the plasmids #459 and #460. The plasmid #207 was cut by PmeI, EcoRI and EcoRV to keep a 4965bp long fragment. Oligonucleotids (819 and 820) were annealed and cut by PmeI and by EcoRV. These cut oligos were ligated to the kept fragment of plasmid #207.

Plasmid #459: This plasmid was needed to drive cell type specific (interneurons) expression by mDLX enhancer of channelrhodopsin variant CoChR. Made out of #457, served as backbone, and #441, mDLX-CoChR-TS-EYFP-ER sequenced was kept; both cut by MLuI and PmeI and finally ligated.

Plasmid #460: This plasmid was needed to drive cell type specific (interneurons) expression by mDLX enhancer of channelrhodopsin variant Chrimson. Made out of #457, served as backbone, and #442, mDLX-Chrimson-TS-EYFP-ER sequence was kept; both cut by MLuI and PmeI and finally ligated.

2.3.6 Primers

Table 4 includes all primers for PCR amplification and Tab. 5 primers needed for cloning of plasmids. All primers were ordered from Eurofins Genomics.

Table 4 Primers for PCR

Number	Type	Sequence (5'->3')	Annealing Temp. (°C)	Application	Product size (bp)
607	for.	GCTGCTTTAATGCCTTTGTATCATGCTATT	59.8	GFP genotyping	528
619	rev.	TCGAGGTCGACGGTATCGAT			
258	for.	GAGGTCCAGAGGCGAAGAAT	52.5	Sexing	for females: 452/202 for males: 418
259	rev.	ACCGCCTTATCTCTGCATCA			
380	rev.	CCACACATGAAAACCACCCAA			
92	for.	(CT)T(GT)CCAAG(AG)ATGAGAAACTG [YTKCCAAGRATGAGAAACTG]	55.0	Sexing	for females: 355/389 for males: 355
93	rev.	TCTGCATCACTAAA(GT)CCTTT			
514	for.	AAGAGCAGGATCACCAGCGA	64.0	eYFP genotyping	954
849	rev.	GCTCAAGGGGCTTCATGATG			
620	for.	CCAAGCTGAAGGTGACCAAG	59.4	mcherry genotyping	505
621	rev.	TCCACGATGGTGTAGTCCTC			

Thermal protocols for the amplification different fragments via PCR are listed in Tab. 11 (see 2.9.4) together with the corresponding master mix compositions.

Table 5 Primers for cloning
The binding parts of the sequence are displayed in capital letters in sequences and parts, which were added via amplification like restriction sites are displayed in small letters.

Number	Sequence (5'->3')
85	AGCTGCTCTGTTGTGATATTGA
86	CCTTGCTTCTTGTTCCGCTT
88	CGGGGATCCGCCGCTCTGCTTCTGGAAGCGT
89	TAGTTCGAAAGCTGCTCTGTTGTGATATTGA
90	TTAATTAAGGGTTAACGGCGTACGGCCTTGCTTCTTGTTCCGCTT
91	CGTACGCCGTTAACCCTTAATTAACCCGGCTGGCATAAAAGTCAGGG
136	CGCGGATCCGCCACCATGATGCAAGAATCTGGG
137	GCGGAATTCCTACTTATCGTCGTCATCCTTGTAATCTTCTATGTCCTC
141	TGGACCGGTGCCACCATGGGCAAGCCCATCCCTAAC
144	TTAGAATTCTCAAGCTTCGAACTTGGGAAG
145	TTAGAATTCTCAGGCACCGGGCTTGCGGGT
146	TGGACCGGTGCCACCATGGGCAAGCCCATCCCTAAC
174	GCCGAATTCACGACGGCTAGCCACCTTCCTTTTCTTTTTTGG
175	TGGGCTAGCGAGTTCTCTAGAGGCAGTGGAGAGGGCAGAGGA
177	GGCCTGACAAAGAGCTCATG
193	CGCGAATTCATGATGCAAGAATCTGGGAC
194	CGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCTTCTATGTCCTCATTTACAGG
195	CTCCTCGGTCTCGATTCTACGGGCAGTGGAGAGGGCAGAGGA
197	AGAACTAGTTCAGGCACCGGGCTTGCGGGT
455	AATTCTTCGAACACGTGACGCGTG
456	CTAGCACGCGTCACGTGTTCGAAG
760	gatGCTAGCACTAGTCATATGGGTACCAAGAGCAGGATCACCAGCGA
761	atcGTTTAAACTTACACCTCGTTCTCGTAGCAGAA
770	gatACTAGTATGCTGGGAAACGGCAGCGC
771	atcGGTACCTGCTACTACCGGTGCCGCCAC
772	gatACTAGTGCCACCATGAGCAGACTGGTCGCCGC
773	atcGGTACCCACTGTGTCCTCGTCCTCCTCCT
819	gatGATATCACGCGTTTCGAAGTTTAAACgat
820	atcGTTTAAACTTCGAAACGCGTGATATCatc

2.3.7 Enzymes

All restriction enzymes for cloning were purchased from New England Biolabs (Ipswich, USA) and Fast Digest enzymes from Fermentas (St. Leon-Rot, Germany). DreamTaq DNA polymerase (Thermo Fisher Scientific, USA) or laboratory made recombinant Taq polymerase was used for PCRs. For ligation T4 Ligase enzyme was applied from Promega (Madison, WI, USA). Recombinant Proteinase K (PCR Grade) was purchased from Roche (Switzerland) to extract DNA from tissue samples.

2.3.8 Rhodopsins

An overview about all rhodopsins, which were part of any experiment of this study, can be found in Tab. 6.

Table 6 Rhodopsins used for manipulation experiments in this study.

Rhodopsin	Origin	Ex. wavelength (nm)
red light drivable rhodopsin (Chrimson)	*Chlamydomonas noctigama*	590
large-current channelrhodopsin (CoChR)	*Chloromonas oogama*	475

2.3.9 Kits

Any commercially available kit, used in this project for different purposes, can be found in Tab. 7.

Table 7 Commercially available kits, which were used for the experiments.

Name	Company	Application
Nucleo Spin Tissue XS	Macherey Nagel	DNA extraction from small samples (i.e. sperm)
NucleoSpin Gel and PCR Clean-up	Macherey Nagel	DNA extraction of gel fragments
NucleoSpin Plasmid EasyPure	Macherey Nagel	Mini preparation of plasmid DNA
Nucleo Bond Xtra Midi Kit	Macherey Nagel	Midi preparation of plasmid DNA
PAS staining system	Sigma-Aldrich	PAS staining for PGCs
Monarch Genomic DNA Purification Kit	New England BioLabs	DNA extraction from tissue samples
DIG High Prime Labeling and Detection Starter Kit	Roche	DIG labeling of DNA probe

2.3.10 Software & internet sources

Used software and internet sources are noted in Tab. 8.

Table 8 Software and internet sources.

Name	Link to website	Application
Reverse complement	www.bioinformatics.org/sms/rev_comp.html	Reverse complement sequences
Primer 3	http://frodo.wi.mit.edu/primer3/	Primer design tool
MWG	www.eurofinsdna.com	Primer ordering
CLUSTALW (European Bioinformatics Institute)	www.ebi.ac.uk/Tools/msa/clustalw2/77	Alignments of DNA sequences
NCBI (Ntl. Center for Biotech. Information)	www.ncbi.nlm.nih.gov	BLAST, literature; ORF finder; DNA,
Zebra finch BLAT Search	https://genome.ucsc.edu/cgi-bin/hgBlat	Blat against zebra finch
Tm calculator Thermo Fisher	https://www.thermofisher.com/de/de/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/tm-calculator.html	determination of annealing temperature for primers
SnapGene	https://www.snapgene.com/	Maps of plasmids; restriction sites
Rstudio (Version 4.0.3)	https://www.rstudio.com/	Statistical analysis; graph design
Fiji (ImageJ 1.52p)	https://fiji.sc/	Quantification of fluorescence in tissue samples

2.3.11 Statistical Analysis

All statistical analysis has been conducted applying R Studio (version 4.0.3) using the following packages: readxl, ggpubr, ggplot2, rstatix, dplyr and forcats. To test whether data were normally distributed the Shapiro-Wilk normality test was performed. A comparison of means of unpaired and normally distributed data was performed by applying the Welch Two Sample t-test; if data did not show normal distribution the Wilcoxon rank sum test was used instead.

2.4 Transgenic zebra finches via injection into embryos

2.4.1 Plasmid preparation for Lentiviruses

Plasmid preparation to obtain an appropriate amount of plasmids for virus production was carried out applying Nucleo Bond Xtra Midi Kit (Macherey-Nagel, Germany) following manufacture's protocol.

2.4.2 Viral constructs

Viruses were either purchased from the viral core facility of the Charité Berlin (AAVs and Lenitviruses) or self-produced (Lentivirus production, see 2.4.3).

2.4.3 Lentivirus production

The generation of recombinant lentiviruses, with the aim to manipulate stage x embryos of zebra finches, was done by using the protocol from (Lois et al., 2002). Some modifications were carried out as follows.

HEK293-T cells, approximately $5x10^6$ in 10mL medium for each of the 24 cell culture petri dishes (10cm diameter CELL+ from Sarstedt, Nümbrecht, Germany), were seeded in medium (10% fetal calf serum, FCS, in DMEM with Glutamax and 1g/L glucose, without antibiotics) at 37°C. On the day of transfection HEK293-T cells were co-transfected with all viral constructs. Per dish the transfection mixture contained 10µg viral transfer vector (#137 or #459), 2µg envelope vector pVSV-G (#14), 6/3µg packaging vectors (#15 pLP1/#16 pLP2), 500µL 2x BBS (1x BBS pH 6.7: 2x BBS pH7.2), 50µL 2.5M $CaCl_2$, 429 µL MQ water. First water and vectors were mixed and incubated at 37°C. Then, 2x BBS and afterward $CaCl_2$ were added. Directly after adding the last component the mixture was vortexed and eight-minute incubation in a pre-warmed water bath took place. Medium from cells was removed and the transfection mixture was added dropwise. Finally, new medium (10% FCS DMEM with Glutamax and 1g/LD-Glucose and Pyruvate, without antibiotics) was added carefully.

The next day medium was removed, and new medium was added (2% FCS DMEM with Glutamax and 1g/L D-Glucose and Pyruvate, Containing 1% penicillin/streptomycin of 100x stock solution from Gibco, Carlsbad, California). Lentiviral particles were collected 48h post transfection. For this, the culture supernatant was transferred to fresh tubes to clear by centrifugation (500xg for 7min at room temperature, RT) and filtration (45µm pore vacuum filter with CA membrane from Corning, New York, USA). Filters were equilibrated with media before

filtering the virus-containing medium. Tubes for ultracentrifugation were prepared (cleaning with 70% ethanol, EtOH, and rinsing with medium for removing any traces of alcohol). After transferring the virus-containing medium to prepared tubes ultracentrifugation (25,000 rpm, 2h, at 4°C) in an Optima L-80XP centrifuge (from Beckmann Coulter, Krefeld, Germany) with rotor SW32 was started for the concentration of virus particles. Medium could be discarded without touching the pellet. To free the pellets from rests of medium the tubes were then turned around and put on Kim wipes (Kimtech/Kimberly-Clark, USA) for 10min. Subsequently the pellets were covered with 1mL DMEM with Glutamax overnight as the procedure of virus collection was repeated on the next day (after supernatant up take for the first virus collection, fresh medium was given to the cells and incubated again for the continuation of virus production). Finally, virus pellets were re-dissolved in 20µL of 1xHBSS without magnesium and calcium (Gibco, Carlsbad, California) and aliquots of 3µL in Eppendorf tubes were shock frozen in liquid nitrogen and stored at -80°C for later use.

2.4.4 Titration of lentiviruses

To determine the virus titer one day before titration HEK293-T cells were seeded ($4x10^5$ cell per 6-well (CELL+, Sarstedt, Germany). For infecting the cell with the produced virus (1µL of undiluted and of diluted virus -1:10, 1:50, 1:250, 1:500, 1:1,000- was added to the cell culture medium (with antibiotics) of the corresponding well. Infected cells were quantified by flow cytometry with a FACScalibur (Beckton Dickinson, Heidelberg, Germany) 72h post infection.
In this project, all produced viruses encoded the green fluorescent protein (GFP), so the same wavelength (530nm) was applied to quantify the infected cells. The titer was calculated by the division of GFP positive cells through the total number of seeded cells (as described above approx. $4x10^5$ per well) and multiplied by the dilution factor. The percentage of fluorescent cells in the 1:10 and 1:100 dilutions were considered to calculate/determine the virus titer, as titers normally are around 10^6. Usually, the virus titer ranged from 1-$3x10^6$/µL.

2.5 Sperm samples

2.5.1 Sperm collection

Sperm samples may provide information about sperm quality (morphology and swimming velocity e.g.), and quantity. In this case we also wanted to ascertain whether the transgene was detectable in sperm. Different approaches for avian sperm collection have been described: cloacal/abdominal massage, female dummy and sampling from faeces (Immler & Birkhead, 2005). A study demonstrated that by offering a dummy female only few males provided sperm (Girndt et al., 2017). Moreover, they revealed differences in sperm morphometry when comparing fecal (shorter head and midpiece) to abdominal massage and therefore recommend the last variant. Therefore, sperm from zebra finch males for this project was collected by applying cloacal massage (Kucera & Heidinger, 2018; Samour et al., 1986; Samour, 2002, 2004). This method is fast and feasible without much practice but requires two experimenters. One holds the bird in the left hand and applies cloacal massage with the right hand. The other collects the ejaculate with a pipette fitted with a 10 or 20µL pipette tip when the clear drop appears at the opening of the cloaca. The ejaculate was transferred into an Eppendorf tube containing sperm extender (Lake & Ravie, 1984) or buffer (depending on which experiments followed).
Usually, it takes almost two weeks for avian PGCs (progenitors of the sperm cells) to differentiate into functionally sperm (Bhat & Maiti, 1988; Jones & Lin, 1993; Lin & Jones, 1992). Therefore, collection of sperm samples started around two weeks after manipulating male testes. DNA was extracted from the sperm samples to check via PCR for the presence of the reporter gene mRNA (i.e. eGFP or eYFP, which is coupled to the transgene) that had been introduced into the testes via injection and electroporation beforehand.

2.5.2 Assessment of sperm quality

To check whether the sperm of a male zebra finch was of high quality 1µL of each sperm sample was put on a slide to be used for microscopy (Axiovert S100, Zeiss, Germany). Mostly microscopic analysis was done at a magnification of 5x or 10x in phase contrast. All samples were qualitatively checked for their viability, swimming movements and morphology. Regarding morphology the majority should be assigned to normally shaped sperm, which means all parts of a sperm cell should be present, an intact head, which is crucial for the fertilization process and not more than one head (see Fig. 14). Dead sperm could not be included in any further manipulation experiments, but still be utilized for DNA extraction.

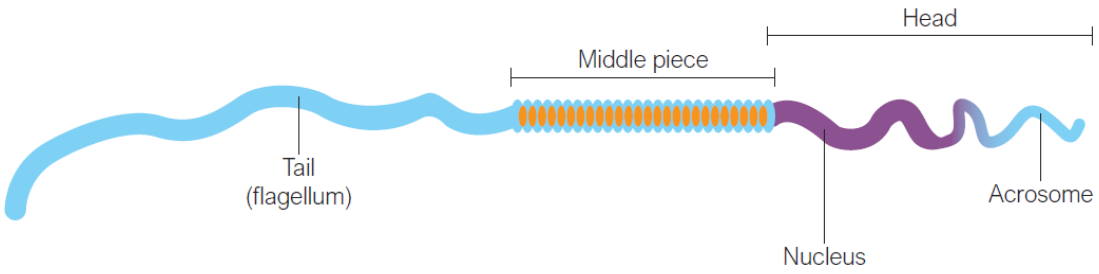

Figure 14 Schematic structure of a sperm cell.
Different parts of the sperm cell are depicted. Tail for swimming behaviour, midpiece containing many mitochondria for energy delivery, nucleus carrying the genetic information and acrosome for acrosome reaction to fertilize the ovum.

2.5.3 DNA extraction from sperm samples

For DNA extraction from sperm, the samples were collected in 1xPBS (as described above; 2.5.1) and if necessary stored at -80°C. In contrast to Kucera et al., 2018 (QIAamp DNA Micro Kit) a different kit from Macherey Nagel (Nucleo Spin Tissue XS, #740901.50) was chosen to extract DNA from sperm and manufacture's protocol had been slightly modified as follows: a sperm sample from one ejaculate after cloacal massage in 20µL 1xPBS, 60µL T1 buffer for pre-lysis, after adding ethanol 2x 5s vortex, 8µL buffer for elution, 5min at 80°C 450rpm for alcohol removal (Fig. 15).

1 Prepare sample			sperm in 20µL 1xPBS
2 Pre-lyse sample			60µL T1 8µL Proteinase K 56 °C, 1–4h
3 Lyse sample			80µL B3 70 °C, 15min
4 Adjust binding conditions			80µL ethanol Vortex 2x 5s
5 Bind DNA	 		Load lysate 11,000 x *g*, 1min
6 Wash silica membrane	 	1st wash 2nd wash	50µL B5 11,000 x *g*, 1min 50µL B5 11,000 x *g*, 2min
7 Elute DNA	 		8µL BE 11,000 x *g*, 1min
8 *Optional:* *Remove residual ethanol*			*Optional:* *80 °C,* *5min*

Figure 15 Extraction for small sized samples of genomic DNA by NucleoSpin® Tissue XS kit.
Here used to isolate DNA from zebra finch sperm samples (protocol modified from Macherey Nagel).

2.6 *In vivo* microinjection followed by electroporation of zebra finch testes

In Fig. 16 an overview about the main steps of the method '*In vivo* microinjection with following electroporation of testes' to generate transgenic zebra finches is demonstrated.

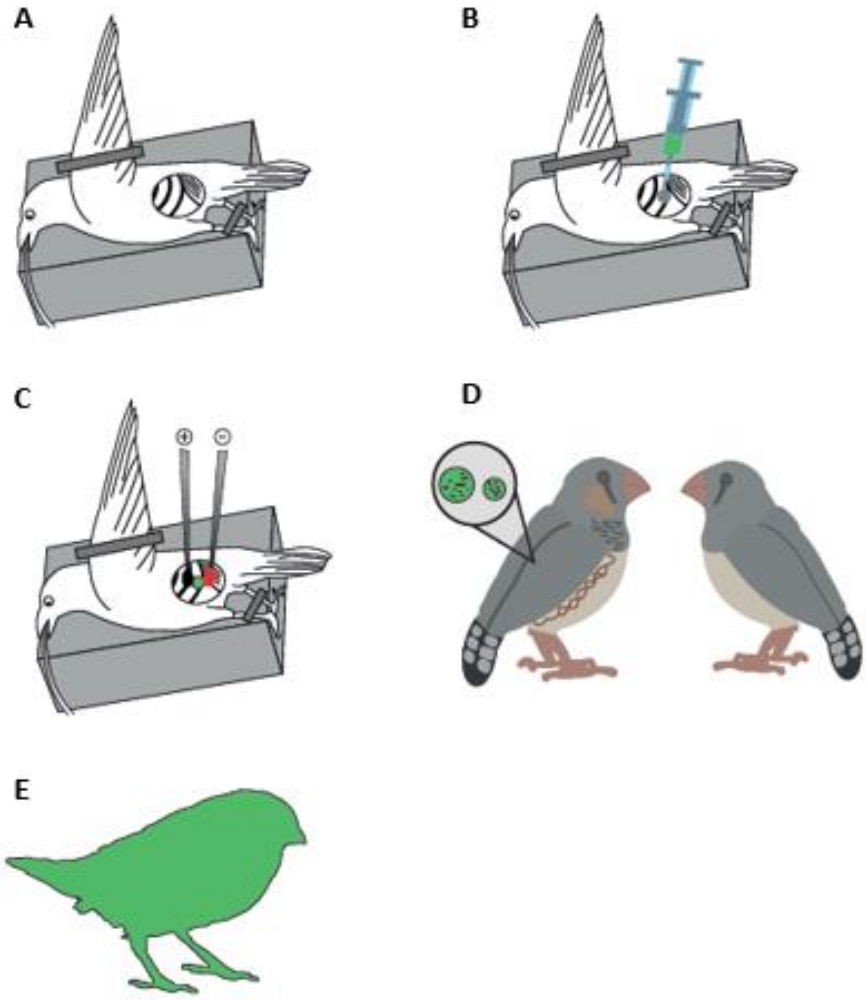

Figure 16 Schematic workflow of 'In vivo microinjection and electroporation of zebra finch testes'.
A: Laparotomy via incision between the last two ribs, B: Microinjection of desired construct into testis, C: electroporation of injected testis, D: Crossing of manipulated male for reproduction, E: Generation of transgenic F1.

2.6.1 Plasmid preparation

Plasmid preparation was conducted as described in 2.4.1.

2.6.2 Anesthesia of male zebra finches for *in vivo* microinjection of testes

30 minutes before anesthesia was initiated, birds received an intramuscular injection of analgesic (dose 5µL/g body weight of 5mg/mL carprofen solution; Rimadyl zoetis, Germany) as pre-operative pain prevention. For initiation of anesthesia 2.5l/min O_2 and 2.5-3.5% Isoflurane were mixed and provided by an evaporator (Dräger, Trajan 808) to the beak of the bird via pipe system for approx. 5-10min in the maintenance phase (duration of surgery) 2.5l/min O_2 + 0.8-2% Isoflurane were supplied. To wake up birds from anesthesia isoflurane was reduced and O_2 level raised. Analgetic treatment was continued for the next 3 days after surgery with Meloxicam (0.33 µL/g body weight) intraoral by pipette.

2.6.3 Laparotomy

Aiming to manipulate testes, first of all it is necessary to get access to the gonads. This part is easier in mice as the testes are located outside the body contrary to zebra finches, where testes are located inside the body. Manipulation happens in anesthetized birds using isoflurane. The birds had to be placed and fixed on the right side to ensure access to the left testis (Fig. 19 A). Removing some obstructing feathers is optional before making a cut in the skin to guarantee a better sight towards the underlying ribs. The skin was disinfected with 70% EtOH. As soon as the last rib and the prior rib are identified a small incision between them is required to get finally access to the gonads. The left testis is easily visible at the dorsal end of the body. Closely posterior to the left testis and a bit deeper inside the body, the right testis should be visible as well. At this point any manipulation (i.e. microinjection or electroporation) of the gonads can be carried out. Afterwards the outer skin layer, which had been shifted to the sides for the procedure, are reconstituted and the cut is covered by collodion (Fluka, Switzerland) to promote healing.

2.6.4 *In vivo* microinjection of zebra finch testes

After achieving access to the gonads of the bird by laparotomy, the desired construct (either reporter gene constructs or Hypbase constructs were introduced to the center of the testis/testes by applying glass capillaries and an injector (MO-10, Narishige, Japan). Some seconds after injection, the capillary was removed. The waiting prevented the solution from escaping via the injection site. Afterwards an electroporation step followed (see 2.6.5).

2.6.5 *In vivo* electroporation of zebra finch testes

After microinjecting, custom-made electrodes were applied to testes and several electro pulses at different sites of the tissue were conducted (30, 40 and 50V were tested). The dimensions of the electrodes are adjusted and were specifically ordered for the usage of very small animals like in this case zebra finches (see Fig. 17). Finally, electrodes were removed, and the birds were prepared for their anesthesia recovery as described in 2.6.2 and 2.6.3.

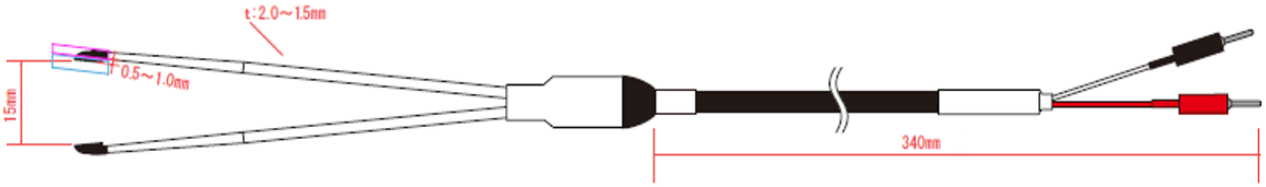

Figure 17 Dimensions of custom-made electrodes for* in vivo *electroporation of zebra finch testes.
Custom-made electrodes for this project to allow access to the gonads of male zebra finches. Electroporation was carried out after* in vivo *microinjection of testes with construct of interest to get manipulated spermatogonia and finally manipulated sperm for the generation of transgenic offspring.

