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Transmission of the EGFP Transgene in Zebrafish Progeny

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Abstract

Zebrafish (*Danio rerio*) are widely used as model organisms in biomedical research, particularly in studies involving mutagenesis and transgenesis. Transgenic zebrafish, which express fluorescent proteins from a single transgene copy, are commercially available and exhibit distinct phenotypic traits. To investigate the inheritance pattern of the fluorescent phenotype, crossbreeding was performed between transgenic and wild-type individuals. The results revealed a marked difference in transgene inheritance depending on parental origin, with maternal transmission resulting in a significantly higher frequency of EGFP-positive offspring. Additionally, the appearance of wild-type individuals in the F1 generation from fluorescent parents suggests heterozygosity in the parental generation. These findings provide insights into the inheritance dynamics of the EGFP transgene and the genetic composition of the parental stock.

Keywords: Zebrafish, *EGFP*, inheritance, fluorescent phenotype

Introduction

The primary reason for using zebrafish in research is the high percentage of human genes that have zebrafish orthologue (Postlethwait et al. 1998), their well studied embryonic and physiological development, and the relatively easy conditions they require in aquatic cultures. Previous studies that put zebrafish in the spotlight, have enabled researchers to study the molecular mechanisms responsible for some neurodegenerative diseases (Guo 2004), muscular dystrophy (Bassett & Currie 2004), regeneration of the heart muscle (Poss et al. 2002, 2003), hematopoietic and immune diseases (Trede et al. 2004), cancer (Patton & Zol 2004), and more. Although the zebrafish are a modern cornerstone as a model organism, their significance is elevated through transgenesis with fluorescent genes. These genes can be used as reporter genes in specific tissues to monitor gene expression, or specific localization of a protein in a cell. The transgene encodes a specific fluorescent protein that is easily detectable to the naked eye, due to the transparency of the body of the early embryos. Due to advantages like these, the transgenic zebrafish are used to develop and optimize various number of methodologies and technologies regarding their growth and physiology and consequently, to produce transgenic lines that will express these transgenes *in vivo* the same way they express their own, constitutive genes (Linney et al. 1999).

The method of transgenesis via microinjection into early – stage embryos carries certain degree of instability, because it is considered that from this first generation the inheritance can be with unpredictable patterns. As a result of uneven integration of the transgene into the genome, each individual fish is considered to have a unique genotype, even if they all express the fluorescent protein. After this generation, the transgene is considered to be stabilized if it's passed on to the subsequent offspring (Mosimann et al. 2011).

The main goal of this experiment is to determine the inheritance pattern and frequency of the *EGFP* (Enhanced Green Fluorescent Protein) transgene in the first filial generation of a commercially acquired zebrafish population, based on phenotypic traits, and without any prior information on their genotype.

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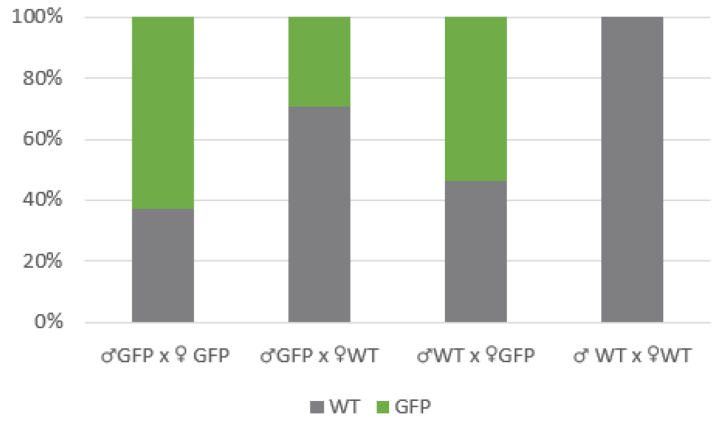


Fig. 1. Percent of wild-type and transgenic phenotypes in the offspring from four different crossbreeding groups

Materials and Methods

Adult *Danio rerio* individuals used in this study were commercially acquired from certified suppliers. Both wild-type and transgenic lines expressing Enhanced Green Fluorescent Protein (EGFP) were included. The transgenic fish were obtained already carrying the EGFP transgene; no additional procedures of transgenesis or microinjection were performed as part of this study. Fish were maintained under standard laboratory conditions, in accordance with established zebrafish care protocols (Westerfield 2000).