2.6.6 Quantification of fluorescence in zebra finch testes after manipulation

To determine best parameters for the tested electroporation parameters, fluorescence of tissue sections from manipulated males were quantified using the image analysis software Fiji (ImageJ 1.52p). Images were opened in the Fiji software and threshold was adjusted to 18-100, and particles of sizes between 0.002-1 (pixel^2) were counted (without edges of the counting window) to consider all fluorescent cells.

2.7 Histology

Imaging after histology was carried out either at binoculars (DFC420 C, Leica, Germany) for lower magnifications or different microscopes for higher magnification (Axiovert S100, Zeiss, Germany or confocal microscopy (TCS SP8, Leica, Germany) for visualizing the location of fluorescent dyes with higher resolution.

2.7.1 Sacrifice of birds through overdose of anesthesia and perfusion of birds for organ removal

Before sacrificing, birds were transferred to an adjoining room to prevent stress for the rest of the colony. There birds were anesthetized by isoflurane and died from inhalation of isoflurane overdose. To determine unequivocally that the bird had died before perfusion commenced, we monitored the absence of breathing, heartbeat, reflexes and muscle tonus.
Perfusion assures that blood is removed from the brain and cells. Moreover, their content is fixed quickly. The abdomen of sacrificed birds was opened with surgical scissors to get access to the heart. First a small incision into the right atrium was made to allow blood to leave the vascular system. Immediately following a total of 29mL of 1xPBS was pumped (Minipuls 3, Gilson, USA) via the left atrium of the heart from a 30 gauge butterfly needle (Venofix, Braun, Germany) for 7min (flow rate 19.2 rpm). Next 20mL of 4% PFA (paraformaldehyde in 0.1M PB) ran through (by a Minipuls 3 pump, Gilson, USA) the vascular system to fix organs. The required organs were then dissected and post-fixed in 4% PFA in 0.1M PB for one day. PFA was then exchanged by 1xPBS before the tissue was cut in order to perform immunohistochemistry (IHC) or other staining.

2.7.2 Immunohistochemistry

To confirm gene expression in gonad and brain, tissues were first cut into slices. Either frozen (unperfused) tissue, embedded in TissueTek (Sakura, Germany), was cut in 20µm sections on a Cryostat Type HM 560 M (Microm, Germany) or fixed (by 4% PFA in 0.1M PB) tissue was cut in 40µm sections on a vibratom VT1000 S (Leica, Germany) and attached to superfrost microscope slides (Menzel-Gläser, Thermo Scientific, USA). If not already done, tissue was fixed in 4% PFA in 0.1M PB for at least 5min. Then tissue was permeabilized by three washing steps in PBS/0.3% Triton X.

Next, slices were blocked with 1x Roti-Immunoblock (Carl Roth, Germany) in 1xPBS for a minimum time of 1h at room temperature. The incubation with the diluted first antibody (ab) (see Tab. 3) was done overnight. All antibodies were diluted in 1xPBS. The next day, slices were washed three times with PBS/0.3% Triton X before applying the diluted secondary antibody for 2h, then washed again three times with 1x PBS. All washing steps had a minimum duration of 5min. Finally, slices were stained with DAPI (Serva, Germany) for one minute before being mounting using mounting medium Shandon Immu-Mount (Epredia, Switzerland). In some slices the signal was enhanced with a biotinylated ab and streptavidin ab. In these cases, another incubation step with a third streptavidin ab was necessary (ab dilution in 1xPBS for 2h incubation and washing steps in between).
All antibodies used in this project are summarized in table 3.

2.7.3 Periodic Acid-Schiff staining

To identify PGCs in blood samples and to distinguish them from other cell types Periodic Acid-Schiff staining was used. PGCs possess many vesicles containing polysaccharides, which stain pink after PAS. Blood smears were dried on slides as preparation for Periodic Acid-Schiff staining (PAS). Then cells were fixed in a formalin-ethanol fixative solution for 1min. Afterwards, the following protocol was conducted (Tab. 9).

Table 9 Periodic Schiff acid staining.
Manufacture's standard PAS protocol; here for PGC identification

step	**time**	**solution**
Fixation	1min	fixative
Rinse	1min	tap water
Immerse	5min	Periodic acid
Rinse	1min	distilled water
Immerse	15min	Schiff's reagent
Wash	5min	tap water
Couterstain	90s	hematoxylin solution Gill no. 3
Rinse	15-30s	running tap water
air dry	as necessary	-

Finally, slides were mounted with Mowiol and checked for PGCs under a microscope.

2.8 PGC cultivation, manipulation & re-injection into recipient embryos

In order to manipulate PGCs, these cells had to be isolated (see 2.8.1). Cultivation protocol followed Gessara et al., 2021 with a previously published medium composition (Whyte et al., 2015). Manipulation was achieved by infecting PGCs by a lentiviral (see 2.8.2). Modified PGCs were later harvested for reinjection into a recipient embryo to generate transgenic founders. Before harvesting, PGCs were checked under a microscope for fluorescence, so successful transduction could be assessed (Fig. 18).

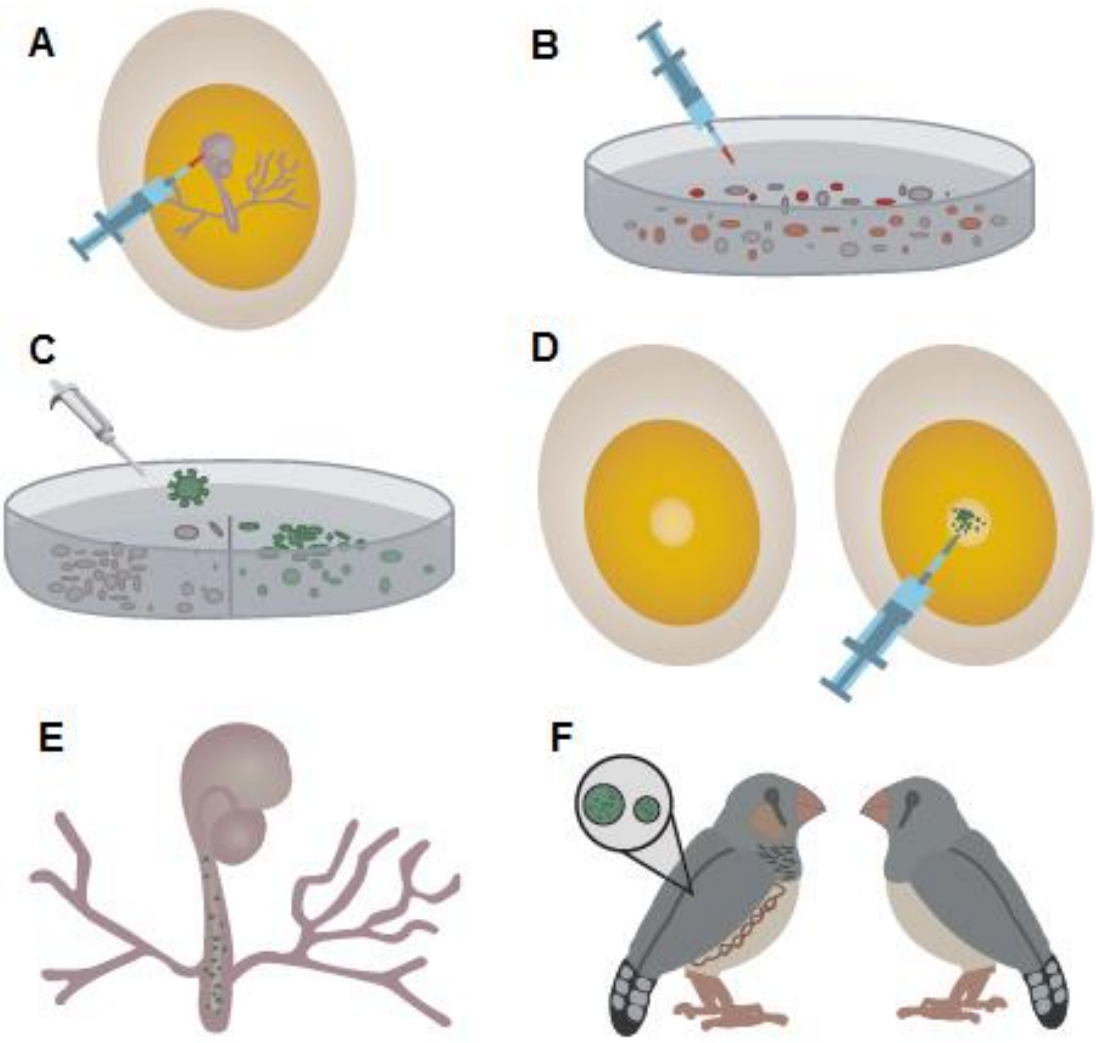

Figure 18* In vitro *manipulation of PGCs for injection into stage x embryo to generate transgenic zebra finches. Schematic workflow of the method 'manipulated PGC derived transgenesis for zebra finches'. A: blood extraction from HH14/16 embryos; B:* in vitro *cultivation of blood containing cells (including PGCs); C: PGCs built cell clumps (around 7div) and were transduced by lentiviral construct (green) at; D: injection of manipulated PGCs (green) into stage x embryo; E: migration of manipulated PGCs (green) to the gonads; F: hatched founder individual (carrier of the manipulation, see green testes) is crossed to WT bird for reproduction to obtain transgenic progeny (adopted from (Gessara et al., 2021).

2.8.1 PGC culture from embryonic blood

Zebra finch eggs were placed in an incubator (ProCon CTD7 or EHRET BSS 300) set to 38°C to develop until stage HH13-15. Under binoculars the egg was opened, the shell and the membrane were removed to get access to the embryo (Gessara et al., 2021). With pulled mircocapillaries made of glass (PCR Micropipets 1-5µL, Drummond, USA) 1-3µL of blood were extracted from the vascular system and then transferred into wells of a 48 well cell culture plate filled with 200µL culture medium. Every day 150µL medium was replaced. The duration of cultivation lasted for one to two weeks maximum as cells begin to undergo cell death. Further experiments with PGC were usually organized to happen at the maximum amount of PGCs (between 7 and 10 div). For medium composition, see 2.3.2.

2.8.2 Infection of PGCs

PGCs were infected 2 days before the date of injection (between 7 and 10 div as these cells cannot be kept for longer than one month in culture and cell death starts by 14 div). 1µL Virus was added to the cell culture medium. One day after infection medium was exchanged. Two days after infection, PGCs were checked for fluorescence under the microscope to ensure success of transduction.

2.8.3 Injection of transduced PGCs into recipient embryo

Cells were harvested, washed with 1xPBS (centrifugation at 2,500xg for 5min) and treated with papain to get single cells (Papain treatment: 200µL Papain solution for cell pellets, incubation at 37°C for 30min). Papain required preparations in advance: 4mg Papain (lyophilized, Worthington Biochemical Corporation, USA) were mixed with 2mL KO DMEM without Ca^{2+} (custom made, Gibco, Thermo Scientific, USA), incubated for 30min at 37°C to activate the enzyme and a filtration through sterile filter (0.2µm diameter, Filtropur S0.2; Sarstedt, Germany) was conducted afterwards. After centrifugation and another washing step, the cells could be re-suspended in culture medium (amount or dilution to 500 cells/µL). 1µL of the transduced PGCs were injected by microcapillaries into a stage x recipient embryo.

2.8.4 Post manipulation incubation and cross fostering

After closing the manipulation window in the shell by albumen and a patch of shell (from another egg), manipulated eggs could air dry and were then placed back into the incubator (conditions see 2.2.4). Eggs were transferred to foster parents after heartbeat was visible (around HH16) for further development. If timing allowed re-placing manipulated eggs in their origin cage (biological parents), this was preferred.

2.9 Genotyping of embryos & zebra finches

2.9.1 Collection of tissue samples from zebra finches

Developing embryos were collected on day 8 or 9 after oviposition (2/3 of embryonic development in relation to 14 days of incubation until hatching; in accordance with §14 of the German animal protection/ordinance for experimental animals no authorization required) to to genotype tissue samples. Two samples were put into Eppendorf reaction tubes and stored at -80°C until further experiments started. For examination of testis, manipulated birds were beforehand scarified as described in 2.7.1 and then gonads were dissected.

2.9.2 DNA Extraction

DNA was extracted either with Monarch Genomic DNA Purification Kit (New England BioLabs, USA) according the manufacturer's protocol or using the following protocol for DNA extraction of tissue (Phenol chloroform extraction). A tiny piece of embryonic tissue was incubated for at least 3h or overnight in 225µL DNA extraction buffer and 25µL Proteinase K for each sample shaking at 56°C and 350rpm. The next day enzymes were inactivated and proteins removed by chloroform/isopropanol precipitation by adding 250µL Phenol/Chloroform/Isoamylalcohol (25:24:1, Carl Roth, Germany) followed by centrifugation for 5min at 15,000rpm for phase separation. Supernatant (200µL) was transferred to a new tube and digested with 5µL RNaseA (10mg/mL) for 30min at 37°C to degrade RNA. Again, Phenol/Chloroform/Isoamylalcohol (25:24:1) was added -here 205µL- followed by centrifugation as described above. To enhance later visibility of DNA pellet 1µL Glycogen (20mg/mL) was added to the supernatant. Then 150µL Isopropanol and 15µL 3M sodium acetate solution (pH5.2) were added to the mixture and it was immediately vortexed. A 15 to 30min long centrifugation step followed resulting in a DNA pellet. Supernatant was discarded and pellets were washed with 1mL 70% EtOH once.

The pellets were dried in order to let traces of alcohol evaporate. DNA was solved in 50µL 1xTE (pH7.6-8.0) and concentration was measured to estimate required volumes of each sample for further experiments.

2.9.3 Spectrophotometric determination of DNA and mRNA concentration

Nucleic acids -DNA and mRNA- of all experiments for this study were measured by UV spectroscopy at wavelength 260/280nm with Nanodrop (PEQLab Nanodrop, Spectrophotometer ND-1000) to determine the concentration in every sample (input of 2µL per sample).

2.9.4 Polymerase chain reaction from genomic DNA

PCR was needed to amplify DNA fragments mostly for genotyping potential transgenic founders or their offspring. Depending on the construct of the transgene used for manipulation, specific primers were picked (Fig. 19 and 20).

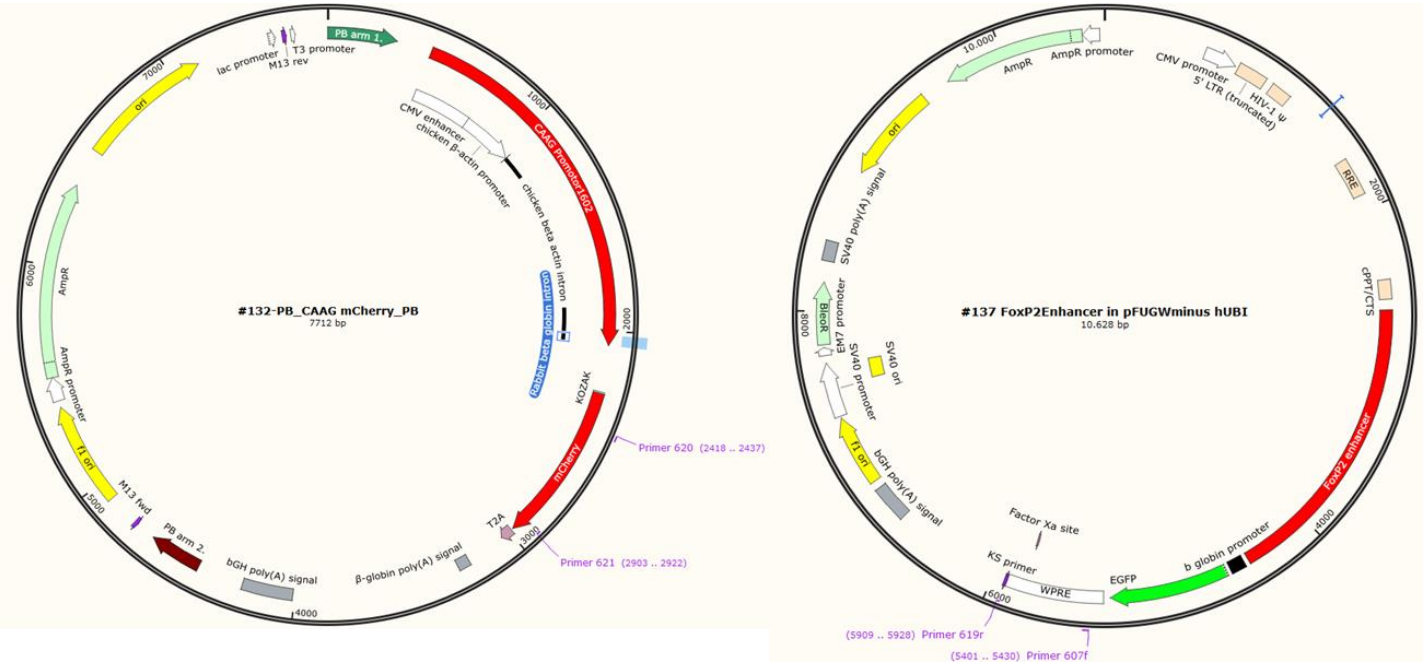

Figure 19 Plasmid map of #132 CAAG mcherry and #137 pFUGW FoxP2enhancer.
For genotyping manipulated birds where #132 (L.) or #137 (R.) should be introduced, a fragment of mcherry or EGFP was amplified using the primers 620 and 621 or 607 and 619 (made by SnapGene).

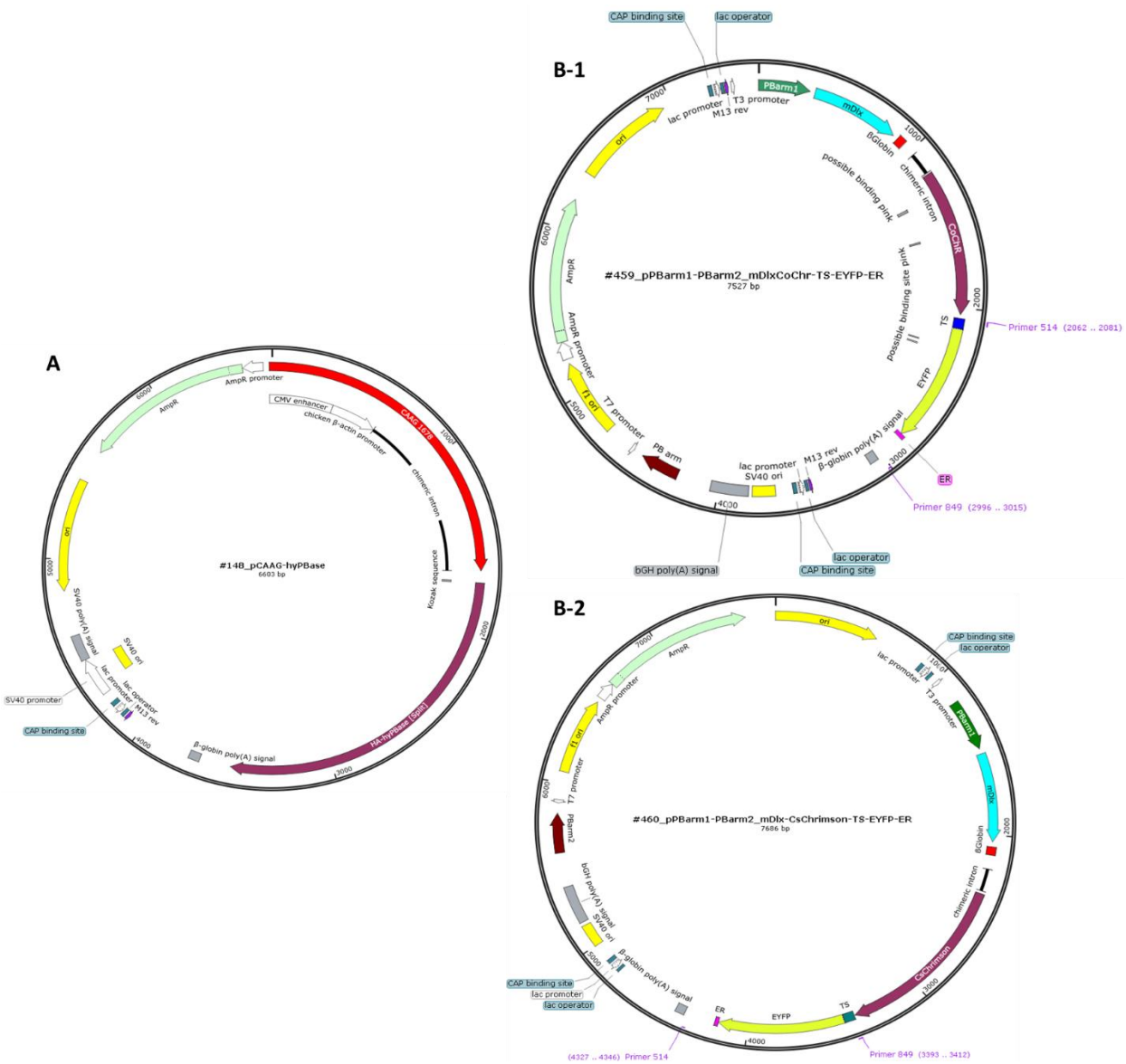

Figure 20 Plasmid maps for manipulation experiments with channelrhodopsin variants.
Two constructs were co-injected, when applying* in vivo *microinjection and electroporation for the expression of channelrhodopsin. To achieve stable integration a transposase (HypBase) was required (A) and additionally a channnelrhodopsin variant was chosen; either CoChR (B-1) or Chrimson (B-2). Genotyping was performed by detecting the shared reporter eYFP by the primers 514 and 849 (made by SnapGene).

Reactions were performed in a total volume of 25µL placed in Multiply µStrip Pro 8-strips (Sarstedt, Germany). All components of Master mixes and the corresponding thermal programs for every PCR are listed in Tab. 10.

Table 10 Different PCR protocols.
For every PCR (for the amplification of fragment from different target genes) the thermal profile and the master mix compositions are shown (A: eGFP; B: eYFP; C: CoChR; D/E: Sexing). A-C were used for genotyping of potentially transgenic birds; D/E for sex determination of zebra finches.

A

substance		**conc.**	**volume (µL)**	**final conc.**
Dream Taq buffer	incl.			
20mM $MgCl_2$		10x	2.50	1x
		20mM		2mM
For. Primer		10µM	1.00	0.4µM
Rev. Primer		10µM	1.00	0.4µM
dNTP's		je 10mM	0.50	0.2mM
Dream DNA Taq Polymerase		5U/µL	0.125	0.625U/sample
DNA (500ng)/water			19.875	500ng/sample
total volume			25.00	
step		**number of cycles**	**temp.**	**time**
initial denaturation		1x	95°C	3'
Denaturation			95°C	30''
Annealing		40x	59.8°C	30''
Elongation			72°C	30''
final elongation		1x	72°C	10'
expected product size:			528bp	

B

substance		**conc.**	**volume (µL)**	**final conc.**
Dream Taq buffer	incl.			
20mM $MgCl_2$		10x	2.50	1x
		20mM		2mM
For. Primer		10µM	1.00	0.4µM
Rev. Primer		10µM	1.00	0.4µM
dNTP's		je 10mM	0.50	0.2mM
Dream DNA Taq Polymerase		5U/µL	0.125	0.625U/sample
DNA (500ng)/water			19.875	500ng/sample
total volume			25.00	
step		**number of cycles**	**temp.**	**time**
initial denaturation		1x	95°C	3'
Denaturation			95°C	30''
Annealing		40x	64.0°C	30''
Elongation			72°C	60''
final elongation		1x	72°C	10'
expected product size:			954bp	

C

substance		conc.	volume (µL)	final conc.
Dream Taq buffer	incl.			
20mM $MgCl_2$		10x	2.50	1x
		20mM		2mM
For. Primer		10µM	1.00	0.4µM
Rev. Primer		10µM	1.00	0.4µM
dNTP's		je 10mM	0.50	0,2mM
Dream DNA Taq Polymerase		5U/µL	0.125	0.625U/sample
DNA (500ng)/water			19.875	500ng/sample
total volume			25.00	
step		**number of cycles**	**temp.**	**time**
initial denaturation		1x	95°C	3'
Denaturation			95°C	30''
Annealing		40x	65.1°C	30''
Elongation			72°C	54''
final elongation		1x	72°C	10'
expected product size:			894bp	

D

substance		conc.	volume (µL)	final conc.
Dream Taq buffer	incl.			
20mM		10x	2.50	1x
		20mM		2mM
Primer #258		100µM	0.25	1µM
Primer #259		100µM	0.125	0.5µM
Primer #380		100µM	0.125	0.5µM
dNTP's		je 10mM	0.50	0.2mM
Dream DNA Taq Polymerase		5U/µL	0.125	0.625U/sample
DNA (500ng)/water			21.375	500ng/sample
total volume			25.0	
step		**number of cycles**	**temp.**	**time**
initial denaturation		1x	95°C	3'
Denaturation			95°C	30''
Annealing		40x	52.5°C	30''
Elongation			72°C	30''
final elongation		1x	72°C	10'
expected product size:			females: 452/202bp; males: 418bp	

E			
substance	**conc.**	**volume (µL)**	**final conc.**
TDMH		4.80	1x
for. primer	10µM	0.50	0.1923µM
rev. primer	10µM	0.50	0.1923µM
Lab.-made DNA Taq Polymerase	5U/µL	0.100	1U/sample
DNA (500ng)/water		19.100	500ng/sample
total volume		25.00	
step	**number of cycles**	**temp.**	**time**
initial denaturation	1x	94°C	5'
Denaturation		94°C	30''
Annealing	46x	55°C	30''
Elongation		72°C	45''
final elongation	1x	72°C	10'
expected product size:	females: 389/355bp; males: 355bp		

All PCRs were run on T100 Thermo cyclers (BioRad, USA).

2.9.5 Agarose gel electrophoresis

Agarose gels (SeaKem LE Agarose, Lonza, Switzerland) with a concentration of 1-2% (w/v) in 1xTAE buffer were normally used for the separation of PCR products by electrophoresis. Therefore, 3.5µL of EtBr were put in 60mL of agarose gel and the mixture could cool down before pipetting samples (20µL) into the wells. If necessary 6x loading dye was added to the samples in advance. Otherwise, PCR buffer already contained a loading dye (see Material and Methods).

Electrophoresis ran at 70V for the first 10min and was then continued at 120V (BioRad PowerPac200, USA). The time of the run depended on the expected product size and the size as well as the percentage of the gel. DNA was visualized by UV illumination of the gel (biostep Argus X1 software & Dark Hood DH-40/50, Germany; Olympus Camedia C-5050 Zoom).

2.9.6 Isolation of DNA from agarose gel

Band signals of interest identified in agarose gel after PCR were cut out and then DNA was isolated by applying the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel) according to manufacturer's protocol. Finally, DNA concentration was measured (as described in 2.9.3.).

2.9.7 Cloning of PCR products

If DNA concentration of isolated PCR products was not sufficient for direct sequencing, TA cloning was conducted. First PCR product DNA (having A tail) was ligated (Tab. 11) into pGemTeasy (digested with restriction enzyme and T added) plasmid backbone and transformation into competent TOP10 cells (Tab. 12) followed.

Table 11 Ligation of PCR products into pGemTeasy.
The amount of PCR product is depending on its concentration & its length (size of plasmid divided by size of the product gives the amount in ng, which is needed for ligation).

substance	size	amount (ng)	volume (µL)
10x T4 DNA Ligase buffer			2
pGEM T_Easy	3015bp	50	1
PCR product	....bp	...	...
T4 DNA Ligase (1U/µL)			1
water			...
sum			20

Table 12 Transformation of Top10 cells.

step	time
thaw competent bacteria (50µL aliquot, stored at -80°C)	-
incubate on ice	30'
heat shock at 42°C	60''
incubate on ice	2'
add 0.25mL SOC medium without antibiotics & incubate at 37°C	60'
streak out bacteria on LB plates with antibiotics	-
incubation at 37°C	overnight

Plates containing IPTG allow for blue/white selection of grown clones. Clones of interest (recombinant clones) should be colored white as the inserted sequence disrupts the gene for ß-galactosidase of the lac operon. The enzyme is inactive and is therefore not able to cleave X-Gal, which would lead to blue-coloured clones (because of the resulting byproduct). For further cell growth, several clones were picked and placed into tubes filled with 5mL of TB medium overnight while shaking. The next day, DNA was isolated from the cell suspension applying NucleoSpin Plasmid EasyPure Kit (Macherey-Nagel) and DNA concentration was measured (as described in 2.9.3.) in order to prepare samples for sequencing.

2.9.8 Sanger Sequencing of PCR products

For successful sequencing, the required amount of DNA depended on the concentration of DNA and the PCR product size length. DNA samples were sent for Sanger sequencing to

Microsynth Seqlab (Göttingen, Germany) together with one of the primers used in the original PCR. Results were provided in form of fasta files. For analysis, the sequences were aligned to the corresponding plasmid or/and the zebra finch genome with the help of CLUSTALW (European Bioinformatics Institute) or/and Zebra finch BLAT.

2.9.9 Slot Blot

A second method for the determination of the transgene integration was conducted to confirm the results of the genotyping PCR and histology as illustrated in Fig. 21.

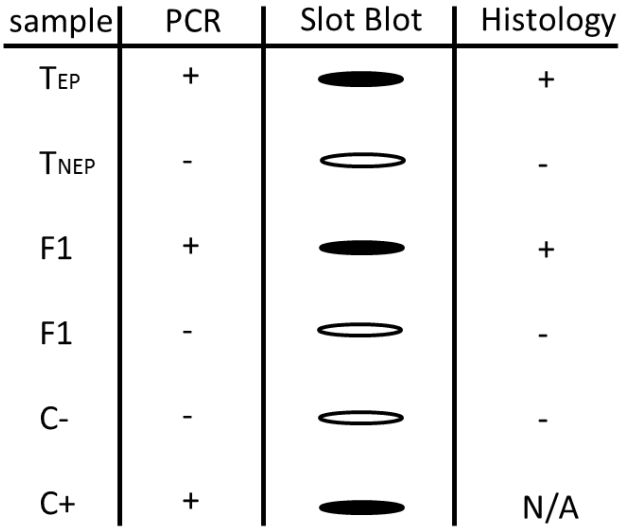

Figure 21 Expected results after* in vivo *microinjection and electroporation for different methods to detect the introduced transgene.
Transgene detection might be conducted by different methods, like PCR genotyping, Slot Blot and histology. Results of different methods should confirm findings. Expected results for different samples and methods are demonstrated here. Transgene transmission of founder individuals to the next generation is not expected to occur each time. Only a proportion of transgenic chicks (carrier of the transgene) is expected. For manipulated testes (TEP) and carrier of the transgene belonging to F1 generation of manipulated founder the amplification of the transgene, a signal in Slot Blot and fluorescence in tissue samples are expected. A positive control (C+, plasmid DNA carrying the transgene) should result in a signal in PCR and Slot Blot. For untreated testes (TNEP), non-carrier of the transgene belonging to F1 generation of manipulated founder and negative control (C-, wildtype birds) no transgene amplification, no signal in Slot Blot and no fluorescence in tissue sections should be observed. Filled ellipse: signal in Slot blot, unfilled ellipse: no signal for Slot Blot.

Applying Slot blot procedure also allowed unraveling the presence of the transgene in the genomic DNA of all individuals. To perform slot blot, genomic DNA was extracted as previously described (2.9.2). Samples were then transferred to a membrane where the transgene could be specifically detected by hybridization of the corresponding probe (complementary to the transgene).
Specific eYFP and mcherry probes were produced, and DIG labeled for later detection of the transgene in genomic DNA by Slot Blot.

The samples were prepared for transgene detection via Slot blot by denaturation: A mix consisting of 80µL DNA in elution buffer or water, 10µL 4M NaOH and 10µL 100mM EDTA (for each sample) was incubated for 10min. at 100°C. The nylon membrane (Roche 1141724001) was humidified with a.d. and placed in the slot blot apparatus PR648 (Hoefer, Austria), which has to be connected to a vacuum pump. First, the membrane was washed with 500µL per slot applying a vacuum of 13-25cmHg intensity. Then, prepared samples, 500µL 0.4 M NaOH and 500µL 2x SSC, were given into the slots one after the other and each was sucked off separately to attach the DNA to and to rinse the membrane afterwards. By applying UV light (crosslink), the membrane was dried. The hybridization of the probe was conducted overnight at hybridization temperature (for eYFP assessed to 54°C according to the GC content of the sequence). After hybridization, several washing steps were performed: two times with 200mL WP1 (40mL 20x SSC, 4mL 10%SDS, 356mL water) buffer for 5min at RT, two times with 200mL WP2 buffer (2mL 20x SSC, 4mL 10%SDS, 394mL water) heated to 68°C for 15min and once with 100mL 1xMABT + 0.3%Tween20 for 2min at RT, always while shaking. Before the detection of the probe was conducted the membrane was blocked for 30min in blocking solution (Blocking reagent, Roche 110690176001). Then, the membrane was incubated in 40mL antibody solution (1/20,000 in blocking solution) to allow antibody binding to the probe. Again, the membrane was washed twice with 100mL 1xMABT + 0.3%Tween20 for 15min at RT. The equilibration of the membrane was conducted applying 50mL NT buffer for 3min. 0.5-1mL of CPD Star solution (Roche, Switzerland) was put on the membrane for 5min and a Super RX film (#4741008389) was exposed to the membrane for 5min before film development (Hyper processor, amersham pharmacia biotech, UK).

If a second detection with a different probe (here with a plasmid carrying FoxP2; #303) was conducted, the membrane was stripped after the first detection via chemiluminescence as follows: the membrane was washed in a.d. for 1min, twice in 0.2M NaOH/0.1% SDS each time for 15min at 37°C and finally in 2xSSC for 5min. For the second detection, the protocol was re-commenced at the hybridization step.

3 Results

3.1 *In vivo* microinjection and electroporation of testes

After ensuring access to the gonads (via laparotomy), zebra finch male's testes were manipulated by microinjecting plasmids and electroporating them in order to generate genetically modified spermatogonia. In the first part of the project, fluorescent constructs were introduced, and the success of the newly established technique was confirmed by histology (see 3.1.1). Therefore, testes were embedded in TissueTek and shock frozen to -80°C to prepare cryoslicing. Slices of 20µm thickness were cut, attached to glass slides and used for microscopy and immunohistochemistry to detect introduced genetic constructs. Another prove of successful application could be reached by genotyping via PCR from tissue samples of manipulated animals (see 3.1.2). All applied parameters for the *in vivo* microinjection and electroporation of testes are described in 2.6.4 (injection volume) and 2.6.5 (voltage). These preliminary experiments were made to clarify the feasibility of the approach in zebra finches and for choosing the most efficient parameters for further experiments. Results based on the microinjection and electroporation of zebra finch males' testes are divided as follows: 1) birds carrying reporter constructs (to prove feasibility of this method for zebra finches; see 3.1.1) as illustrated in Fig. 22A - C-a) birds where constructs carried a channelrhodopsin variant and a reporter (for future functional studies; see 3.1.2) illustrated in Fig. 22C-b.

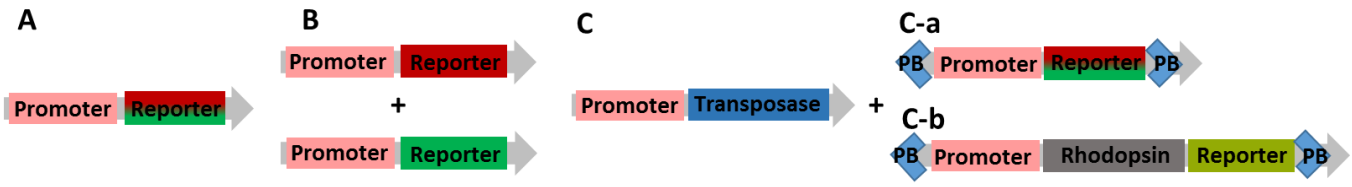

Figure 22 Schematic structure of constructs for manipulation by* in vivo *microinjection and electroporation of testes.
First, a construct carrying a strong promoter and a fluorescent reporter gene (e.g. mcherry or eGFP) is chosen to clarify feasibility of the method in zebra finches (A). Then, it was tested, if two constructs might be introduced at the same time, resulting in co-localization of different reporters (B). Later, intending to guarantee stable integration of desired genetic material into the host genome a second construct, coding for a transposase (C) is applied in combination with first a reporter construct (C-a) or later with a functional channelrhodopsin variant including an eYFP reporter (C-b). C-a and C-b constructs are flanked by PB arms to allow recognition by the transposase.

3.1.1 *In vivo* microinjection and electroporation of testes with reporter

Zebra finch males were manipulated applying the method '*In vivo* microinjection and electroporation testes' with the aim to generate transgenic songbirds. For this part, genetic

constructs (plasmids) carrying mcherry or eGFP were utilized to detect this reporter gene by histology and/or genotyping.

Access to the gonads was required for the application of this technique, as in birds these are located inside the body in contrast to mice. Therefore, a small incision between the last two ribs was needed to enter the body cavity. Then, the testis was injected by a lentiviral construct and fast green to visualize successful injection (Fig. 23).

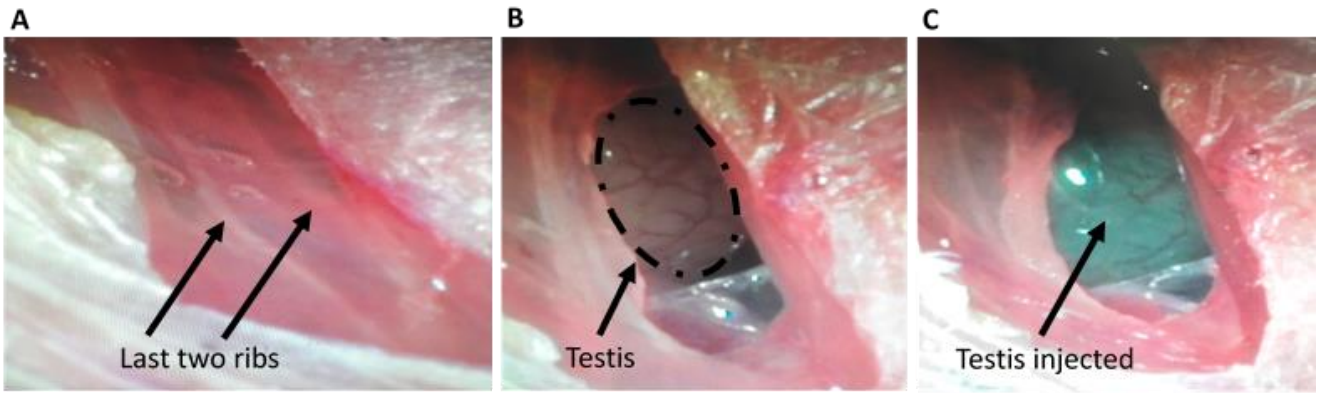

Figure 23 Laparotomy &* in vivo *microinjection of left zebra finch testis.
Site for laparotomy is demonstrated (A). Small incision was carried out between the last two ribs to get access to the left testis (B). Testis was* in vivo *microinjected with a lentiviral construct & fast green (C) prior to the following electroporation step.

3.1.1.1 Successful electroporation of testes *in vivo* applying reporter gene

Because previously this method had only been shown in mice and zebra finches have different testes dimensions than mice, I first determined the optimal injection volume and electroporation voltages for birds (Tab. 13). Males were sacrificed 2-5 days after electroporation and histological analysis showed that the lower voltages and smaller injection volumes were as efficient as larger volumes and voltages. None of the voltages resulted in tissue damage.

Table 13 Test for best parameters when manipulating zebra finch testes* in vivo *by microinjection and electroporation.

Volt	n	Vol. (µl)	damaged tissue	Fluorescence (percentage in tissue)
30	6	5	No	25.93
40	6	5	No	6.95
50	6	5	No	4.57
30	6	10	No	10.55
40	6	10	No	5.59
50	6	10	No	22.89

Reporter gene expression was only visible in the injected testis and not in the contralateral uninjected (control) testis (Fig. 24).

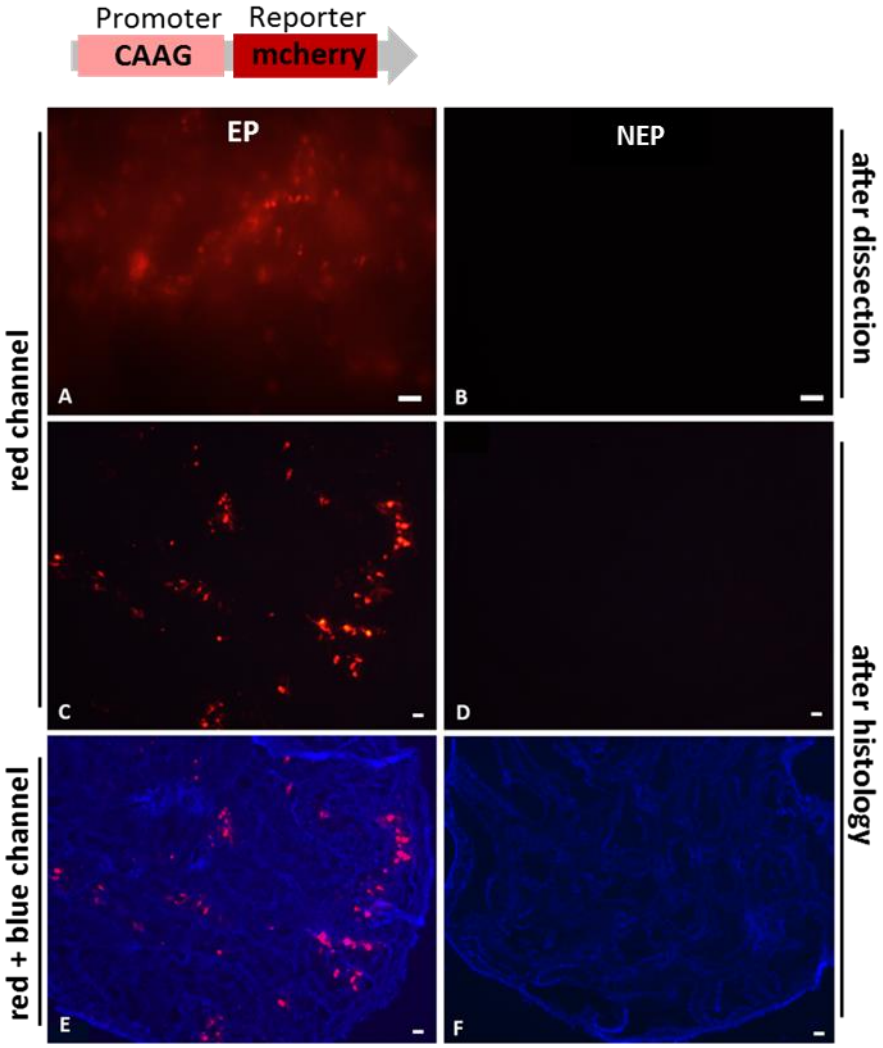

Figure 24 Fluorescence in testis shortly after* in vivo *microinjection and electroporation.
Unilateral* in vivo *microinjection with #132 (CAAG mcherry) and electroporation was applied (parameters: 30V, 8 pulses, 4 times, 5µL injection volume, 4µg/µL concentration of the construct). Only the left testis was treated (EP), and the right testis remained untreated (NEP). Testes dissection was conducted 2 days after manipulation. Left and right testis after dissection but before cryostat slicing (A/B) as well as left (C, E) and right (D, F) testes after cryostat slicing and DAPI staining: mcherry expression shown in red channel (C/D) and together with DAPI as overlay of red and blue channel (E/F)). Fluorescence is exclusively detected for the manipulated left testis. Scale bars: 100µm (A-B) and 50µm (C-F).

Results were compared to previously published mouse results as no data for songbirds were available so far. In Fig. 25, results from two published studies applying the same method in mice (Usmani et al., 2013; Michaelis et al., 2014) and the results of the recent project in a songbird species are demonstrated, suggesting that similar findings could be achieved in zebra finches. Fluorescence in treated and dissected zebra finch testis (before histology) is detected, as for Usmani et al. in 2013. Furthermore, testis shows few green cells in the outer layer of

cross sections from seminiferous tubules as shown in Usmani et al. (after tissue slicing). Higher magnification of the sections exposes similar fluorescence pattern inside the tissue as in published results from Michaelis et al., 2014.

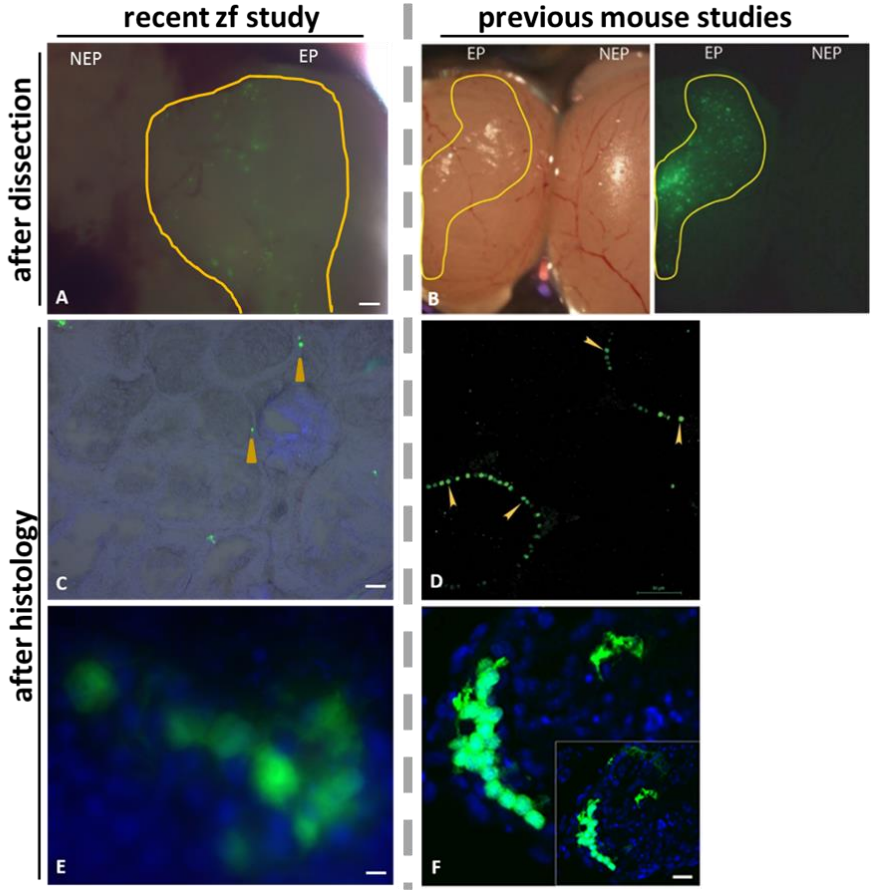

Figure 25 Comparing fluorescence of* in vivo *microinjected and electroporated testes from other publications to treated animals from the recent study.
Recent study: Left testis of was microinjected with CAAG eGFP construct (5µl) and electroporated. Zebra finch testis was fixed 2 days after manipulation (40V) and 20µm thick cross sections of seminiferous tubules were stained by DAPI (cell nuclei in blue). EGFP expression in testis is visible without any signal enhancement before and after slicing, implying successful manipulation (green dots inside the tissue; A: both testes after dissection; C/E: treated testis after slicing; scale bar: 100µm/50µm).
Testis treatment from Usmani et al., 2013: Mouse testis were fixed 50 days after manipulation (50-90V) and 4µm thick cross sections of seminiferous tubules were obtained. Green indicates successfully manipulated cells (B: dissected testes in bright field (L.) and under UV light (R.), D: treated testis after slicing and immunostaining against eGFP, scale bar: 50µm)
Testis treatment from Michaelis et al., 2014: Mouse testis was fixed 3 days after manipulation (40V) and 5µm thick cross sections of seminiferous tubules were stained by To-Pro-3 (cell nuclei in blue). Green indicates successfully manipulated cells (F; scale bar: 20µm). EP standing for electroporated and NEP for not electroporated.

Next, I tested whether two different constructs could be introduced into the testes simultaneously, resulting in co-expression (Fig. 26). This was a prerequisite for the planned experiment that require long-term expression caused by stable integration into the host genome via transposition.

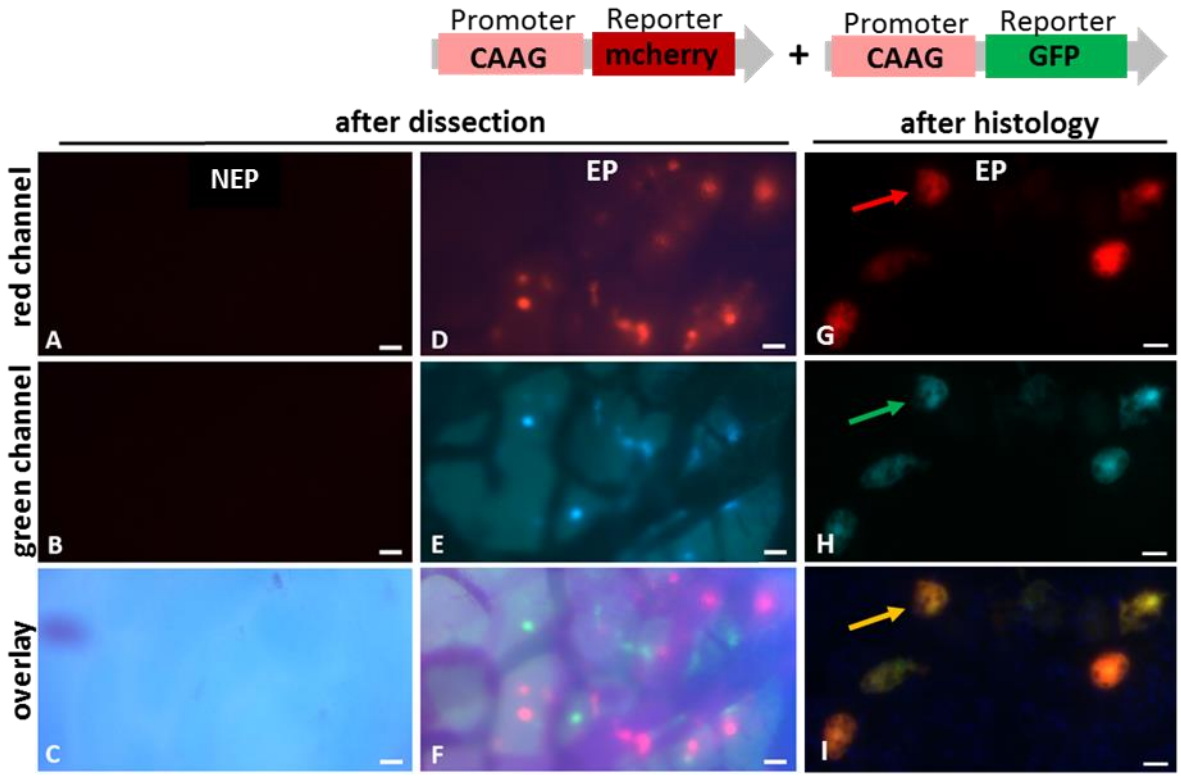

Figure 26 Testis after* in vivo *microinjection and electroporation using two constructs with different fluorescent reporters at the same time.
Unilateral* in vivo *microinjection with #132 & #316 (CAAG mcherry & CAAG eGFP) and electroporation (parameters: 50V, 8 pulses, 2 times, 5µL injection volume, 10µg/µL concentration of the construct) was applied. Only the left testis was treated, and the right testis remained untreated. Testes dissection was conducted 2 days after manipulation. Left (D-F) and right testis (A-C) after dissection and before cryostat slicing: mcherry and eGFP expression shown in red channel (A, D) and in green channel (B, E), both depicted as merged images (C, F). Left testis is additionally shown after cryostat slicing and DAPI staining: mcherry and eGFP expression shown in red or green channel (G, F) respectively and as merged images (I). Fluorescence of both reporters (mcherry and eGFP) was exclusively detected for the manipulated left testis. Co-transfection of two constructs owing different reporter genes resulted in cells expressing both (yellow cells in I). Scale bars: 100µm (A-F) and 20µm (G-I).

Since these experiments showed that the new method was feasible in zebra finches, I tested how long the expression lasted in the testis, when additionally, HypBase construct was applied. In Fig. 27, fluorescence was still detected under the microscope for the treated testis (left) and confirmed the presence of the introduced constructs more than 5 months after the manipulation event.

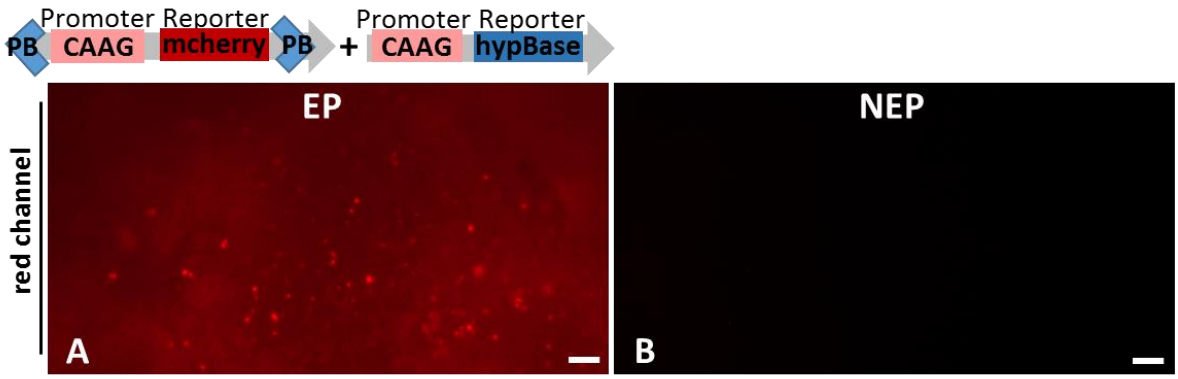

Figure 27 Long-term expression in testis after* in vivo *microinjection and electroporation.
Unilateral* in vivo *microinjection with #132 and #148 (CAAG mcherry and CAAG HypBase) and electroporation (parameters: 40V, 8 pulses, 4 times, 5µL injection volume, 10µg/µL concentration of the construct). Only the left testis was treated (EP) and the right testis (NEP) remained untreated. Testes dissection was conducted 168 days after manipulation. Left (A) and right testis (B) are shown in red channel for mcherry expression. Fluorescence of the reporter (mcherry) is still visible more than 5 months after the manipulation. Scale bar: 100µm.

To determine whether transfected testes cells resulted in mature spermatozoa that carry the transgene and are capable to release manipulated spermatids into the lumen, I examined transfected testes at higher magnification and noticed staining that is consistent with fluorescing sperm about to be released into the lumen. A bundle of stained heads (here in blue after DAPI staining) close to the lumen are characteristic for such release (Fig. 28 A). Additionally, in Fig. 28 C spermatids could be observed while releasing sperm cells into the lumen of the seminiferous tubules accompanied by red fluorescence.

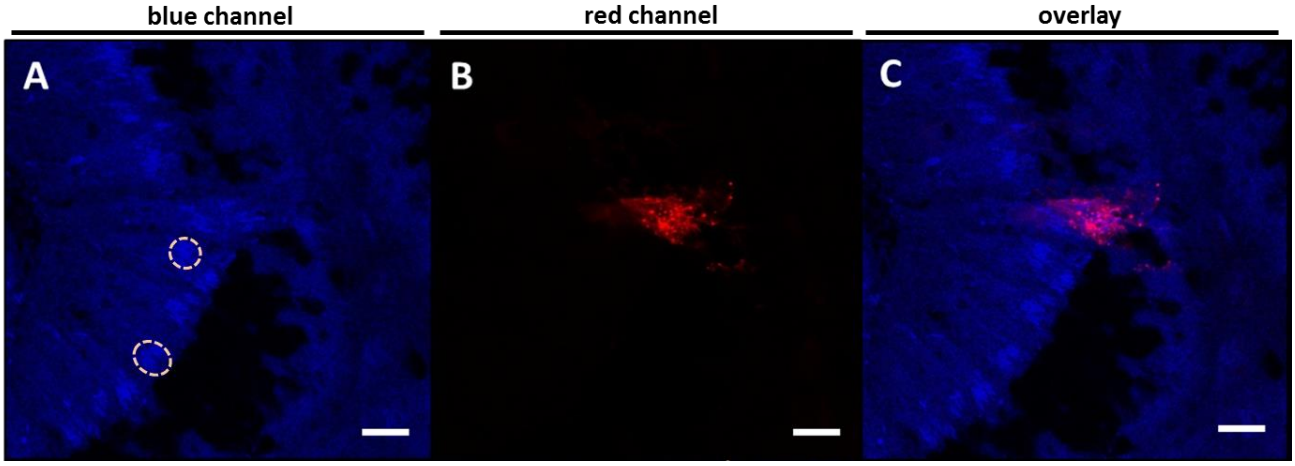

Figure 28 Immunhistochemistry of testes after* in vivo *mircroinjection and electroporation.
Unilateral* in vivo *mircroinjection and electroporation with #132 CAAG mcherry and #148 CAAG HypBase (parameters: 50V, 8 pulses, 4 times, 10µL injection volume, 4µg/µL concentration of the construct). Only the left testis was treated, and the right testis remained untreated. A: testis in blue channel (dashed line indicating sperm about to be released into the lumen); B: testis in red channel; C: as merged image. Scale bar: 100µm (A-C). Red fluorescence pointing out manipulated cells.

3.1.1.2 Transgene detection in different tissue types of males treated with reporter gene construct

Genotyping via PCR

PCR-genotyping of tissue samples from the transfected testes revealed the introduced construct/reporter gene in the transfected testes and not in the uninjected control side (i.e. mcherry; see Fig. 28).

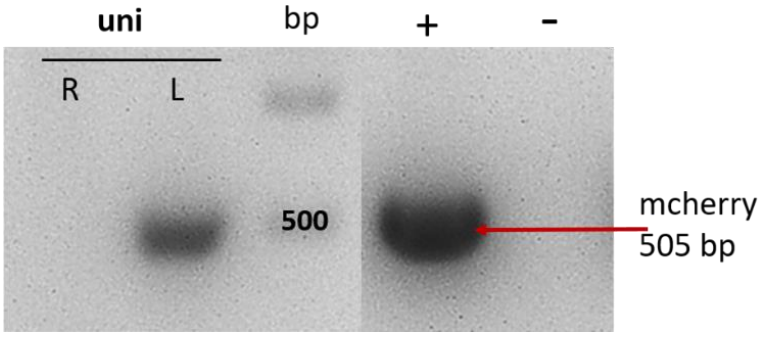

Figure 29 PCR Genotyping of testes from manipulated males.
Genotyping via mcherry PCR for testes of manipulated male (unilateral treatment; -uni-). Photograph of agarose gel after electrophoretic separation (stained by ethidium bromide). Controls are depicted as '+' for positive control (plasmid DNA #132 CAAG mcherry) and as '-' for negative control (water). For the shown samples only the right (R.) and untreated testis is genotyped negative whereas the left (L) and treated one is positive screened for the transgene (signal same seized as mcherry control).

To further determine whether the transgene could be detected in the ejaculate of manipulated males, I applied cloacal massage. Of ejaculates from two birds, one yielded in a PCR that had a signal at the same height as the control plasmid (Fig 30).

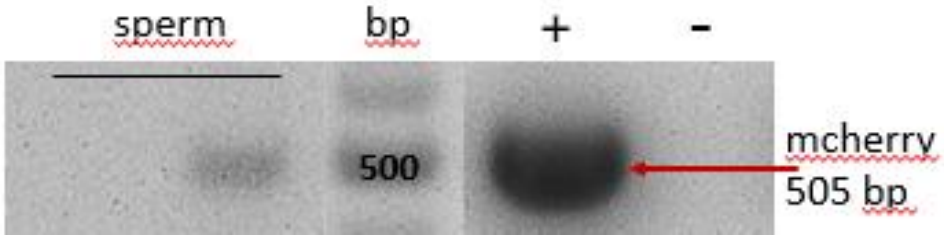

Figure 30 PCR Genotyping of sperm from manipulated males.
Genotyping via mcherry PCR for sperm samples of manipulated males (collected by cloacal massage). Photograph of agarose gel after electrophoretic separation (stained by ethidium bromide). Controls are depicted as '+' for positive control (plasmid DNA #132 CAAG mcherry) and as '-' for negative control (water). Sperm of two different males are depicted. The first one without a signal (genotyped negative) whereas the second is positive tested (signal same seized as mcherry control).

Unfortunately, cloacal massage did not reliably result in sufficient material to extract DNA from each bird (Tab. 14).

Table 14 Overview of PCR genotyping of sperm samples.
Genotyping PCR was performed on DNA samples from sperm samples using primers to detect mcherry.
N/A: no data due to insufficient amount of material

bird	times tested	PCR
1	1	-
2	1	-
3	0	N/A
4	0	N/A
5	1	+
6	0	N/A

Because our experiments showed that in principle injection and electroporation of zebra finch testes results in sperm carrying the transgene, I let manipulated males pair with WT females to screen their offspring for transmission of the transgene. To reduce the number of experimental animals, fertilized eggs were only incubated up to day 9 after oviposition. Tissue samples of embryos were dissected to extract DNA and perform genotyping PCR of the reporter from the introduced construct (i.e. mcherry). An example for positive genotyping is presented in Fig. 31 (second sample).

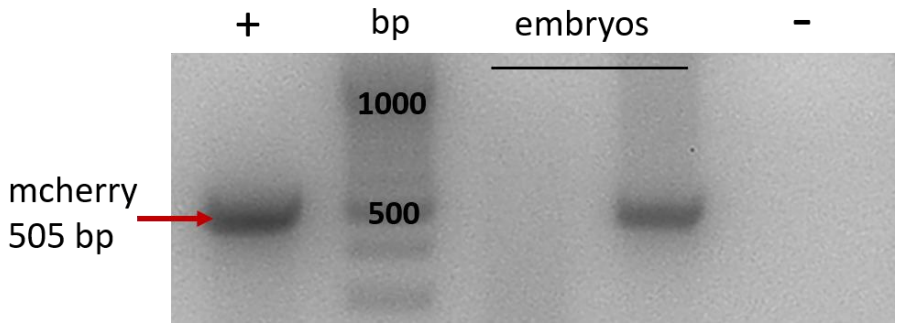

Figure 31 PCR genotyping of progeny from manipulated males.
Genotyping via mcherry PCR for embryonic tissue (samples derived from offspring of manipulated males). Photograph of agarose gel after electrophoretic separation (stained by ethidium bromide). Controls are depicted as '+' for positive control (plasmid DNA #132 CAAG mcherry) and as '-' for negative control (water). Two different samples from embryonic tissue are shown; the first one genotyped negative and the second one positive (signal same seized as mcherry control).

These results lead to the assumption that the method '*In vivo* microinjection and electroporation of testes' works in zebra finches and is a promising approach for the generation of transgenic songbirds. Therefore, further experiments concentrated on introducing functional constructs instead of only reporter genes.

In sections of all animals, manipulated with reporter constructs only, fluorescence was detected under the microscope (see Tab. 13). Some of these animals were later additionally screened for the transgene by PCR or Slot Blot (Tab. 15). Three out of five animals were PCR positive, whereas for Slot Blot only one of 13 was screened negative for the transgene. Among

them two received both genotyping variants as the amount of DNA was sufficient to perform PCR and Slot Blot. In one animal both results were negative, but for the other sample PCR showed no signal and Slot Blot did.

Table 15 Overview about genotyping results of males' testes manipulated by reporter constructs.

genotyped by PCR			genotyped by Slot Blot		
total	positive	negative	total	positive	negative
5	3	2	13	12	1

3.1.2 *In vivo* microinjection and electroporation of testes with channelrhodopsin constructs

Given the promising results for the previous experiments, I used the mDLX-channelrhodopsin plasmids that – if transgenesis was successful – would be expected to only be expressed in the interneurons of nucleus HVC. This would then allow optogenetic experiments to further assess the role of these neurons in song learning by the collaboration partners (Vallentin et al., 2016). Potential founder males were named by a two-letter code (e.g. AT) for better differentiation. At the beginning, offspring of potential founders should be tested during their embryonic phase to reduce the number of experimental animals. Later, if positive screened offspring was detected, hatching could have been permitted to raise living offspring that would be carrier of the transgene. When PCR-genotyping of these offspring was not as reliable as expected, I increasingly also sacrificed o sacrificed the founders themselves and checked for presence of the transgene in the testes.

3.1.2.1 Transgene detection in different tissue types of males treated with channelrhodopsin constructs

PCR genotyping of embryonic tissue from progeny of manipulated males revealed positive signal. I examined different clutches of potential founder males to check if transmission was restricted to the first clutch or occurred in later clutches as well (Fig. 32).

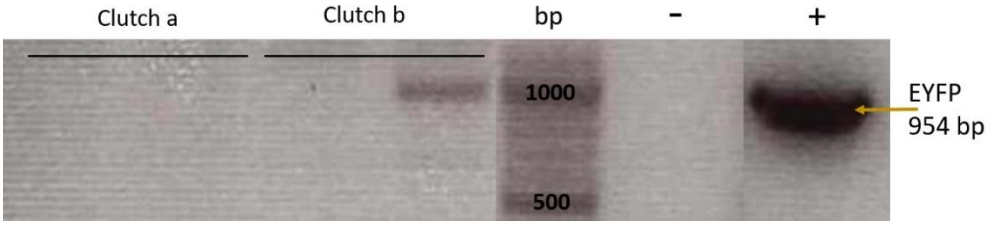

Figure 32 Genotyping via PCR from genomic DNA of embryonic tissue samples.
Genotyping via eYFP PCR from genomic DNA. All embryos of manipulated males were screened for the transgene. Tissue samples were dissected until day 9 of egg development to isolate DNA. PCR was performed on DNA samples using primers to detect eYFP. Photograph of agarose gel after electrophoretic separation (stained by ethidium bromide). Controls are depicted as '+' for positive control (plasmid DNA #459) and as '-' for negative control (water). Here, birds from different ancestries were tested. Individuals from the same clutch are connected by lines. Not every clutch yielded in transgenic hatchlings (clutch a) and in others some individuals carried the transgene (clutch b).

An overview about all PCR based genotyping results of embryonic descendants from manipulated males is illustrated in Fig. 33. Not only the first clutches of potential founders yielded in embryos, which seem to carry the transgene, but later clutches of the same ancestry resulted also in individuals, which were genotyped positive (by PCR). The ratio of positive genotyped progeny varies across clutches. According to these results, for the manipulated males the transmission ranged from 13.3 to 38.7% (AU: 19/49, AQ: 3/15, AT: 8/21).

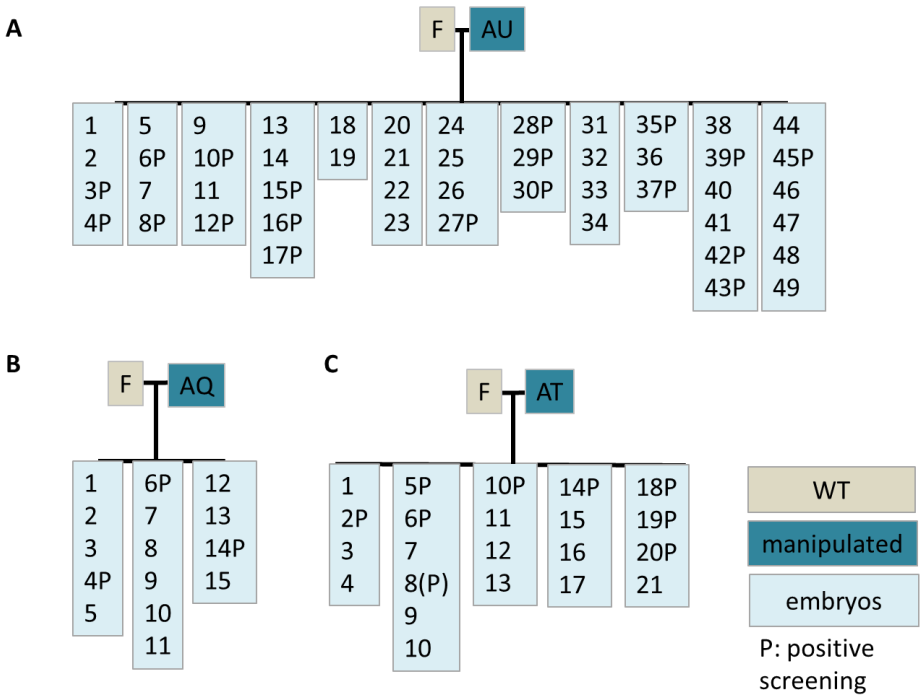

Figure 33 Overview about genotyping of clutches from manipulated zebra finch males using* in vivo *mircroinjection and electroporation of testes.
Males (M) were* in vivo *mircroinjected and electroporated (A: AU, B: AQ, C: AT). For reproduction, males were crossed to WT females (F). Shown are genotyping results (PCR) of embryonic samples from several clutches of different potential founders. Positive screening for the transgene is marked by a 'P'. One box stands for one clutch. Grey indicates Wildtype birds, dark blue manipulated males and middle blue embryonic progeny.

These results are additionally summarized in Tab. 16 and transgene transmission rate is determined for the first generation. Transmission ratio ranges from 0 to 31.2% depending on the potential founder male (mean for birds with CoChR construct is 31.2% and for Chrimson construct 16.0%.

Table 16 Overview of PCR genotyping results from embryos of manipulated males.
Genotyping PCR was performed on DNA samples from embryonic tissue samples (up to 9 days after oviposition) using primers to detect eYFP. (In brackets the number of positive screening events is shown, including very weak signals, which were not counted for further calculations.)

		embryonic progeny		
male	construct	total no.	positive	percentage
AT	CoChR	21	7(8)	33.3
AS	CoChR	0	0	-
AU	CoChR	49	19	38.7
AV	CoChR	8	0(2)	-
AY	CoChR	5	0	-
AZ	CoChR	10	0	-
total	**CoChR**	**94**	**22**	**31.2**
AO	Chrimson	14	1	7.1
AR	Chrimson	4	1	25.0
AP	Chrimson	0	0	-
AQ	Chrimson	15	2(3)	13.3
AW	Chrimson	10	2	20.0
AX	Chrimson	7	1(2)	14.3
total	**Chrimson**	**50**	**7**	**16.0**

PCR genotyping of sperm showed signal for manipulated male itself (AQ) (Fig. 34).

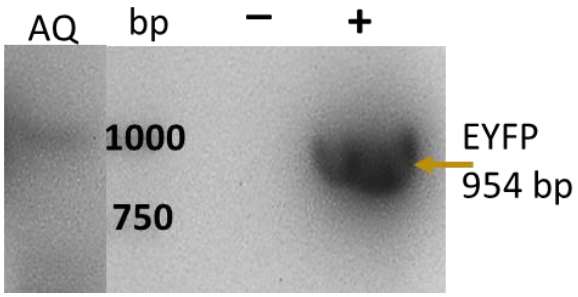

Figure 34 PCR genotyping from sperm samples.
Genotyping via eYFP PCR for sperm samples (collected by cloacal massage) of manipulated male (AQ; carrying mDLX Chrimson). Photograph of agarose gel after electrophoretic separation (stained by ethidium bromide). Controls are depicted as '+' for positive control (plasmid DNA #459) and as '-' for negative control (water). The tested ejaculated showed a positive signal (same seized as eYFP control).

All genotyping results for ejaculates of manipulated males are summarized in Tab. 17. Only two manipulated males showed a positive screening (AU, AQ). Several of their tested offspring (embryonic tissue samples) was genotyped positive, too (Tab. 16 and Fig. 33). For other the

potential founders the presence of the transgene in a sperm sample could not be detected, although some of their embryos had positive PCR results after genotyping (see AT, AU, AO, AR, AX, and AW, Tab. 16). Some birds were tested several times: However, screening was not positive each time (e.g. 3 out of 4 tests were transgene positive in the case of AQ; i.e. 75.0%).

Table 17 Overview of PCR genotyping results from sperm samples of manipulated males.
Genotyping PCR was performed on DNA samples from sperm samples using primers to detect eYFP.

construct	male/ancestry	times tested	positive	%
CoChR	AT	4	0	-
CoChR	AV	1	0	-
CoChR	AY	1	0	-
CoChR	AZ	4	0	-
CoChR	AU	2	1	50.0
Chrimson	AQ	4	3	75.0
Chrimson	AO	2	0	-
Chrimson	AW	3	0	-

Some manipulated males were sacrificed after reproduction to examine their gonads. In Fig. 35 genotyping of testes from manipulated male AU itself is shown. In both samples the transgene was detected by PCR genotyping.

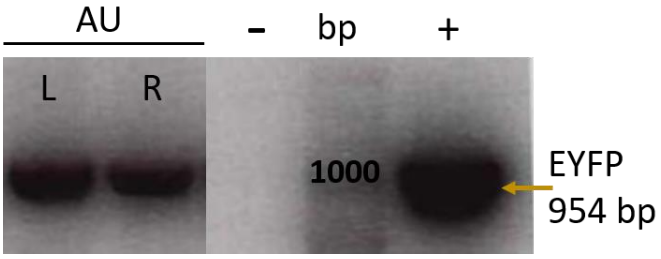

Figure 35 PCR Genotyping of testes from manipulated male.
Genotyping via eYFP PCR for testes of manipulated male AU (bilateral). Photograph of agarose gel after electrophoretic separation (stained by ethidium bromide). Controls are depicted as '+' for positive control (plasmid DNA #459) and as '-' for negative control (water). Both treated testes were genotyped positive (signal same seized as control).

Finally, Tab. 18 gives an overview about all PCR genotyping results of manipulated males; including sample types (testis and/or sperm). For three birds, PCR genotyping was positive in the testis and one of them had additionally a signal in the Slot Blot (AS). The transgene was also detected in sperm of two birds. Unfortunately, it was often not sufficient material to conduct both genotyping approaches.

Table 18 Overview about PCR genotyping results of different sample materials from males manipulated by* in vivo *microinjection and electroporation of testes (CoChR or Chrimson).

male	construct	testis	sperm
AT	CoChR	N/A	-
AS	CoChR	+	N/A
AU	CoChR	+	+
AV	CoChR	N/A	-
AY	CoChR	N/A	-
AZ	CoChR	N/A	-
AO	Chrimson	N/A	-
AP	Chrimson	N/A	N/A
AR	Chrimson	+	N/A
AQ	Chrimson	N/A	+
AW	Chrimson	N/A	-
AX	Chrimson	N/A	N/A

3.1.2.2 Confirming PCR genotyping by sequencing

After PCR genotyping PCR products of embryos were sent for sequencing. Sequences of four descendants could be generated and aligned to a transgene-specific sequence i.e. eYFP (Fig. 36 and Tab. 19).

```
AT       -----------ATCAACGTGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCA  49
eYFP     ACCAGATCGACATCAACGTGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCA  60
                    **************************************************

AT       TCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCG  109
eYFP     TCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCG  120
         ************************************************************

AT       AGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGC  169
eYFP     AGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGC  180
         ************************************************************

AT       CCGTGCCCTGGCCCACCCTCGTGACCACCTTCGGCTACGGCCTGCAGTGCTTCGCCCGCT  229
eYFP     CCGTGCCCTGGCCCACCCTCGTGACCACCTTCGGCTACGGCCTGCAGTGCTTCGCCCGCT  240
         ************************************************************

AT       ACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCC  289
eYFP     ACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCC  300
         ************************************************************

AT       AGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGT  349
eYFP     AGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGT  360
         ************************************************************

AT       TCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACG  409
eYFP     TCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACG  420
         ************************************************************

AT       GCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGG  469
eYFP     GCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGG  480
         ************************************************************

AT       CCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACG  529
eYFP     CCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACG  540
         ************************************************************

AT       GCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGC  589
eYFP     GCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGC  600
         ************************************************************

AT       TGCTGCCCGACAACCACTACCTGAGCTACCAGTCCGCCCTGAGCAAAGACCCCAACGAGA  649
eYFP     TGCTGCCCGACAACCACTACCTGAGCTACCAGTCCGCCCTGAGCAAAGACCCCAACGAGA  660
         ************************************************************

AT       AGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGG  709
eYFP     AGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGG  720
         ************************************************************

AT       ACRAGCTGTACAAGTTCTGCTACGAGAACGAGGTGTAAGTTTAAACGATAAACGCGGCCG  769
eYFP     ACGAGCTGTACAAGTTCTGCTACGAGAACGAGGTGTAAGTTT------------------  762
         ** ***************************************

AT       CCAATTCACTCCTCAGGTGCAGGCTGCCTATCAGAAGGTGGTGGCTGGTGTGGCCAATGC  829
eYFP     ------------------------------------------------------------  762
```

Figure 36 Sequence alignment of AT offspring (embryo) to eYFP template sequence.
PCR product (tested positive in genotyping PCR) of AT offspring was purified and sequenced (Sanger method). Alignment of the sequenced PCR product (here named 'AT') and the template sequence (here named 'eYFP') was generated by CLUSTALW. Asterisks indicate the same base at a particular position. Thus, the identity of the sequences is very high.'-' means that there is no sequence of the test sample which could be aligned, and spaces stand for base differences between the sequences.

A summary of all sequencing results of samples, which were already genotyped positive in an eYFP genotyping PCR, is displayed in Tab. 19. All obtained sequences could be aligned to the template sequence of eYFP, noticing that several individuals being descendants of the same manipulated male (AU) and individuals from different potential founders were tested. Unfortunately, it was not feasible to extract an appropriate amount of DNA from a PCR product of sperm samples from manipulated birds, so no sequencing data for an ejaculate was available.

Table 19 Overview of sequence alignments to template after eYFP genotyping PCR.
Samples were screened by the transgene via eYFP genotyping PCR, PCR products were cut out, purified and sequenced (Sanger sequencing). Afterwards, resulting sequences were aligned to the template plasmid carrying eYFP (#459) to check the consensus sequence. All samples with symbol 'L' are embryonic tissue derived and 'S' stands for DNA from sperm. No results could be generated for samples without sufficient DNA amount (N/A). '+' confirming the alignment of the sample to the query sequence. In brackets the information about the father is given.

sample ID	ancestry	aligned to EYFP	length of align. (bp)
L1576	AT	+	751
L1373	AU	+	666
L1405	AU	N/A	N/A
L1407	AU	+	843
L1408	AU	+	564
S179	AU	N/A	N/A

3.1.2.3 Alternative transgene detection

With Slot Blot, a second method for the detection of the transgene in manipulated birds or their offspring was conducted and should confirm the results of the genotyping PCR and histology as illustrated in Fig. 21 (see 2.9.9).
An example for Slot Blot is demonstrated in Fig. 37. Signals for the control plasmids (mcherry carrying plasmid detected by mcherry probe) of different concentration are visible, so the detection of specific DNA sequences by a complementary probe via Slot blot was achieved. Slot blot on different sample types was performed (testis, embryonic tissue). The presence of the transgene could be detected for treated testes (lack of signal for unmanipulated testis).

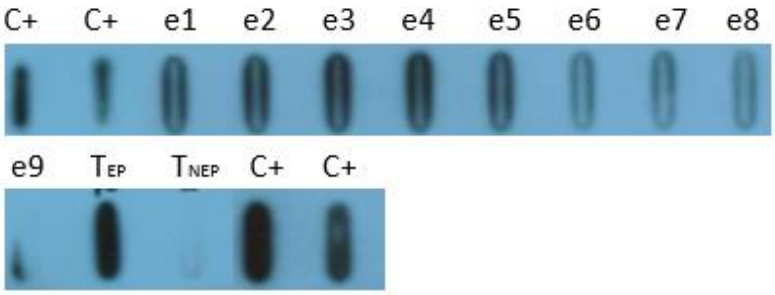

Figure 37 Determination of transgene integration by slot blot analysis of manipulated males (by* in vivo *microinjection and electroporation of testes) and their offspring.
Males were* in vivo*, electroporated and crossed to WT females after approx. one month for reproduction. To extract genomic DNA, embryonic tissue samples were collected for each individual of all potential founders, whereas testes were used for manipulated males themselves. Slot Blot was performed to detect the presence of the transgene. Controls are marked as 'C' and samples either embryo or testis derived as 'e'/'T'. mcherry could be detected in corresponding controls: C+ (mcherry; left 100pg and right 10pg). Samples from embryonic tissue (e1-e9) and untreated testis (TNEP) did not show any signal, whereas one testis of a manipulated male (TEP) did.

In Tab. 20 all Slot blot results for manipulated males and their offspring are summarized. The testes of four manipulated males were examined and three of them did show a signal in the slot blot, meaning the detection of the transgene. Just few experimental animals were checked by this method, as sometimes no more tissue was left after PCR genotyping and some were not sacrificed yet, because no further offspring production would be possible. Some of the generated progeny was screened with this approach but did not result in any positive signal. Apparently, AT could not transmit the transgene as he himself had no signal in the Slot blot genotyping. So, results of AT (all Slot Blot negative) progeny is plausible. However, the opposite is not true for AU. Although in his testes the transgene was detected, embryonic offspring was never screened positive.

Table 20 Overview of Slot Blot results from* in vivo *microinjected and electroporated males and their progeny.
Males were* in vivo *microinjected (with either a construct for Chrimson or CoChR), electroporated and crossed to WT females after approx. one month for reproduction. To extract DNA, embryonic tissue samples were collected for embryonic offspring or of all potential founders, whereas testes were used for manipulated males themselves. Slot Blot was performed to detect the presence of the transgene. Results are marked by '+' for positive and by '-' for negative screening. All examined testes were genotyped positive by Slot blot, but no positive signal was obtained in the filial generation.

male	construct	testes	no. embryos	result
AT	CoChR	-	12	-
AS	CoChR	N/A	N/A	N/A
AU	CoChR	+	29	-
AV	CoChR	N/A	2	-
AY	CoChR	+	N/A	N/A
AZ	CoChR	+	N/A	N/A
AO	Chrimson	N/A	1	-
AP	Chrimson	N/A	1	-
AR	Chrimson	N/A	N/A	N/A
AQ	Chrimson	N/A	8	-
AW	Chrimson	N/A	2	-
AX	Chrimson	N/A	1	-

3.2 *In vitro* manipulation of PGCs for generating transgenic zebra finches

In order to generate transgenic songbirds by the manipulation of PGC. This cell type was extracted from zebra finch embryos, cultured to multiply them and manipulated (by viral transduction) *in vitro* before harvesting and re-injecting them into recipient embryos as described in 2.8.

3.2.1 PGC cultivation and identification

Blood was extracted from HH13-15 embryos an cultured in well plates containing special culture medium (Whyte et al., 2015). The cells were incubated for propagation of target cells, PGCs, and for later manipulation (around 7 to 10 div). In Fig. 38 extracted blood cells and cultured PGC are represented. Directly after extraction, the distinction of PGCs from other blood cells is difficult as they are occurring occasionally (Fig. 38 A). The discrimination might be done by size or their characteristic morphology (polysaccharide containing vesicles and cellular protrusions). After several div, PGCs form grape shaped cell clumps, which are easily recognized indicating their cell growth (Fig. 38 B/C). Periodic Schiff acid staining served as method for identification of PGCs. Because of their huge amount of polysaccharide containing vesicles, they display a very specific staining contrary to other cell types. Fig. 38 D points out PGCs after periodic Schiff acid staining, which facilitates recognition of their morphologic characteristics. Additionally, immunohistochemistry was conducted to identify PGCs as they express several pluripotency and stem cell markers. In Fig. 38 F, immunostaining against SSEA-1 -a surface marker for PGCs- is displayed. SSEA-1 is a carbohydrate epitope, which is expressed by avian PGCs during embryonic development, when migration to the gonads happens (D'Costa and Petitte, 1999; Jung et al., 2019). In Fig. 38 E DAPI staining of the same cells is demonstrated. Only one of the two presented cells show a signal in the red channel (see arrows in Fig. 38 E & F).

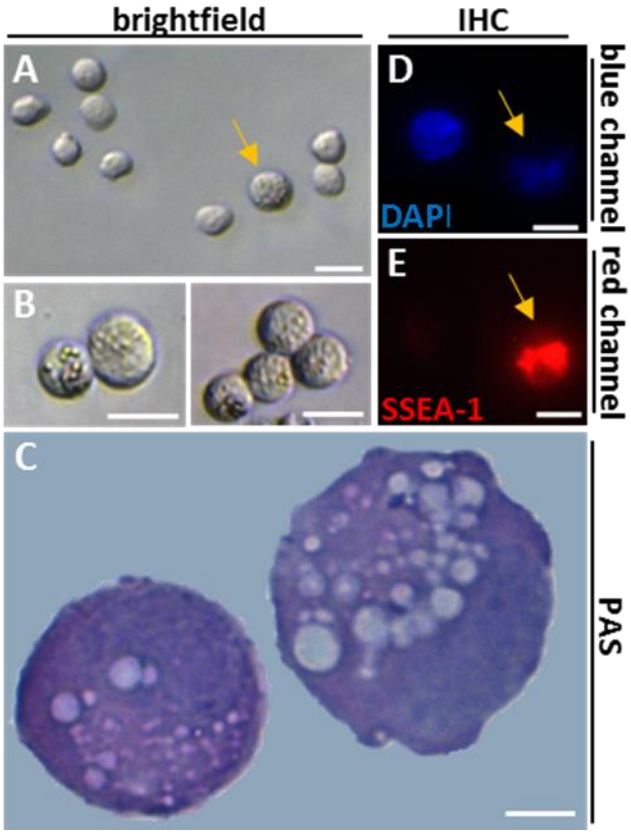

Figure 38 Characterization of zebra finch PGCs.
A: Blood cells directly after extraction (yellow arrow points at PGCs) and B/C: Accumulation (grape-shaped) of PGCs at 3 div; scale bars: 20µm. D: PGCs after PAS staining; scale bar: 5µm. E/F: To characterize the extracted blood cells immunohistochemistry was performed. IHC was conducted against SSEA-1, a specific marker for PGCs, (primary antibody), followed by Alexa 568 (secondary antibody, in red channel; F) to identify PGCs (yellow arrow) and cell nuclei were visualized by DAPI staining (in blue; E); scale bar: 50µm. Two cells are visible, but only the right one expresses the marker, indicating the PGC cell type.

3.2.2 Infection of PGCs by lentivirus

After 7 to 10 div, PGCs were infected by adding lentivirus (either a virus carrying a CAG promoter and a GFP reporter or virus carrying an mDLX enhancer and the channelrhodopsin variant, CoChR, as well as the reporter) to the cell culture medium. 48h after transduction, cells were checked under the microscope for green fluorescence. Fluorescent cells were proving the success of infectivity of the virus and the expression of the introduced reporter gene (Fig. 39 A-C). Additionally, immunostaining was performed on infected cells to verify the correct cell type (Fig. 39 D-I). Infected cells did also display the PGC marker SSEA-1.

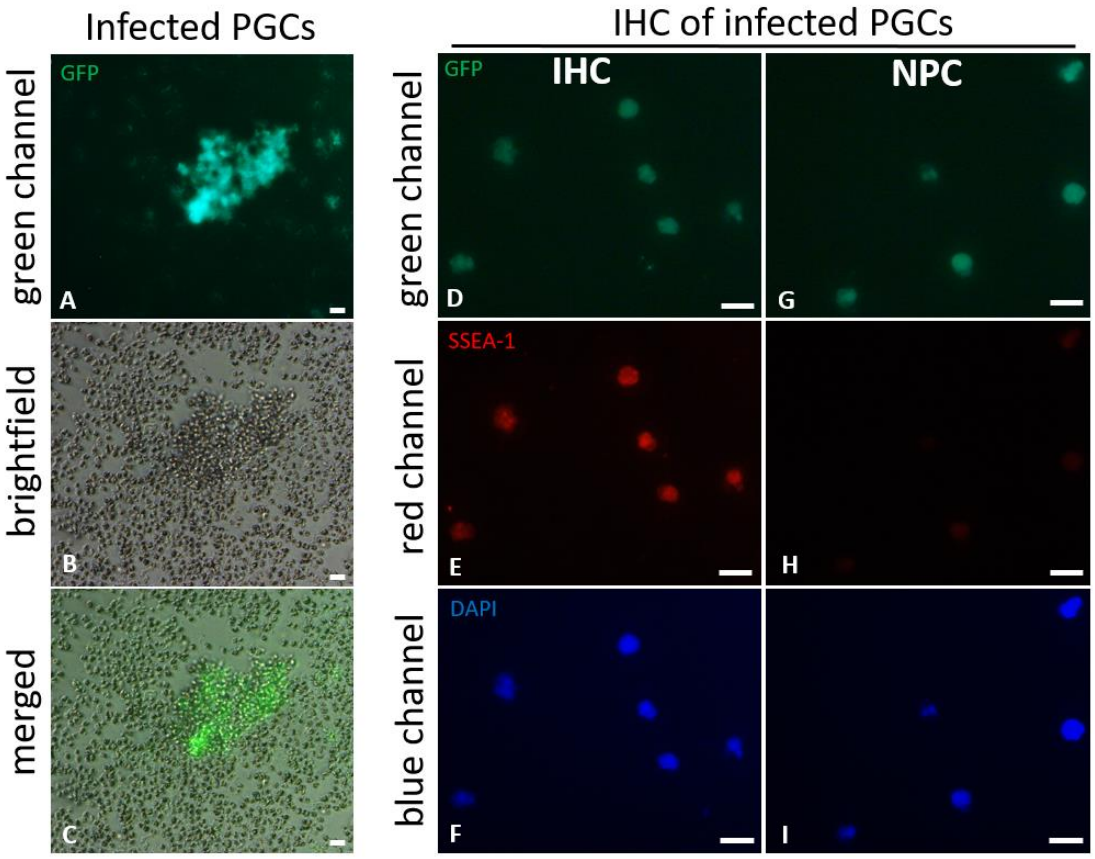

Figure 39 GFP expressing PGCs after lentiviral transduction.
L.: PGCs were cultured and lentivirus 1161b was added 7 div. Microscopy was conducted 2 days after infection. PGCs are shown in green channel (A), in brightfield (B) and both channels merged (C); scale bar: 20µm. R.: Immunostaining against SSEA-1 was applied to detect PGCs, infected by lentivirus 1161b carrying GFP. Treatment of cells like described above. Primary Ab: anti SSEA-1 and secondary Ab: Alexa 568; a no primary antibody control (NPC) was performed to ensure binding specificity additionally. D/G: green channel; E/H: red channel; F/I: blue channel (DAPI); scale bar: 10µm. Fluorescent reporter of the viral construct was visualized in treated cells after microscopy and GFP expressing cells did also express a PGC specific marker (SSEA-1) indicating that the target cells were infected.

3.2.3 Injection of manipulated PGCs into embryos

Successfully transduced PGCs could be harvested, eventually treated with papain to obtain single cells and used for injection into recipient embryos. These embryos were incubated until reaching 2/3 of embryonic development, dissected to collect tissue samples for later genotyping (see 3.2.4).

Development of manipulated embryos

In Fig. 40 the development of injected stage x embryos (injecting virus infected PGCs) are summarized. In Fig. 40 A all treated eggs (n=385) are combined. The total amount of eggs with development (217/385; 56.4%) are illustrated (left-hand). In B and C eggs were plotted construct wise, so 205 eggs for the GFP group and 180 for the CoChR group are shown. Development could be observed for 58.5% (120/205) in case of the GFP and 53.9% (97/180)

for the CoChR construct. The development process was also followed on a daily basis (see Fig. 40 A right-hand for all injected eggs and for GFP or CoChR in C). Eggs, which did not develop after manipulation or which stopped developing, were removed from the incubator and opened to confirm the lack of an embryo or to examine the egg for any obvious developmental failure. Most eggs, that were not alive at days 9 of embryonic development, did not develop at all. In fewer cases the embryonic development in eggs was interrupted.

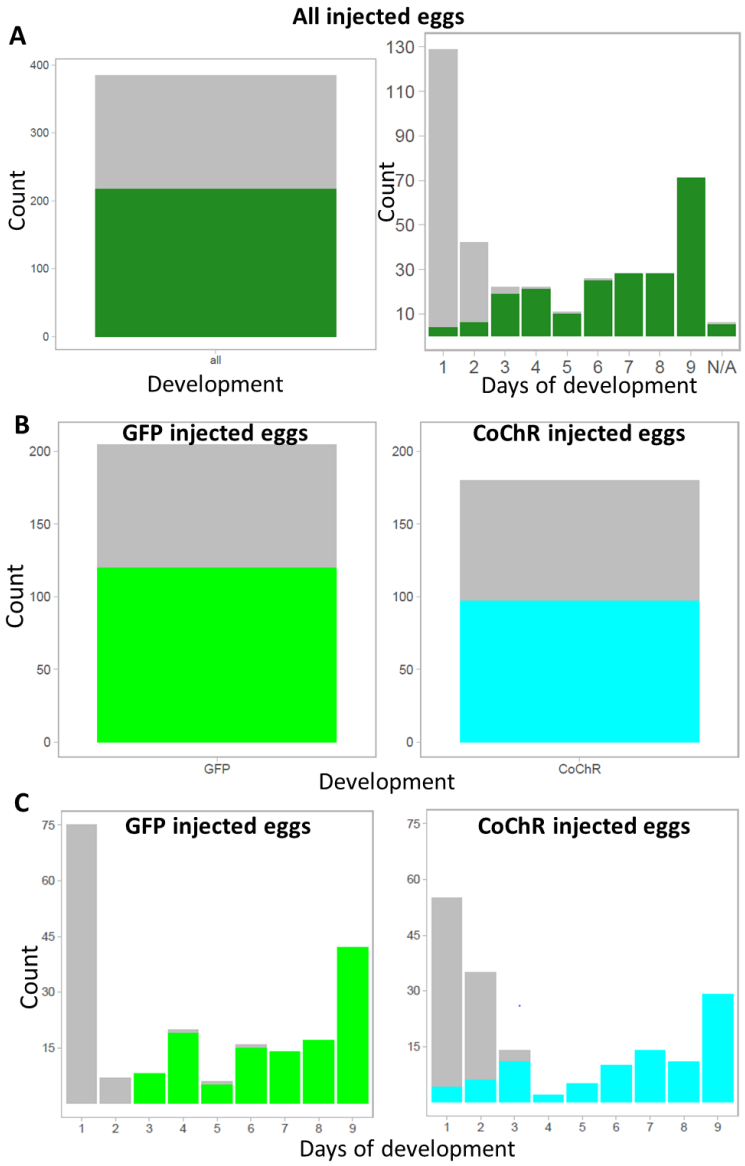

Figure 40 Development of embryos after* in ovo *injection of virus transduced PGCs.
Zebra finch WT embryos of stage x were injected with modified PGCs, afterwards sealed and incubated. PGCs were beforehand collected from blood of WT embryos (stage HH 13-15),* in vitro *cultured (for approx. 10 div), infected by different viral constructs (lentivirus either carrying GFP or CoChR). Egg development is shown by color: grey standing for no visible development, and light colored for development of embryos. All injected eggs are shown in A (independent from construct), whereas in B and C eggs are split according to the applied viral construct, GFP (left/green) and CoChR (right/cyan). In B total numbers are depicted and in C development is documented on a daily basis. In C 5 values of CoChR could not be considered as exact day of development could not be assigned.

Failure of embryonic development after in ovo *manipulation*

There are many reasons why an unmanipulated embryo or an injected embryo might not develop. In the cases, in which it was possible to observe potential reasons for the stop of embryonic development, this was documented to discover the potential weaknesses of the procedure and of course for future improvements (Fig. 41). Four observations could be made, when of embryonic development failed after manipulation: air bubble(s) in the egg, a damaged eggshell, embryos, which were attached to the shell inside, and harmed yolk. The last one was the most often to cause the stop of embryonic development. Only in 45 out 385 cases (17.3%) a reason could be documented, so the main part of failure for injected eggs remained unclear.

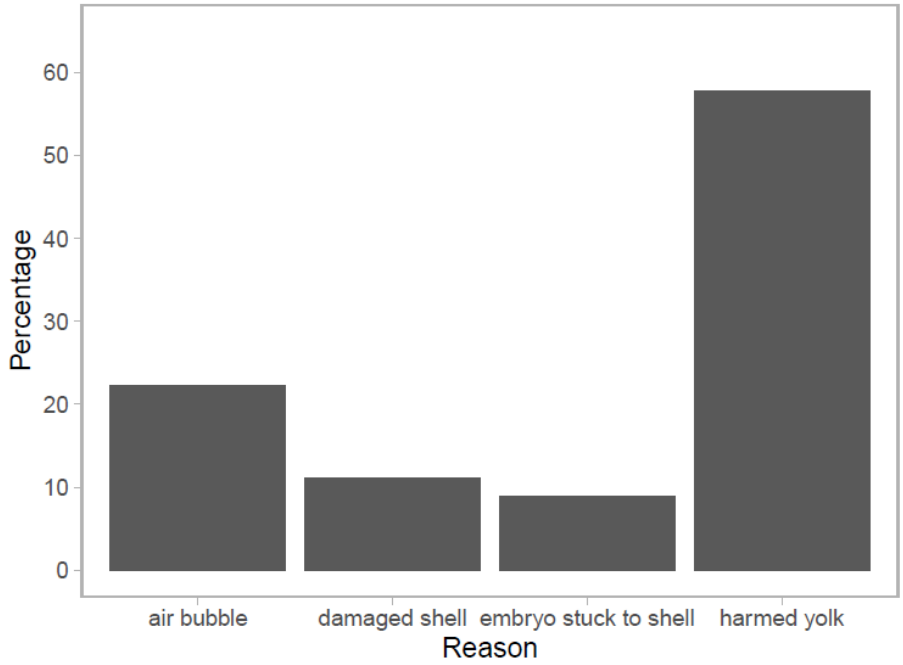

Figure 41 Reasons for failure of embryonic development after* in ovo *manipulation.
Reasons for failure of embryonic development after* in ovo *manipulation of stage x embryos are shown, if known (n=45 of a total of 385 manipulated eggs). PGCs were cultured from blood and beforehand collected from WT zebra finch embryos (stage 13-15), transfected with lentivirus (carrying an eGFP or CoChR construct), harvested and injected into WT zebra finch embryos (of eggs at stage x). Most frequent cause for stop of embryonic development was harming the yolk.

3.2.4 Different treatments for manipulated eggs: Chicken membrane & papain treatment

During the project manipulated eggs and cultured PGCs were treated differently:

At the beginning manipulated eggs were closed by placing a piece of eggshell on the window glued to the egg by albumen and is further called the ‘no’ group (n=74). In most of the previous studies were a lentivirus was injected into stage x embryos, this was the method to close the eggshell (Abe et al., 2015; Agate et al., 2009; Liu et al., 2015). In other eggs a chicken membrane was put on the window before the eggshell piece imitating the inner egg membrane

of the egg as was applied in Gessara et al., 2021. This chicken membrane treated group, 'ch', is the smallest among them (n=17). Finally, the manipulated PGCs were treated with papain, an enzyme, that transformed cell accumulations into single cells for easier injection. This was adopted from the Gessara study also. This treatment was applied in combination with the chicken membrane (the 'ch/pap' group) and was the biggest data set (n=294). In Fig. 43 the impact of the different treatments in relation to the development of the manipulated eggs is examined. The different treatment concerning manipulation window closing and egg placing during the manipulation procedure are illustrated in Fig. 42.

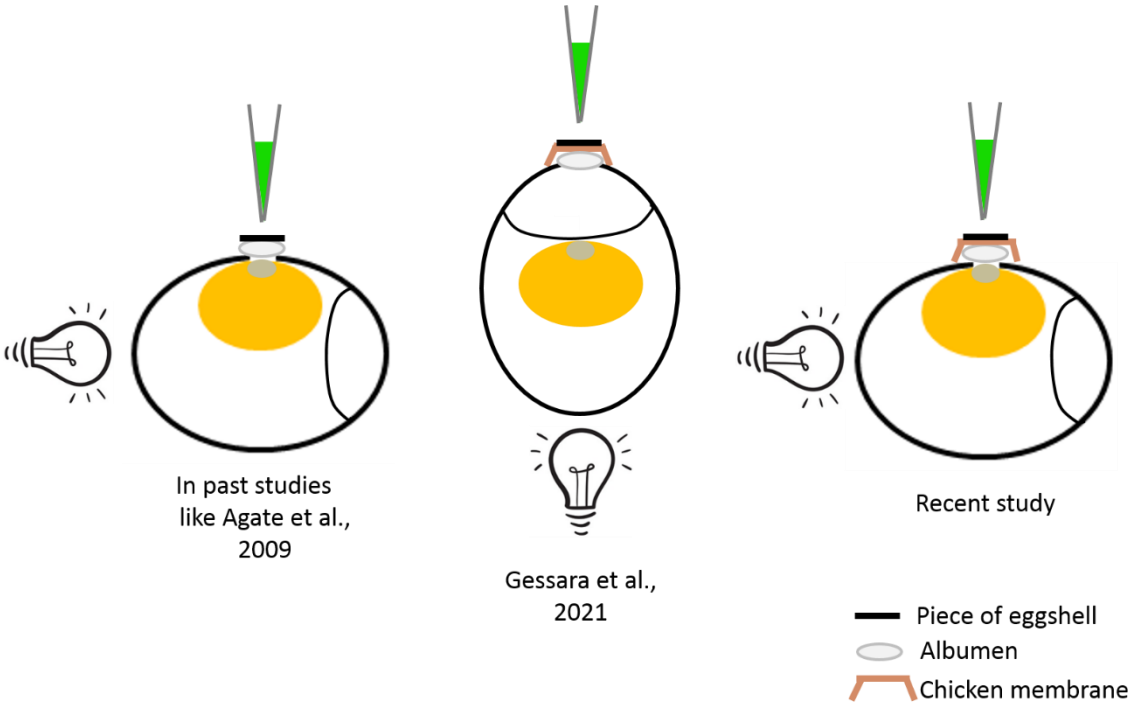

Figure 42 Different options for egg placing and window closing after* in ovo *manipulation of zebra finch stage x embryos. There is a variation in egg placing and closing window in the shell among studies, in which stage x embryos of zebra finches were manipulated to obtain transgenic songbirds. In past studies applying the direct injection of lentivirus into the early embryo, the manipulation window was closed by adding albumen and a piece of shell from a donor egg. The light source was placed beside the egg, which was lying in a horizontal position. Later, another PGC based approach was published by Gessara et al., 2021 and there, eggs were placed with the air chamber upwards and light came from below. The window was not only closed by albumen and shell, but additionally by a piece of membrane from a chicken egg. Injection happened through the inner membrane situated between the air chamber and the liquid interior of the egg. In this recent study, eggs were placed as described for Agate et al., but mostly covered by chicken membrane additionally to albumen and shell as described in the publication from Gessara et al., 2021.

Development of injected eggs was found for all three subgroups: no= 47/74 (63.5%), ch= 13/17 (76.5%), ch/pap= 157/294 (53.4%) with different ratios, heeding varying samples size between the differently treated groups. In Fig. 43 groups of different treatments were compared according to their development. All groups were not normally distributed (Shapiro Wilk test: p no=2.425^{7}; p ch<2.2^{16} & p ch/pap<2.2^{16}), so Kruskal Wallace test (pairwise Wilcoxon rank sum

test) was chosen to check for differences between the groups. No significant difference could be detected when comparing each treatment group with each other (pairwise Wilcoxon rank sum test: p no vs. ch=1; p no vs. ch/pap=1; p ch vs. ch/pap=0.57).

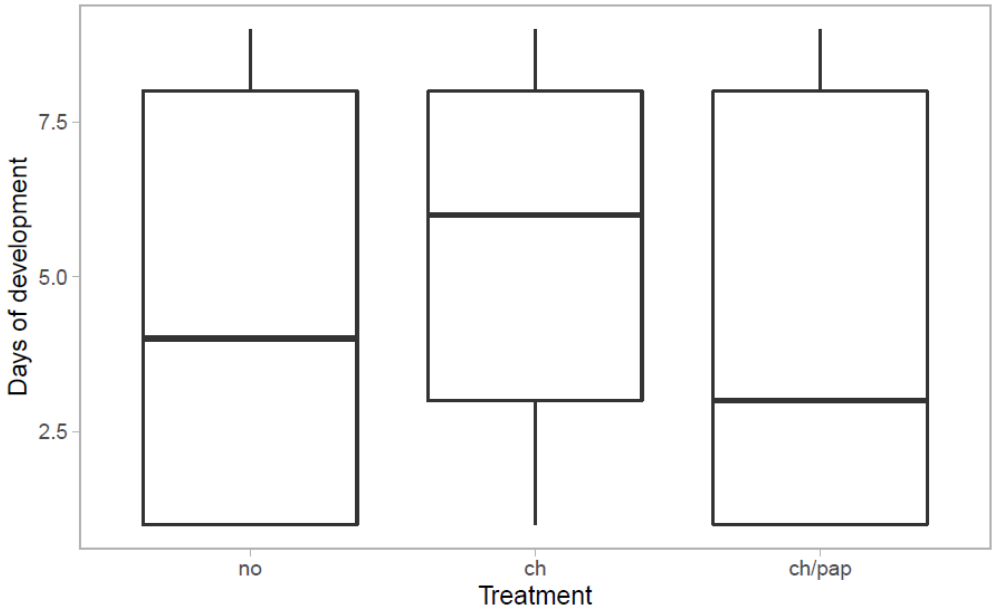

Figure 43 Impact of chicken membrane and/or papain treatment for development of* in ovo *injected embryos.
Zebra finch WT embryos of stage x were injected with modified PGCs, afterwards sealed and incubated. PGCs were beforehand collected from blood of WT embryos (stage 13-15),* in vitro *cultured (for approx. 10div), transfected by different lentiviral constructs. For most* in ovo *injected embryos covering of the manipulation window in the eggshell was not only done by albumen and a piece of eggshell (n no=74), but additionally by a patch of chicken membrane below the shell patch (n ch=17). Furthermore, for the majority off eggs, PGCs were treated with papain to prevent cell clumps and to get single cells before the injection into the embryos, n ch/pap=294). There was no evidence of advantages for embryonic development after applying any treatment (combination): Kruskal Wallis test (pairwise Wilcoxon rank sum test) revealed no difference between the groups (p no vs. ch=1; p no vs. ch/pap=1; p ch vs. ch/pap=0.57). All groups are not normally distributed (Shapiro Wilk test p no=2.425^{7}; p ch & p ch/pap<2.2^{16}).

3.2.5 Genotyping of PGC injected embryos

Genotyping was performed to detect the transgene and therefore determine, whether manipulation was successful or not. Tissue samples of manipulated embryos were used to extract the required DNA. A fragment of the potentially introduced transgene should be amplified by PCR and visualized via agarose gel electrophoresis as describe in 3.1 including Fig. 29 as an example for a genotyping PCR result.
In Tab. 21 all PCR genotyping results of manipulated embryos are summarized. 8 out of 54 (14.8%) examined samples were genotyped positive for the transgene.

Table 21 PCR genotyping results for embryos injected with modified PGCs.
Overview of PCR genotyping results after injection of transduced PGCs with lentiviral constructs into stage x embryos. Several samples of embryonic tissue from manipulated eggs were screened positive for the transgene.

no. embryos	pos. PCR	neg. PCR
54	8	46

Because the slot blot method is less likely to produce false positives, embryos were tested this way during the later stages of this thesis. Unfortunately, none of the 21 embryos (7x GFP and 14x CoChR) analyzed, were tested positive for the transgene.
Genotyping by Slot Blot did not result in any potential transgenic founder individual.
Not all manipulated eggs could be genotyped due to lack of development or lack of sufficient raw material (i.e. for embryos at very early developmental stages).

4 Discussion

4.1 *In vivo* microinjection and electroporation of zebra finch testis

The method '*In vivo* microinjection and electroporation of testes' was successfully applied to generate transgenic mice (Dhup & Majumdar, 2008; Huang et al., 2000; Majumdar et al., 2009; Michaelis et al., 2014; Usmani et al., 2013; Yomogida et al., 2003) in one generation and did not affect the fertility of the animals. Here, this approach was adapted for zebra finches, particularly the injection volume and the voltage for electroporation as well as the access to the testis due to differences in anatomy and size. The weight of testis for mice differs from zebra finches: 0.119g (Taylor & Breed, 2000) vs. 0.06g (Birkhead et al., 1999). In this study, the manipulation of zebra finch testis did apparently not impair fertility, as all males except one reproduced successfully and hatchlings developed normally.

4.1.1 Histology of *in vivo* manipulated testes

To check for the feasibility of the transgenesis approach '*In vivo* microinjection and electroporation of testes', applied for the first time in a songbird, groups of 6 birds each were treated by different parameters. At the same time best conditions should be determined for follow up experiments with the same technique. On the testes of these manipulated birds, histology was performed afterwards in order to detect the presence of the transgene inside spermatogonial cells (fluorescence was confirmed) and to check if this technique is harmful for the tissue, as it could influence fertility. No morphological abnormalities were detected after manipulation of testes. When giving manipulated birds the opportunity to breed and reproduce, all experimental animals except one could fertilize their partners, as developing eggs were observed for all. One couple did not reproduce for unknown reasons. Consequently, testes manipulation per se did not impair male's fertility. Results uncovered the general feasibility of this approach to avian species as treated testes showed fluorescent cells after introducing a reporter with a strong promoter shortly after the manipulation event and absence of any fluorescent signal in the untreated control testes (before and after tissue cutting; see Fig. 24). In all groups, which were treated by different parameters (injection volume and voltage) fluorescence could be observed, but best parameter combination was achieved by 5µl injection volume and 30 Volt (Tab. 13). Moreover, expression lasted for 168 days post electroporation (Fig. 27), indicating that spermatogonia were successfully targeted. Otherwise, testes would lose fluorescence after all manipulated cells entered the differentiation process to build mature

spermatozoa (spermiogenesis), because spermatogonia are the only cells, which are able to undergo homonymous cell division for self-maintenance. Spermiogenesis in zebra finches (Passeriformes) is assumed to last shorter than for non-passerine birds (around 12 days; Lin & Jones, 1992, Jones & Lin, 1993, Bhat & Maiti, 1988), so by the latest after 2 weeks no more manipulated cells should be detectable in the testes of manipulated males, if no integration of the transgene had occurred. In addition, I could show that the introduction of two different constructs at the same time is possible. Microscopy revealed that cells expressed both constructs (carrying different reporters) at the same time (Fig. 26). This was important as stable integration should be achieved by transposition of the gene of interest and therefore a second plasmid containing the information for the hyperactive Piggybac transposase was necessary. Immunostaining (against mcherry or GFP) clearly demonstrated the expression of the introduced reporter inside the tissue of manipulated male zebra finches (Fig. 28). There was no signal in control testis (right) compared to the treated one (left) of the same animal, proving that with the method it is possible to introduce genetic material.

4.1.2 Genotyping by PCR vs. Slot Blot

Establishing a reliable and reproducible method for genotyping of potentially transgenic animals is crucial in order to ensure the success of the applied method for the generation of transgenics. Genotyping by PCR was chosen in many other published studies, which aimed to generate and detect transgenic animals. Among them also studies concerning zebra finches (Abe et al., 2015; Agate et al., 2009; Liu et al., 2015) as well as studies concerning mice (Michaelis et al., 2014; Usmani et al., 2013) that was adopted to establish *in vivo* microinjection an electroporation testes for zebra finches. In the present study, results from PCR did not deliver reliable results. Subsequently, an alternative way was established to clarify the presence of the transgene. Slot blot genotyping (also used in Usmani et al., 2013) did not detect transgenic offspring, so transgene transmission of manipulated birds to their descendants could not be shown yet. However, positive genotyping for treated testes of potential founder males was found and confirmed the results of histological data except for one male. Unfortunately, no sperm sample were examined using Slot Blot, as ejaculates do not lead to an adequate quantity of DNA, although it would be interesting to know if the transgene could be detected in the sperm samples of manipulated males.

4.1.3 Final conclusions on *in vivo* microinjection and electroporation of testes in zebra finches

Concluding with *in vivo* microinjection and electroporation of testes for the generation of transgenic songbirds, this approach seems not exclusively a promising way for the generation of transgenic mice, but also for zebra finches. For future studies in zebra finches, one should maybe rather choose linearized constructs like in Dhup and Majumdar (2008), Majumdar et al. (2009) and Usmani et al. (2013) as there transgenic sperm and offspring was successfully generated, although in Yomogida et al. (2003) they claimed that with linearized vectors reduced expression was observed few days after electroporation, whereas with circular constructs expression remained for 35 days after manipulation. In mice Sertoli cells were more easily manipulated than germ cells, therefore Michaelis et al. (2014) recommended to co-transfect testes with two constructs (target and control plasmid, each having a different reporter). It is still unclear, if this is the case for zebra finches, too. Nevertheless, this is a good suggestion to prove, if the procedure is working and did successfully work out in one of the here conducted experiments (see Fig. 26, co-localization of two reporters inside testes after injecting two constructs followed by electroporation). Another study (Dhup & Majumdar, 2008) removed one testis (hemi-castration) or manipulated both testes (Yomogida et al., 2003) to raise the proportion of/chances for transgenic sperm cells in the ejaculates. Here in this study, the second variant was followed, but to date there is no evidence, which variant leads to a higher amount of transgenic sperm and thus a higher probability for transgenic offspring. It is questionable which way is best, as the left avian testis is in most cases of smaller size (Lake & Ravie, 1984) and thus suggested to be less deciding for reproduction. A common hypothesis is that the left testis is normally ‘inactive’ and becomes only ‘active’, if necessary. This means when the left testis is, no matter for what reason (in case of injury i.e.), not functional, so the right testis would serve as back up (Møller, 1994). It has not been clarified yet, if manipulation, lesion or removal of the left testis causes the activation of the right testis. Hemi-castration would prevent the mixture of ejaculates by unmanipulated sperm from the right testis, if unilateral treatment was chosen, but means at the same time a more invasive intervention for the animal. The bilateral manipulation could achieve manipulated spermatogonia in the right testis, too and so reduce the proportion of sperm cells lacking the transgene and consequently increase the chance for transgenic offspring and would be less invasive as hemi-castration. It cannot be predicted which choice would be more advantageous.

There were other options conceivable, i.e. the crossings of family members from different founders, carrying the same construct. These could be crossed to raise the proportion of transgenic hatchlings within a clutch. Challenging could be the timing (achieving sexual maturity at different time) and eventually missing compatibility (concerning sex and construct

variants), depending on the experimental design, but imaginable for future projects. Another consideration would eventually be beneficial: if the manipulated sperm could be separated from the not manipulated sperm (only possible, if constructs carry a detectable reporter like a fluorescent dye), this could be then introduced via artificial insemination into the cloaca of several females being in their reproductive period and so all of the following progeny should carry the transgene. This procedure would accelerate projects as transgenic offspring is produced fast, at a higher rate (as no WT sperm is present for fertilization) and following experiments could consequently start earlier. In many species sperm cells of ejaculates were distinguished (Y chromosome from X chromosome carrying sperm, as well as sperm expressing fluorescence) by fluorescence activated cell sorting (FACS) (Garner et al., 2013). It would be worth to test selection of manipulated sperm by FACS for zebra finches, too. Especially, as for mice there was already fluorescent sperm detected after making use of the *in vivo* microinjection and electroporation of testes method (Usmani et al., 2013). In the recent study, functional constructs were restricted to expression in interneurons (due to mDLX enhancer), so this option was not given. Another point is, that in general expression in sperm might be low and reduced to essential genes only as the main function is to meet the ovum for fertilization. In the past, artificial insemination has already been successfully applied to many avian species, especially in the poultry industry (Samour et al., 2002; Samour et al., 2004). The difficulties lie in the circumstances of the female. Naturally, both partners of zebra finch couples share all tasks according brood care, but then of course there is always the opportunity that her male initiated copulation and therefore his WT sperm is introduced as well. This would again reduce the chances for transgenic offspring. If at least a manipulated male would be her companion, it would not be as worse as with a WT male. The best match would be an infertile male (naturally occurring or achieved by castration) or a same-sex couple with two females as such pair bonds are equally stable to male-female couples (Bailey & Zuk, 2009; Elie et al., 2011). Thus, it might be not easy to find females, which lay eggs and would take care of the clutch as well as for the later hatched nestlings without a present male partner.

With these findings, an alternative approach for the generation of transgenic songbirds is presented. This new and fast way for the manipulation of testes (originally published for mice in Usmani et al., 2013) was established in a songbird species (zebra finch) assuming that this technique leads easier and faster to founder individuals as the target cells of the experiment belong directly to the germline, which is essential when a transgene is aimed to be transmitted to the next generation.

4.2 *In vitro* manipulation of PGCs for generating transgenic zebra finches

The extraction and cultivation of PGCs from circulating blood from early zebra finch embryos were achieved as described in Gessara et al, 2021. Furthermore, the infection of PGCs by lentivirus and the injection of these manipulated cells into recipient stage x embryos was successfully conducted. Although applying the published protocol, the outcome of both studies differed greatly.

4.2.1 Development of manipulated eggs

In the present study development success differed from the previous study of Gessara. I found that after 9 days of incubation approx. half of the manipulated eggs developed (56.4% independent from construct) whereas in the previous study from Gessare 45.4% did show development. There are several possible reasons, which could explain this: 1. Injection method, 2. method of closing the egg after manipulation, 3. incubation of eggs after manipulation and 4. differences in lentiviral constructs used.

Since point 4 is unlikely to be the case as lentiviral manipulation with different promoters in zebra finch embryos was already shown (Abe et al., 2015; Agate et al., 2009; Gessara et al., 2021; Liu et al., 2015), I will concentrate on the other three. To point 1: Gessara used a different orientation of the egg and pierced the membrane without opening it (Fig. 45). This might result in lower incidence of introducing air, and less likely harming of the yolk, which I observed in some of my eggs (Fig. 44). The present experiments were conducted before the report of Gessara was published and I only found this difference out at the end of my thesis, so I could not determine whether changing this variable played a decisive role. Therefore, I chose at the beginning of the experiments the orientation for injecting eggs as described in earlier publications (Agate et al., 2009; Velho & Lois, 2014), where successful manipulation of stage x embryos was achieved.

2.: Closing the manipulated eggs with a piece of chicken membrane before placing the shell might improve development and prevent sticking of the embryo to the shell. However, I did not see a large difference between the two methods in my hands (Fig. 46) and thus consider this less likely. More important seems to me placing the egg while air-drying in a way that the embryo is not directly located under the patch as then embryos stuck more often to the inside of the shell (personal observation).

And last: In the study from Gessara et al., incubation was first continued in an incubator for 72h at 38°C (for Agate: 1-3 days at 37-38°C and 40-50% humidity) before placing eggs in

foster nests. No details were available for humidity (in case of Gessara et al.) and for turning rates (for both Agate et al. and Gessara et al.) inside the incubator. Foster parents might not treat manipulated eggs the same way as their own eggs, which is suggested by some publications (Golüke et al., 2016). I did not systematically investigate this option, but it could have played a role and personal observations in the lab's own breeding colony hint as well at divergent willingness of birds to care for foreign eggs. Gessara et al. did not report on the details of foster-parents choice, neither Agate et al. did. Another possibility would be the replacement of own eggs by dummy eggs and later exchanging manipulated eggs again (when development after manipulation was visible).

The hatching success in the Gessara study was reported to be 45.4% (10/22) of manipulated developed eggs. As in the recent study development of manipulated eggs was permitted until day 9 of embryonic development only, data of hatching rates are not available for comparison.

4.2.2 Low manipulation success

Although the successful viral transduction was detected via the fluorescent reporter in cultured PGCs, it did not result in transgenic animals. This is odd as in the study of Gessara et al., 2021 all manipulated and hatched embryos became founder individuals producing transgenic offspring. Comparing both studies reveals no difference in the number of injection site. The injected number of PGCs were 300 to 500 for the previous study, here 500 cells were taken. Both studies used a lentiviral construct with GFP as reporter and a strong promoter. The previous study took the human phosphoglycerate kinase promoter, often used for high level expression of transgenes and here CAG, also suitable for long term expression of transgenes, was used as promoter, so promoter switch is not assumed to explain the different outcome in the result of the studies. The titer of the virus for Gessara was around $2x10^8$ transducing units (TU/mL), whereas in the recent project the titer was around $2.4x10^9$TU/mL (eGFP) or $3.6x10^9$TU/mL (CoChR) and microscopy of transduced PGCs showed green fluorescence after successful infection.

One parameter in PGC cultivation differed according to Gessara et al., 2021: The incubation temperature was higher (Gessara: 37°C vs. recent study: 42°C). At the very beginning PGCs were cultured at both temperatures to identify best conditions. This was tested, because of the natural body temperature of zebra finches (42°C; Zann, 1996) and due to lab-intern experiences with other zebra finch cell lines (G266 and ZF-TMA; Itoh and Arnold, 2011). These cells grew better at 42°C and transfection ratios were higher compared to cells cultured at 37°C (unpublished data). It was assumed that this could be the case also for PGCs. Their growth & survival seemed similar successful. These possibilities could have led to lower manipulation

success in the recent project. It would be interesting to repeat the experiments with the original published incubation temperature to find out, if then the manipulation of zebra finch embryo by injecting *in vitro* modified PGCs would result in comparable outcome as for Gessara's study.
Recently published data refuse the assumption PGCs being one homogenous cell type. At least in developmental stage HH28 of embryos, PGCs can be divided into different subgroups. These clusters vary in their expression patterns, suggesting different functionality (Jung, Seo, et al., 2021). In their study PGCs were gonads derived as for other publications of Jung (Jung et al., 2019; Jung, Kim, et al., 2021), whereas in Gessara et al. and in the recent study PGCs were always blood derived (HH13-15). Meaning different studies on PGC manipulation used PGCs from different developmental stages. It is still questionable, if PGCs can be separated into subgroups at this or other developmental stage/s.
In general, this PGCs heterogeneity should be considered when planning experiments, as it might have a big impact on transgenesis approaches, which are based on PGCs. In the actual project this could be relevant as the correctness of embryo staging could have a strong impact on experimental success/outcome.

4.2.3 Chicken membrane or/and papain treatment

Different treatments were applied to subgroups of manipulated eggs: On the one hand, PGCs were enzymatically dissociated by papain treatment (pap) after transduction by lentiviral construct to facilitate injection into fresh embryos and to prevent cell clusters (which are formed during culturing), that might clog capillaries. On the other hand, eggs were not only closed by adding albumen and a patch of eggshell onto the manipulation opening, but additionally covered by a piece of chicken membrane (ch). This could imitate the natural occurring inner membrane, which was removed simultaneously as the shell by getting access to the embryo. Subsequently, this ch treatment simulates the natural state and seems plausible. The third subgroup received both treatments (ch and pap). In the study of Gessara et al. both treatments were applied routinely, however the positive or negative effect of each treatment was not examined individually.

Papain treatment of manipulated PGCs
Papain treatment of modified PGCs as preparation for the injection was also conducted in the experiments of Gessara et. al., 2021 and adopted for the recent study. Proteolytic dissociation is widely used in cell culture. Papain seems more effective and less harmful compared to other proteases in certain tissues (Lam, 1972). Injection of modified PGCs without papain treatment was not performed in the previous study and there was no subgroup with papain treatment

only present in this recent study. However, according to data analysis including the other subgroups (no, ch and ch/pap), there is no evidence that papain treatment (pap) did significantly supports the development of manipulated eggs in a positive way. There was no difference detected between manipulated eggs without any treatment (no), with chicken membrane treatment (ch) or with chicken membrane and papain treatment (ch/pap) (pairwise Wilcoxon rank sum test: p no vs. ch=1; p no vs. ch/pap=1; p ch vs. ch/pap=0.57; see also Fig. 43).

In theory, it seems plausible to inject single cells rather than cell clumps, but if these cell accumulations are not greater than the diameter of the capillary, there exists no risk of blocking the opening of the capillary. PGC diameter measurements lie between 16 and 20µm (Macdonald et al., 2010). Consequently, the measured diameter of the tip should be above these values to ensure passing of cells. Otherwise, the tip of the capillary should be kept small to prevent embryo damages at the injection site.

Chicken membrane for covering opening window

Statistical analysis did not confirm a significant positive effect of chicken membrane treatment for the development of manipulated eggs (pairwise Wilcoxon rank sum test: p no vs. ch=1; p no vs. ch/pap=1; p ch vs. ch/pap=0.57; see also Fig. 43). So, it does not seem to matter, if chicken membrane is applied for closing the manipulation window in the eggshell or not.

4.2.4 Final conclusions for the manipulation by *in vitro* culturing, virus transfection and re-injection into stage x embryos

PGCs offer a great opportunity to generate transgenic zebra finch lines. According to Gessara et al. 2021, lentivirus modified and later injected Primordial Germ Cells can generate transgenic songbird embryos. Their results show highly efficient results considering the hatching rate (45.4%) and the generation of transgenic founder individuals (100%) at best level. Following their protocol, the recent study did result in isolating PGCs from embryonic blood, in culturing this specific cell type as well as to infect these cells by lentivirus and to inject the manipulated cells into early stage embryos. Similar findings were generated according to development of injected eggs (Gessara: 45.4% vs. recent study: 56.4%). Because injected eggs were only incubated until 2/3 of embryonic development, the recent study cannot make a statement to hatching success.

Genotyping by slot blot or PCR revealed no transgenic founder in my hands, indicating that transgenesis does not only offer unbelievable opportunities for research, but at the same time is an extremely challenging task. Referring to the published data from Gessara, this approach

seems to be a promising way to generate transgenic songbirds, but to date no other publication generated a transgenic songbird based on this method. Moreover, Jung et al. investigated in PGC based methods to manipulate zebra finches. They used gonadal derived PGC instead of blood derived one (as Gessara et al., 2021). In two studies they resulted in culturing, manipulating and tracing PGCs settling at the gonadal anlagen, but never documented a hatched transgenic bird (Jung et al., 2019; Jung et al., 2021). There exists no other publication, which resulted in a transgenic songbird making use of the methods from Jung et al. (2019 and 2021). Although there has already be done some research in the cultivation and manipulation of zebra finch PGCs, it is still difficult to implement these results in a general pipeline for the generation of transgenic songbirds. Furthermore, researcher should keep in mind the new discovered heterogeneity of PGCs (Jung et al., 2022) and more studies on this topic could help to optimize PGC based approaches to generate transgenic birds.

5 Outlook

This work provides groundwork for the establishment of a new method in songbird transgenesis. It was clearly demonstrated here that '*In vivo* microinjection and electroporation of testes' leads to zebra finches with manipulated testes. Therefore, this approach seems not exclusively a suitable way to produce transgenic mice, but also for transgenic songbirds. Although transmission of the manipulation was not detected yet, it seems feasible to produce transgenic offspring via '*In vivo* microinjection and electroporation of testes' in the future, as the transgene expression was stable over long time. Beyond this, tissue was not harmed by the procedure and fertility was not affect, which is obviously crucial for natural reproduction and consequently for the generation of transgenic offspring. Follow up studies apparently need to concentrate on the transgene transmission. To improve chances of transgenic progeny the proportion of manipulated cells must be increased, so more sperm cells would carry the transgene.

'*In vitro* manipulation of PGCs and re-injection of them into recipient stage x embryo' is the most promising attempt to generate transgenic songbirds regarding PGCs based approaches. It resulted already in transgenic offspring contrary to Jung et al., 2019, as there no transgenic animal hatched. Furthermore, the founder rate was beyond all percentages reached with the classical 'Lentivirus injection into stage x embryos' (Abe et al., 2015; Agate et al., 2009; Liu et al., 2015). Nevertheless, the present study was not able to repeat it. Surely, there will be efforts in the characterization, the cultivation and the manipulation of PGCs, as for the first-time heterogeneity of PGCs was discovered (Jung et al., 2021). Though it would be interesting to verify, if this is affecting other developmental stages as well, and if these results support the generation in transgenesis as optimal time for PGC extraction and manipulation might be determined. To date, the relevance of this publication for already existing PGC based methods like described in Gessara et al. (2021) or Jung et al. (2019) is still unknown and needs to be investigated.

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List of Abbreviations

A

A	(nucleobase) adenin
aa	amino acids
AAV	adeno associated virus
Ab	antibody
a.d.	distilled water
AFP	anterior-forebrain-pathway
AI	artificial insemination
AP	alkaline phosphatase

B

BCIP	5-bromo-4-chloro-3-indolyl-phosphate
bi	bilateral
BiPOLES	bidirectional Pair of Opsins for Light-induced Excitation and Silencing
BF	bright field
bp	base pairs
BSA	bovine serum albumen
ßGlo	beta globin

C

°C	degrees in Celsius
CA	cellulose acetate
CAAG	CMV early enhancer/chicken β actin promoter
cAMP	Cyclic adenosine monophosphate
CAS	Childhood Apraxia of speech
Cas	Crispr associated
CDH	chicken dead-end homologue gene
CF	cell free
Ch	chicken
CHD	chromo box helicase DNA binding gene
Chrimson	red light-drivable channelrhodopsin
cm	centimeter
(H)CMV	(human) cytomegalovirus
CO_2	carbon dioxide
CoChR	large-current channelrhodopsin
conc.	Concentration
CR	calretinin
Crispr	Clustered Regularly Interspaced Short Palindromic Repeats
CVH	chicken vasa homologue

D

DAPI	4',6-Diamidino-2-phenylindol
DAZL	Deleted in azoospermia like
ddH_2O	double-distilled water
DEPC	Diethylpyrocarbonate
DIG	digoxigenin
div	days of *in vitro* culture
DMF	Dimethylformamide
DNA	desoxyribonucleic acid
dNTP´s	Desoxyribonucleosidtriphosphate
DLM	medial portion of the dorsolateral nucleus of the thalamus
DLX	distal-less homeobox
DMEM	Dulbecco's Modified Eagle Medium
DMRT1	doublesex and Mab-3 related transcription factor 1
DMSO	Dimethylsulfoxid
ds (DNA/RNA)	double strand (-ed DNA/RNA)
DSHB	*Developmental Studies Hybridoma Bank*
DVD	Developmental Verbal Dyspraxia

E

EDTA	Ethylendiamintetraacetate
EGK	Eyal-Giladi and Kochav stage of embryonic development
EMA-1	epithelial membrane antigen 1
EtOH	Ethanol
eGFP	enhanced green fluorescent protein
eYFP	enhanced yellow fluorescent protein

F

FACS	fluorescence activated cell sorting
Fig.	Figure
FCS	fetal calf serum
for.	Forward (Primer)
FOX	Forkhead box
FoxP	Forkhead box protein subfamily P
FOXP1	Forkheadbox protein, subfamily P, member 1
FOXP2	Forkheadbox protein, subfamily P, member 2

G

GABA	gamma-aminobutryric acid
Gad67	pan-interneuron marker
Gag	group specific antigen (HIV-1)
GDNF	glial cell line-derived neurotrophic factor
GFP	green fluorescent protein
GFRA1	GDNF family receptor alpha 1
gRNA	guide RNA
GSC	gonadal stoma cells

H

HBSS	Hank's Balanced Salt Solution
HCl	Hydrochloric acid
HH	Hamburger Hamilton stages for embryonic development of ch/zf
h	hour/s
HEK293T	Human embryonal kidney cells, expressing SV40 large T-antigen
Hiss	heat inactivated sheep serum
hPGK	human promoter encoding for the glycolytic enzyme phosphoglycerate kinase gene
HR	homologous recombination
HVC	used as a proper name
HypBase	Hyperactive PiggyBac Transposase

I

IHC	Immunohistochemistry
Ig	Immunoglobulin
IGTB1	Integrin beta 1
Inj.	injection
IPTG	Isopropyl β-d-1-thiogalactopyranoside

J

K

kb	kilo bases
KE	Family in which the human FoxP2 mutation was first identified
KD	knockdown
KIT	tyrosine-kinase KIT/CD117
KO	Knockout

L

Lab.	laboratory
L	liter
L.	left
LB	lysogeny broth
LMAN	lateral subdivision of the magnocellular nucleus of the anterior nidopallium

M

MACS	Magnetic Activated Cell Sorting
M	Molar
m	meter
mDLX	murine enhancer between DLX (distal-less homeobox) 5 and 6
mM	milli Molar
max.	maximum
min	minutes
mg	milligram
$MgCl_2$	magnesium chloride
mL	milliliter
mRNA	messenger RNA
ms	milliseconds

N

N/A	not available
NaCl	sodium chloride
NBT	nitrotetrazolium blue chloride
NEAA	non-essential amino acids
neg.	negative
ng	nano gram
no.	number
nm	nano meter
nXIIts	the tracheosyringeal portion of the twelfth cranial nerve

O

O_2	oxygen
OFP	orange fluorescent protein
OV	ovalbumin

P

P	positive/positive control (plasmid carrying the sequence, which is aimed to amplify)
p.	page
PAS	Periodic Acid-Schiff stain
PB	PiggyBac
PBS	phosphate-buffered saline
PBST	Phosphate Buffer Saline Tween
PBS-Tx	Phosphate Buffer Saline Triton X
PCR	polymerase chain reaction
pg	picogram
PFA	paraformaldehyde
PGC	primordial germ cells
PHD	post hatching day/s
Pol	DNA polymerase (HIV-1)
pos.	positive
PV	parvalbumin

Q

R

R.	right
RA	robust nucleus of the arcopallium
rev.	reverse (primer)
rev	regulatory protein (HIV-1)
rpm	rounds per minute
RT	room temperature

S

s	seconds
SOB	Super Optimal Broth
Soc	SOB medium, 20mM glucose added
somBiPOLES	soma-targeted variant of BiPOLES

STAGE	sperm transfection assisted gene editing
SSCs	Spermatogonial stem cells
ssDNA	Single stranded DNA
SSEA-1	stage specific antigen 1
SST	sperm-storage tubules
SV40	Simian Vacuolating virus 40

T

Tab.	Table
TAE	TRIS-Acetate-EDTA-buffer
TALEN	Transcription activator-like effector nucleases
Taq.	Polymerase from *Thermus aquaticus*
T	(nucleobase) thymine
Tm	melting temperature (for primers)
Top10	Chemically Competent *E. coli*
TO-PRO-3	carbocyanine monomer nucleic acid stain
Tol2	transposase system
tRNA	transfer RNA
TS	Tension sensor module

U

U	Unit(s)
uni	unilateral
UV	Ultraviolet

V

V	Volt
Vol.	volume
vs.	versus
VSV-G	Vesicular stomatitis virus G protein

W

W	water (negative control)
WT	Wildtype
WPRE	Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element

X

(Stage) X	Hamburger Hamilton staging of embryonic development (stage after oviposition; freshly laid eggs)
x g	times gravity
X-Gal	5-Brom-4-chlor-3-indoxyl-β-D-galactopyranosid

Y

Z

Zf	zebra finch

µ

µg	mircogram
µL	mircoliter
µm	micrometer

References

Abe, K., Matsui, S., & Watanabe, D. (2015). Transgenic songbirds with suppressed or enhanced activity of CREB transcription factor. *Proceedings of the National Academy of Sciences*, *112*(24), 7599-7604.

Adam, I., Scharff, C., & Honarmand, M. (2014). Who is who? Non-invasive methods to individually sex and mark altricial chicks. *Journal of visualized experiments: JoVE*(87).

Agate, R., Scott, B., Haripal, B., Lois, C., & Nottebohm, F. J. P. o. t. N. A. o. S. (2009). Transgenic songbirds offer an opportunity to develop a genetic model for vocal learning. *106*(42), 17963-17967.

Anstrom, K. K., & Tucker, R. P. (1996). Tenascin-C lines the migratory pathways of avian primordial germ cells and hematopoietic progenitor cells. *Developmental dynamics: an official publication of the American Association of Anatomists*, *206*(4), 437-446.

Apfeld, J., &Alper, S. (2018). What can we learn about human disease from nematode C. elegans?. *Disease Gene Identification*, 53-75.

Bacon, C., & Rappold, G. A. (2012). The distinct and overlapping phenotypic spectra of FOXP1 and FOXP2 in cognitive disorders. *Human genetics*, *131*(11), 1687-1698.

Bailey, N. W., & Zuk, M. (2009). Same-sex sexual behavior and evolution. *Trends in Ecology & Evolution*, *24*(8), 439-446.

Bakst, M. R. (1998). Structure of the avian oviduct with emphasis on sperm storage in poultry. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, *282*(4-5), 618-626.

Bennison, C., Hemmings, N., Slate, J., & Birkhead, T. (2015). Long sperm fertilize more eggs in a bird. *Proceedings of the Royal Society B: Biological Sciences*, *282*(1799), 20141897.

Bhat, G., & Maiti, B. (1988). Study of spermatogenesis in a wild bird, the yellow-throated sparrow (Petronia-xanthocollis burton). *Zoologischer Anzeiger*, *221*(5-6), 430-434.

Biegler, M. T., Fedrigo, O., Collier, P., Mountcastle, J., Haase, B., Tilgner, H. U., & Jarvis, E. D. (2021). Induction of an immortalized songbird cell line allows for gene characterization and knockout by CRISPR-Cas9. *bioRxiv*.

Birkhead, T. (1998). Cryptic female choice: criteria for establishing female sperm choice. *Evolution*, *52*(4), 1212-1218.

Birkhead, T., Fletcher, F., & Pellatt, E. J. (1999). Nestling diet, secondary sexual traits and fitness in the zebra finch. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *266*(1417), 385-390.

Birkhead, T., & Hunter, F. (1990). Mechanisms of sperm competition. *Trends in Ecology & Evolution*, *5*(2), 48-52.

Birkhead, T., & Møller, A. (1992). Numbers and size of sperm storage tubules and the duration of sperm storage in birds: a comparative study. *Biological Journal of the Linnean Society*, *45*(4), 363-372.

Birkhead, T., Pellatt, E., Brekke, P., Yeates, R., & Castillo-Juarez, H. (2005). Genetic effects on sperm design in the zebra finch. *Nature*, *434*(7031), 383-387.

Birkhead, T. R., & Fletcher, F. (1995). Male phenotype and ejaculate quality in the zebra finch Taeniopygia guttata. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *262*(1365), 329-334.

Blackbum, D. G. (1999). Viviparity and oviparity: evolution and reproductive strategies.

Bolhuis, J. J., & Gahr, M. (2006). Neural mechanisms of birdsong memory. *Nature reviews neuroscience*, *7*(5), 347-357.

Bolhuis, J. J., Okanoya, K., & Scharff, C. (2010). Twitter evolution: converging mechanisms in birdsong and human speech. *Nature reviews neuroscience*, *11*(11), 747-759.

Brainard, M. S., & Doupe, A. J. (2002). What songbirds teach us about learning. *Nature*, *417*(6886), 351-358.

Calhim, S., & Birkhead, T. R. (2009). Intraspecific variation in testis asymmetry in birds: evidence for naturally occurring compensation. *Proceedings of the Royal Society B: Biological Sciences*, *276*(1665), 2279-2284.

Calhim, S., Immler, S., & Birkhead, T. R. (2007). Postcopulatory sexual selection is associated with reduced variation in sperm morphology. *PloS one*, *2*(5), e413.

Carouso-Peck, S., & Goldstein, M. H. (2019). Female social feedback reveals non-imitative mechanisms of vocal learning in zebra finches. *Current Biology*, *29*(4), 631-636. e633.

Chen, S.-R., & Liu, Y.-X. (2015). Regulation of spermatogonial stem cell self-renewal and spermatocyte meiosis by Sertoli cell signaling. *Reproduction*, *149*(4), R159-R167.

Condro, M. C., & White, S. A. (2014). Recent advances in the genetics of vocal learning. *Comparative cognition & behavior reviews*, *9*, 75.

Cooper, C. A., Challagulla, A., Jenkins, K. A., Wise, T. G., O'Neil, T. E., Morris, K. R., Tizard, M. L., & Doran, T. J. (2017). Generation of gene edited birds in one generation using sperm transfection assisted gene editing (STAGE). *Transgenic research*, *26*(3), 331-347.

Dhup, S., & Majumdar, S. S. (2008). Transgenesis via permanent integration of genes in repopulating spermatogonial cells in vivo. *Nature methods*, *5*(7), 601-603.

Dimidschstein, J., Chen, Q., Tremblay, R., Rogers, S. L., Saldi, G.-A., Guo, L., Xu, Q., Liu, R., Lu, C., & Chu, J. (2016). A viral strategy for targeting and manipulating interneurons across vertebrate species. *Nature neuroscience*, *19*(12), 1743-1749.

Dimitrov, L., Pedersen, D., Ching, K. H., Yi, H., Collarini, E. J., Izquierdo, S., van de Lavoir, M.-C., & Leighton, P. A. (2016). Germline gene editing in chickens by efficient CRISPR-mediated homologous recombination in primordial germ cells. *PloS one*, *11*(4), e0154303.

Do Carmo, S., & Cuello, A. C. (2013). Modeling Alzheimer's disease in transgenic rats. *Molecular neurodegeneration, 8*(1), 1-11.

Doupe, A. J., & Kuhl, P. K. (1999). Birdsong and human speech: common themes and mechanisms. *Annual review of neuroscience*, *22*(1), 567-631.

Eales, L. A. (1985). Song learning in zebra finches: some effects of song model availability on what is learnt and when. *Animal Behaviour*, *33*(4), 1293-1300.

Elie, J. E., Mathevon, N., & Vignal, C. (2011). Same-sex pair-bonds are equivalent to male–female bonds in a life-long socially monogamous songbird. *Behavioral Ecology and Sociobiology*, *65*(12), 2197-2208.

Elie, J. E., & Theunissen, F. E. (2018). Zebra finches identify individuals using vocal signatures unique to each call type. *Nature communications*, *9*(1), 1-11.

Fitch, W. T. (2000). The phonetic potential of nonhuman vocal tracts: comparative cineradiographic observations of vocalizing animals. *Phonetica*, *57*(2-4), 205-218.

Forstmeier, W., Martin, K., Bolund, E., Schielzeth, H., & Kempenaers, B. (2011). Female extrapair mating behavior can evolve via indirect selection on males. *Proceedings of the National Academy of Sciences*, *108*(26), 10608-10613.

Froman, D. P., Pizzari, T., Feltmann, A. J., Castillo-Juarez, H., & Birkhead, T. R. (2002). Sperm mobility: mechanisms of fertilizing efficiency, genetic variation and phenotypic relationship with male status in the domestic fowl, Gallus gallus domesticus. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *269*(1491), 607-612.

Fujimoto, T., Ukeshima, A., & Kiyofuji, R. (1976). The origin, migration and morphology of the primordial germ cells in the chick embryo. *The Anatomical Record*, *185*(2), 139-153.

Garner, D. L., Evans, K. M., & Seidel, G. E. (2013). Sex-sorting sperm using flow cytometry/cell sorting. *Spermatogenesis*, 279-295.

Gessara, I., Dittrich, F., Hertel, M., Hildebrand, S., Pfeifer, A., Frankl-Vilches, C., McGrew, M., & Gahr, M. (2021). Highly efficient genome modification of cultured primordial germ cells with lentiviral vectors to generate transgenic songbirds. *Stem cell reports*, *16*(4), 784-796.

Gilbert, S. (2000). Spermatogenesis. *Developmental biology*, *6*.
Gilbert, S. F. (2000). Developmental Biology. Sunderland, MA. In: Sinauer Associates, Inc.
Girndt, A., Cockburn, G., Sánchez-Tójar, A., Løvlie, H., & Schroeder, J. (2017). Method matters: Experimental evidence for shorter avian sperm in faecal compared to abdominal massage samples. *PloS one*, *12*(8), e0182853.
Griffith, S. C., & Buchanan, K. L. (2010). The zebra finch: the ultimate Australian supermodel. *Emu*, *110*(3), v-xii.
Griffith, S. C., Holleley, C. E., Mariette, M. M., Pryke, S. R., & Svedin, N. (2010). Low level of extrapair parentage in wild zebra finches. *Animal Behaviour*, *79*(2), 261-264.
Griffiths, R., Double, M. C., Orr, K., & Dawson, R. J. (1998). A DNA test to sex most birds. *Molecular ecology*, *7*(8), 1071-1075.
Golücke, S., Dörrenberg, S., Krause, E. T., & Caspers, B. A. (2016). Female zebra finches smell their eggs. *PloS one, 11*(5), e0155513.
Haesler, S., Rochefort, C., Georgi, B., Licznerski, P., Osten, P., & Scharff, C. (2007). Incomplete and inaccurate vocal imitation after knockdown of FoxP2 in songbird basal ganglia nucleus Area X. *PLoS biology*, *5*(12), e321.
Hamdan, F. F., Daoud, H., Rochefort, D., Piton, A., Gauthier, J., Langlois, M., Foomani, G., Dobrzeniecka, S., Krebs, M.-O., & Joober, R. (2010). De novo mutations in FOXP1 in cases with intellectual disability, autism, and language impairment. *The American Journal of Human Genetics*, *87*(5), 671-678.
Han, J. Y. (2009). Germ cells and transgenesis in chickens. *Comparative immunology, microbiology and infectious diseases*, *32*(2), 61-80.
Han, J. Y., & Park, Y. H. (2018). Primordial germ cell-mediated transgenesis and genome editing in birds. *Journal of animal science and biotechnology*, *9*(1), 1-11.
Hauber, M. E., Louder, M. I., & Griffith, S. C. (2021). The natural history of model organisms: neurogenomic insights into the behavioral and vocal development of the zebra finch. *Elife*, *10*, e61849.
Hauser, M. D., Chomsky, N., & Fitch, W. T. (2002). The faculty of language: what is it, who has it, and how did it evolve? *science*, *298*(5598), 1569-1579.
Hemmings, N., & Birkhead, T. (2017). Differential sperm storage by female zebra finches Taeniopygia guttata. *Proceedings of the Royal Society B: Biological Sciences*, *284*(1860), 20171032.
Heston, J. B., & White, S. A. (2015). Behavior-linked FoxP2 regulation enables zebra finch vocal learning. *Journal of Neuroscience*, *35*(7), 2885-2894.
Hisey, E., Kearney, M. G., & Mooney, R. (2018). A common neural circuit mechanism for internally guided and externally reinforced forms of motor learning. *Nature neuroscience*, *21*(4), 589-597.
Holt, W. V., & Van Look, K. J. (2004). Concepts in sperm heterogeneity, sperm selection and sperm competition as biological foundations for laboratory tests of semen quality. *Reproduction*, *127*(5), 527-535.
Horn, D., Kapeller, J., Rivera-Brugués, N., Moog, U., Lorenz-Depiereux, B., Eck, S., Hempel, M., Wagenstaller, J., Gawthrope, A., & Monaco, A. P. (2010). Identification of FOXP1 deletions in three unrelated patients with mental retardation and significant speech and language deficits. *Human mutation*, *31*(11), E1851-E1860.
Houdebine, L.-M. (2007). Transgenic animal models in biomedical research. *Target discovery and validation reviews and protocols*, 163-202.
Houtman, A. M. (1992). Female zebra finches choose extra-pair copulations with genetically attractive males. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *249*(1324), 3-6.
Hrabia, A., Takagi, S., Ono, T., & Shimada, K. (2003). Fertilization and development of quail oocytes after intracytoplasmic sperm injection. *Biology of reproduction*, *69*(5), 1651-1657.

Huang, Z., Tamura, M., Sakurai, T., Chuma, S., Saito, T., & Nakatsuji, N. (2000). In vivo transfection of testicular germ cells and transgenesis by using the mitochondrially localized jellyfish fluorescent protein gene. *FEBS letters*, *487*(2), 248-251.

Hyland Bruno, J., Jarvis, E. D., Liberman, M., & Tchernichovski, O. (2021). Birdsong learning and culture: analogies with human spoken language. *Annual Review of Linguistics*, 449-472.

Immelmann, K. (1969). Song development in the zebra finch and other estrildid finches. *Bird vocalizations*, 61-77.

Immler, S., & Birkhead, T. R. (2005). A non-invasive method for obtaining spermatozoa from birds. *Ibis*, *147*(4), 827-830.

Itoh, Y., & Arnold, A. P. (2011). Zebra finch cell lines from naturally occurring tumors. *In Vitro Cellular & Developmental Biology-Animal*, *47*(4), 280-282.

Ivarie, R. (2003). Avian transgenesis: progress towards the promise. *TRENDS in Biotechnology*, *21*(1), 14-19.

Jarvis, E. D. (2019). Evolution of vocal learning and spoken language. *science*, *366*(6461), 50-54.

Jarvis, E. D., Güntürkün, O., Bruce, L., Csillag, A., Karten, H., Kuenzel, W., Medina, L., Paxinos, G., Perkel, D. J., & Shimizu, T. (2005). Avian brains and a new understanding of vertebrate brain evolution. *Nature reviews neuroscience*, *6*(2), 151-159.

Johnson, A. L. (2015). Reproduction in the female. In *Sturkie's avian physiology* (pp. 635-665). Elsevier.

Jones, R., & Lin, M. (1993). Spermatogenesis in birds. *Oxford reviews of reproductive biology*, *15*, 233-264.

Jung, K. M., Kim, Y. M., Keyte, A. L., Biegler, M. T., Rengaraj, D., Lee, H. J., Mello, C. V., Velho, T. A., Fedrigo, O., & Haase, B. J. T. F. J. (2019). Identification and characterization of primordial germ cells in a vocal learning Neoaves species, the zebra finch. *33*(12), 13825-13836.

Jung, K. M., Kim, Y. M., Kim, J. L., & Han, J. Y. (2021). Efficient gene transfer into zebra finch germline-competent stem cells using an adenoviral vector system. *Scientific reports*, *11*(1), 1-10.

Jung, K. M., Seo, M., Kim, Y. M., Kim, J. L., & Han, J. Y. (2021). Single-Cell RNA Sequencing Revealed the Heterogeneity of Gonadal Primordial Germ Cells in Zebra Finch (Taeniopygia guttata). *Frontiers in cell and developmental biology*, *9*, 791335-791335.

Karagenc, L., Cinnamon, Y., Ginsburg, M., & Petitte, J. (1996). Origin of primordial germ cells in the prestreak chick embryo. *Developmental genetics*, *19*(4), 290-301.

Kucera, A. C., & Heidinger, B. J. (2018). Avian semen collection by cloacal massage and isolation of DNA from sperm. *JoVE (Journal of Visualized Experiments)*(132), e55324.

Kulak, M., Takki, O., & Galkina, S. (2021). Cell culture establishment from zebra finch embryonic fibroblasts. *Adv. Anim. Vet. Sci*, *9*(3), 344-347.

Lai, C. S., Fisher, S. E., Hurst, J. A., Vargha-Khadem, F., & Monaco, A. P. (2001). A forkhead-domain gene is mutated in a severe speech and language disorder. *Nature*, *413*(6855), 519-523.

Lake, P., & Ravie, O. (1984). An exploration of cryoprotective compounds for fowl spermatozoa. *British Poultry Science*, *25*(1), 145-150.

Lam, D. M. (1972). Biosynthesis of acetylcholine in turtle photoreceptors. *Proceedings of the National Academy of Sciences*, *69*(7), 1987-1991.

Leung, M. C., Williams, P. L., Benedetto, A., Au, C., Helmcke, K. J., Achner, M., & Meyer, J. N. (2008). Caenorhabditis elegans: an emerging model in biomedical and environmental toxicology. *Toxicological sciences, 106*(1), 5-28.

Lillico, S. G., McGrew, M. J., Sherman, A., & Sang, H. M. (2005). Transgenic chickens as bioreactors for protein-based drugs. *Drug discovery today*, *10*(3), 191-196.

Lin, J., & Troyer, D. (2014). Testicular Anatomy and Physiology.

Lin, M., & Jones, R. (1992). Renewal and proliferation of spermatogonia during spermatogenesis in the Japanese quail, Coturnix coturnix japonica. *Cell and tissue research*, *267*(3), 591-601.

Liu, L., Cao, F., Cai, K., Zhang, Y., Ding, Z., & Li, J. (2011). Generation of Sperms Containing EGFP-LacZ Following Transfection of Chicken Testis with a Eukaryotic Dual Reporter Vector. *Reproduction in Domestic Animals*, *46*(1), e39-e45.

Lois, C., Hong, E. J., Pease, S., Brown, E. J., & Baltimore, D. (2002). Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *science*, *295*(5556), 868-872.

Lüpold, S., Calhim, S., Immler, S., & Birkhead, T. R. (2009). Sperm morphology and sperm velocity in passerine birds. *Proceedings of the Royal Society B: Biological Sciences*, *276*(1659), 1175-1181.

Macdonald, J., Glover, J. D., Taylor, L., Sang, H. M., & McGrew, M. J. (2010). Characterisation and germline transmission of cultured avian primordial germ cells. *PloS one*, *5*(11), e15518.

Macdonald, J., Taylor, L., Sherman, A., Kawakami, K., Takahashi, Y., Sang, H. M., & McGrew, M. J. (2012). Efficient genetic modification and germ-line transmission of primordial germ cells using piggyBac and Tol2 transposons. *Proceedings of the National Academy of Sciences*, *109*(23), E1466-E1472.

Majumdar, S. S., Usmani, A., Bhattacharya, I., Sarda, K., Gautam, M., Sharma, D., Basu, S., & Dhup, S. (2009). A method for rapid generation of transgenic animals to evaluate testis genes during sexual maturation. *Journal of reproductive immunology*, *83*(1-2), 36-39.

Mendoza, E., & Scharff, C. (2017). Protein-Protein interaction among the FoxP family members and their regulation of two target genes, VLDLR and CNTNAP2 in the Zebra Finch Song System. *Frontiers in Molecular Neuroscience*, *10*, 112.

Meyer, D. B. (1960). Application of the periodic acid-Schiff technique to whole chick embryos. *Stain Technology*, *35*(2), 83-89.

Meyer, D. B. (1964). The migration of primordial germ cells in the chick embryo. *Developmental biology*, *10*(1), 154-190.

Michaelis, M., Sobczak, A., & Weitzel, J. M. (2014). In vivo microinjection and electroporation of mouse testis. *Journal of visualized experiments: JoVE*(90).

Mizushima, S., Hiyama, G., Shiba, K., Inaba, K., Dohra, H., Ono, T., Shimada, K., & Sasanami, T. (2014). The birth of quail chicks after intracytoplasmic sperm injection. *Development*, *141*(19), 3799-3806.

Mizushima, S., Matsuzaki, M., & Sasanami, T. (2017). Handling of Gametes for In Vitro Insemination in Birds. In *Avian and Reptilian Developmental Biology* (pp. 243-257). Springer.

Mizushima, S., Takagi, S., Ono, T., Atsumi, Y., Tsukada, A., Saito, N., Sasanami, T., Okabe, M., & Shimada, K. (2010). Novel method of gene transfer in birds: intracytoplasmic sperm injection for green fluorescent protein expression in quail blastoderms. *Biology of reproduction*, *83*(6), 965-969.

Møller, A. P. (1994). Directional selection on directional asymmetry: testes size and secondary sexual characters in birds. *Proceedings of the Royal Society of London. Series B: Biological Sciences, 258*(1352), 147-151.

Morgan, A. T., Murray, E., & Liegeois, F. J. (2018). Interventions for childhood apraxia of speech. *Cochrane Database of Systematic Reviews*(5).

Morris, D. (1954). The reproductive behaviour of the zebra finch (Poephila guttata), with special reference to pseudofemale behaviour and displacement activities. *Behaviour*, *6*(1), 271-322.

Mossman, J., Slate, J., Humphries, S., & Birkhead, T. (2009). Sperm morphology and velocity are genetically codetermined in the zebra finch. *Evolution: International Journal of Organic Evolution*, *63*(10), 2730-2737.

Mozdziak, P., Angerman-Stewart, J., Rushton, B., Pardue, S., & Petitte, J. (2005). Isolation of chicken primordial germ cells using fluorescence-activated cell sorting. *Poultry Science*, *84*(4), 594-600.

Murray, J. R., Varian-Ramos, C. W., Welch, Z. S., & Saha, M. S. (2013). Embryological staging of the Zebra Finch, Taeniopygia guttata. *Journal of morphology*, *274*(10), 1090-1110.

Naito, M., Tajima, A., Tagami, T., Yasuda, Y., & Kuwana, T. (1994). Preservation of chick primordial germ cells in liquid nitrogen and subsequent production of viable offspring. *Reproduction*, *102*(2), 321-325.

Norton, P., Barschke, P., Scharff, C., & Mendoza, E. (2019). Differential song deficits after lentivirus-mediated knockdown of FoxP1, FoxP2, or FoxP4 in area X of juvenile zebra finches. *Journal of Neuroscience*, *39*(49), 9782-9796.

Nottebohm, F., Nottebohm, M. E., & Crane, L. (1986). Developmental and seasonal changes in canary song and their relation to changes in the anatomy of song-control nuclei. *Behavioral and neural biology*, *46*(3), 445-471.

Oishi, I., Yoshii, K., Miyahara, D., Kagami, H., & Tagami, T. (2016). Targeted mutagenesis in chicken using CRISPR/Cas9 system. *Scientific reports*, *6*(1), 1-10.

Ono, T., & Machida, Y. (1999). Immunomagnetic purification of viable primordial germ cells of Japanese quail (Coturnix japonica). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *122*(2), 255-259.

Park, T. S., & Han, J. Y. (2012a). Genetic modification of chicken germ cells. *Annals of the New York Academy of Sciences*, *1271*(1), 104.

Park, T. S., & Han, J. Y. (2012b). piggyBac transposition into primordial germ cells is an efficient tool for transgenesis in chickens. *Proceedings of the National Academy of Sciences*, *109*(24), 9337-9341.

Park, T. S., Lee, H. J., Kim, K. H., Kim, J.-S., & Han, J. Y. (2014). Targeted gene knockout in chickens mediated by TALENs. *Proceedings of the National Academy of Sciences*, *111*(35), 12716-12721.

Parker, G. (1998). Sperm competition and the evolution of ejaculates: towards a theory base. *Sperm competition and sexual selection*, 3-54.

Petkov, C. I., & Jarvis, E. (2012). Birds, primates, and spoken language origins: behavioral phenotypes and neurobiological substrates. *Frontiers in evolutionary neuroscience*, *4*, 12.

Pfenning, A. R., Hara, E., Whitney, O., Rivas, M. V., Wang, R., Roulhac, P. L., Howard, J. T., Wirthlin, M., Lovell, P. V., & Ganapathy, G. (2014). Convergent transcriptional specializations in the brains of humans and song-learning birds. *science*, *346*(6215).

Prather, J. F., Okanoya, K., & Bolhuis, J. J. (2017). Brains for birds and babies: Neural parallels between birdsong and speech acquisition. *Neuroscience & Biobehavioral Reviews*, *81*, 225-237.

Raven, C. P. (2013). *Oogenesis: The storage of developmental information*. Elsevier.

Reiner, A., Perkel, D. J., Bruce, L. L., Butler, A. B., Csillag, A., Kuenzel, W., Medina, L., Paxinos, G., Shimizu, T., & Striedter, G. (2004). Revised nomenclature for avian telencephalon and some related brainstem nuclei. *Journal of Comparative Neurology*, *473*(3), 377-414.

Rieth, A., Pothier, F., & Sirard, M. A. (2000). Electroporation of bovine spermatozoa to carry DNA containing highly repetitive sequences into oocytes and detection of homologous recombination events. *Molecular Reproduction and Development: Incorporating Gamete Research*, *57*(4), 338-345.

Rinkwitz, S., Mourrain, P., & Becker, T. S. (2011). Zebrafish: an integrative system for neurogenomics and neurosciences. *Progress in neurobiology, 93*(2), 231-243.

Rottmann, O., Antes, R., Hoefer, P., & Maierhofer, G. (1992). Liposome mediated gene transfer via spermatozoa into avian egg cells. *Journal of animal breeding and genetics*, *109*(1-6), 64-70.

Sakata, J. T., & Birdsong, D. (2022). Vocal Learning and Behaviors in Birds and Human Bilinguals: Parallels, Divergences and Directions for Research. *Languages*, *7*(1), 5.

Salter, D. W., Smith, E. J., Hughes, S. H., Wright, S. E., & Crittenden, L. B. (1987). Transgenic chickens: insertion of retroviral genes into the chicken germ line. *Virology*, *157*(1), 236-240.

Salter, D. W., SMITH, E. J., HUGHES, S. H., WRIGHT, S. E., FADLY, A. M., WITTER, R. L., & CRITTENDEN, L. B. (1986). Gene insertion into the chicken germ line by retroviruses. *Poultry Science*, *65*(8), 1445-1458.

Samour, J., Smith, C., Moore, H., & Markham, J. (1986). Semen collection and spermatozoa characteristics in budgerigars (Melopsittacus undulatus). *The Veterinary Record*, *118*(14), 397-399.

Samour, J. H. (2002). The reproductive biology of the budgerigar (Melopsittacus undulatus): semen preservation techniques and artificial insemination procedures. *Journal of avian medicine and surgery*, *16*(1), 39-49.

Samour, J. H. (2004). Semen collection, spermatozoa cryopreservation, and artificial insemination in nondomestic birds. *Journal of avian medicine and surgery*, *18*(4), 219-223.

Schusser, B., Collarini, E. J., Yi, H., Izquierdo, S. M., Fesler, J., Pedersen, D., Klasing, K. C., Kaspers, B., Harriman, W. D., & van de Lavoir, M.-C. (2013). Immunoglobulin knockout chickens via efficient homologous recombination in primordial germ cells. *Proceedings of the National Academy of Sciences*, *110*(50), 20170-20175.

Sehgal, L., Thorat, R., Khapare, N., Mukhopadhaya, A., Sawant, M., & Dalal, S. N. (2011). Lentiviral mediated transgenesis by in vivo manipulation of spermatogonial stem cells. *PloS one*, *6*(7), e21975.

Sin, F., Bartley, A., Walker, S., Sin, I., Symonds, J., Hawke, L., & Hopkins, C. (1993). Gene transfer in chinook salmon (Oncorhynchus tshawytscha) by electroporating sperm in the presence of pRSV-lacZ DNA. *Aquaculture*, *117*(1-2), 57-69.

Sun, P., Zhang, Z., Wu, G., Yan, L., Yuan, F., Zhang, W., Gao, J., Jin, W., & Li, Z. (2012). Transgenic chimera quail production by microinjecting lentiviral vector into the blood vessel of the early embryo. *Animal Science Journal*, *83*(4), 291-298.

Taylor, T., & Breed, W. (2000). Do females determine the breeding system in Spinifex Hopping Mice Notomys alexis?

Teramitsu, I., Kudo, L. C., London, S. E., Geschwind, D. H., & White, S. A. (2004). Parallel FoxP1 and FoxP2 expression in songbird and human brain predicts functional interaction. *Journal of Neuroscience*, *24*(13), 3152-3163.

Trefil, P., Aumann, D., Koslová, A., Mucksová, J., Benešová, B., Kalina, J., Wurmser, C., Fries, R., Elleder, D., & Schusser, B. (2017). Male fertility restored by transplanting primordial germ cells into testes: a new way towards efficient transgenesis in chicken. *Scientific reports*, *7*(1), 1-9.

Usmani, A., Ganguli, N., Sarkar, H., Dhup, S., Batta, S. R., Vimal, M., Ganguli, N., Basu, S., Nagarajan, P., & Majumdar, S. S. (2013). A non-surgical approach for male germ cell mediated gene transmission through transgenesis. *Scientific reports*, *3*(1), 1-8.

Van de Lavoir, M.-C., Diamond, J. H., Leighton, P. A., Mather-Love, C., Heyer, B. S., Bradshaw, R., Kerchner, A., Hooi, L. T., Gessaro, T. M., & Swanberg, S. E. (2006). Germline transmission of genetically modified primordial germ cells. *Nature*, *441*(7094), 766-769.

Velho, T. A., & Lois, C. (2014). Generation of transgenic zebra finches with replication-deficient lentiviruses. *Cold Spring Harbor Protocols*, *2014*(12), pdb. prot084608.

Vick, L., Li, Y., & Simkiss, K. (1993). Transgenic birds from transformed primordial germ cells. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *251*(1332), 179-182.

Wall, R. J. (2001). Pronuclear microinjection. *Cloning & Stem Cells*, *3*(4), 209-220.

Wang, W., Lin, C., Lu, D., Ning, Z., Cox, T., Melvin, D., Wang, X., Bradley, A., & Liu, P. (2008). Chromosomal transposition of PiggyBac in mouse embryonic stem cells. *Proceedings of the National Academy of Sciences*, *105*(27), 9290-9295.

Whyte, J., Glover, J. D., Woodcock, M., Brzeszczynska, J., Taylor, L., Sherman, A., Kaiser, P., & McGrew, M. J. (2015). FGF, insulin, and SMAD signaling cooperate for avian primordial germ cell self-renewal. *Stem cell reports*, *5*(6), 1171-1182.

Wood, W. E., Roseberry, T. K., & Perkel, D. J. (2013). HTR2 receptors in a songbird premotor cortical-like area modulate spectral characteristics of zebra finch song. *Journal of Neuroscience*, *33*(7), 2908-2915.

Woolley, S. C., & Doupe, A. J. (2008). Social context–induced song variation affects female behavior and gene expression. *PLoS biology*, *6*(3), e62.

Xiao, L., Chattree, G., Oscos, F. G., Cao, M., Wanat, M. J., & Roberts, T. F. (2018). A basal ganglia circuit sufficient to guide birdsong learning. *Neuron*, *98*(1), 208-221. e205.

Yasuda, Y., Tajima, A., Fujimoto, T., & Kuwana, T. (1992). A method to obtain avian germ-line chimaeras using isolated primordial germ cells. *Reproduction*, *96*(2), 521-528.

Yomogida, K., Yagura, Y., Tadokoro, Y., & Nishimune, Y. (2003). Dramatic expansion of germinal stem cells by ectopically expressed human glial cell line-derived neurotrophic factor in mouse Sertoli cells. *Biology of reproduction*, *69*(4), 1303-1307.

Zann, R. A. (1996). *The zebra finch: a synthesis of field and laboratory studies* (Vol. 5). Oxford University Press.

Zhao, W., Garcia-Oscos, F., Dinh, D., & Roberts, T. F. (2019). Inception of memories that guide vocal learning in the songbird. *science*, *366*(6461), 83-89.

Appendix

App.1 List of all plasmids

Name	No.	Description	Reporter	Resistance	Source
pVsVg (pHCMV)	#14	Viral vector envelope vector expressing the vesicular stomatitis virus glycoprotein	-	Ampicillin	Custom made
pLP1	#15	Viral vector packaging vector (gag/pol)	-	Ampicillin	Invitrogen
pLP2	#16	Viral vector packaging vector (rev)	-	Ampicillin	Invitrogen
pGL4.13minusSv40	#30	VLDLR promoter	-	Ampicillin	Lab. intern cloning (Ursula Kobalz)
pCR4Blunt-TOPO	#34	VLDLR promoter	-	Kanamycin	Lab. intern cloning (Ursula Kobalz)
pGL4.13	#53	Luciferase reporter vector	-	Ampicillin	Promega
pFUGW-BstBILinker	#62	BstBILinker	eGFP	Ampicillin	Lab. intern cloning (Ursula Kobalz)
pcDNA3.1+FoxP1-FLAG	#86	Zf FoxP1-FLAG	-	Ampicillin, Neomycin, Kanamycin	Lab. intern cloning (Ursula Kobalz)
px459_pspCas9	#127	Kozak-FLAG-SV40NLS-cas9-NucleoplasminNLS-T2A-Puro	-	Ampicillin, Puromycin	Addgene 48139
pCrispr_PTPN5	#129	Kozak-V5-SV40NLS-cas9-SV40NLS-T2A-OFP	OFP	Ampicillin	Invitrogen
pPB-CAAG mcherry-PB	#132	recognition of PB arms by HypBase	mcherry	Ampicillin	Lab. intern cloning (Sina Girra)
pCR4Blunt-TOPO FoxP2enhancer	#135	FoxP2enhancer	-	Kanamycin	Lab. intern cloning (Ursula Kobalz)
pFUGW FoxP2enhancer	#137	FoxP2enhancer+ß-Globin	eGFP	Ampicillin/Bleomycin	Custom made based on (Lois et al., 2002)
pCAAG-FRT-RAGE-eGFP	#140	FRT-RAGE-FRT-eGFP	eGFP	Ampicillin	The Hebrew University of Jerusalem
pNgn1 D2 enhancer.FLPo	#141	Ngn1 D2 enhancer+FLPo	-	Ampicillin	The Hebrew University of Jerusalem
pPB-PB-LoxP-RAGE-LoxP-MBmcherry-T2A	#146	PBarm1-PBarm2-LoxP-RAGE-LoxP-MBmcherry-T2A	mcherry	Ampicillin	The Hebrew University of Jerusalem
pCAAG hypBase	#148	mammalian expression vector for hypBase	-	Ampicillin	The Hebrew University of Jerusalem
pPB-PB-CAAG-LoxP-RAGE-LoxP-GFP	#152	LoxP-RAGE-LoxP-GFP.S65T	GFP	Ampicillin	The Hebrew University of Jerusalem
pX459-hCas9 NheI EcoRI	#177	Kozak-V5-SV40NLS-cas9-SV40NLS	-	Ampicillin	Lab. intern cloning (Ursula Kobalz)
pX459-hCas9 NheI Puromycin EcoRI	#179	Kozak-V5-SV40NLS-cas9-SV40NLS-T2A-Puro	-	Ampicillin, Puromycin	Lab. intern cloning (Ursula Kobalz)

pPB-CAAG-FoxP1V5-T2APuro-PB	#207	PBarm1-CAAG-FoxP1V5-T2APuro-PBarm2	-	Ampicillin, Puromycin	Lab. intern cloning (Ursula Kobalz)
pAAV mDLX-ChR2-Fishell3	#218	mDLX-ChR2-Fishell3	mcherry	Ampicillin	Addgene #83898
pPB-CAAG eGFP-PB	#316	recognition of PB arms by HypBase	eGFP	Ampicillin	Lab. intern cloning (Sina Girra)
pAAV synP-FLEX-TVA66T-eGFP-B19G	#387	synP-FLEX-TVA66T-eGFP-B19G	eGFP	Ampicillin	Addgene #64097
pAAV mDLX-TVA66T-eGFP-B19G	#389	mDLX-TVA66T-eGFP-B19G	eGFP	Ampicillin	Lab. intern cloning (Ursula Kobalz)
pcdna3.1-ChRime-TS-eYFP-ER	#432	ChRime-TS-eYFP-ER	eYFP	Kanamycin, Neomycin	AG Hegemann MDC
pCMV-CoChR-mScarlet	#434	CoChR-mScarlet	mScarlet	Ampicillin, Kanamycin, Neomycin	AG Hegemann MDC
pCMV-CsChrimson-mCerulean3-0	#435	CsChrimson-mCerulean3.0	mCerulean3.0	Kanamycin, Neomycin	AG Hegemann MDC
pAAV mDLX-TS-eYFP-ER	#436	mDLX-TS-eYFP-ER	eYFP	Ampicillin	AG Hegemann MDC
pAAV mDLX-CsChrimson-TS-eYFP-ER	#441	mDLX-CsChrimson-TS-eYFP-ER	eYFP	Ampicillin	Lab. intern cloning (Ursula Kobalz)
pAAV mDLX-CoChR-TS-eYFP-ER	#442	mDLX-CoChR-TS-eYFP-ER	eYFP	Ampicillin	Lab. intern cloning (Ursula Kobalz)
pPB-PB	#457	PBarm1-PBarm2	-	Ampicillin	Lab. intern cloning (Ursula Kobalz)
pPB-mDLX-CoChR-PB	#459	interneuron specific expression of CoChR	eYFP	Ampicillin	Lab. intern cloning (Ursula Kobalz)
pPB-mDLX-Chrimson-PB	#460	interneuron specific expression of Chrimson	eYFP	Ampicillin	Lab. intern cloning (Ursula Kobalz)