The primary strategy to achieve the goal of the experiment was to perform crossbreeding between wild-type and transgenic fish in different ratios (Table 1). Four tanks were used for this purpose, with identical conditions as follows: 10 liters of deionized water, heater (28 ºC – optimal temperature for breeding), thermometer, air pumps, electronic lighting timer (16 hours day/8 hours night). On the bottom of the tanks there was a layer of smooth glass stones that imitated a natural breeding ground and also prevent egg and embryo predation by adult fish.

The first two tanks, or the experimental groups, gave insight into differences in inheritance of the transgene, if the transgene is passed maternally or paternally in the offspring. The third and fourth tank, or the control groups, were used to asses the presence of possible heterozygotes in the parental generation and also to rule out previous possibilities of mosaicism in the first filial generation.

The collection of eggs was usually on every two or three days, so the fish can adequately recover after every breeding process. The eggs and embryos were siphoned and 50% of the tank water was replaced. All collected material in this process was a subject to microscopic analysis with light microscope or stereomicroscope. The fertilized embryos were kept in a beaker with deionized water at 28ºC supplemented with 2-3 ml of embryo medium per liter water (stock solution of 4g NaCl per 100 mL) The mortality rate of *Danio rerio* is usually high, so in each breeding cycle, only around 10-15% of embryos survive. These embryos were analyzed and marked upon detection, but the earliest stage of development where the pigment is clearly indicated, is at least 72 hours post fertilization (hpf).

All collected data was subjected to statistical analysis in order of proper mathematical interpretation of the results. The statistical test used was the chi (χ^2) test with one degree of freedom DF=1. A statistically significant difference between observed and expected values was indicated when the χ^2 value was greater than 3.841 ($p = 0.05$).

Results

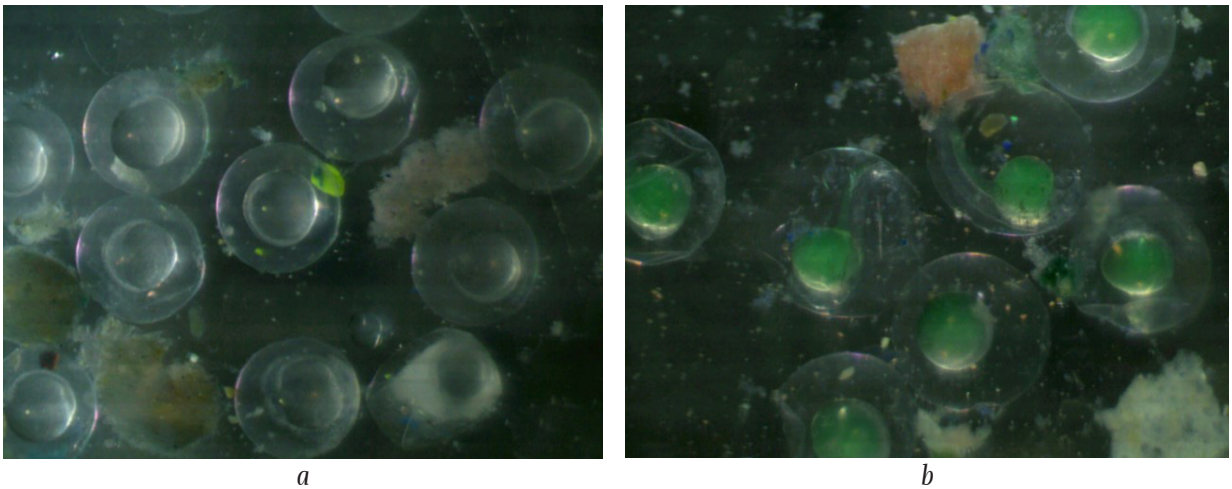
The offspring from both of the tanks that contained the experimental groups, included both wild-type and fish with expression of GFP. This was a clear evidence that the inheritance of the transgene is not sex-linked. However, there was a significant difference in

Table 1. Experimental grouping of zebrafish in 4 tanks based on sex and phenotype

Tank	Female phenotype	Number of females	Male phenotype	Number of males	Total number of fish
1	WT	5	GFP	3	8
2	GFP	5	WT	3	8
3	WT	5	WT	3	8
4	GFP	5	GFP	3	8

the number of GFP positive offspring in the first filial generation (Figure 1). The tank in which the transgenic females were crossed with wild-type male zebrafish, had a larger percentage of GFP positive offspring than the group from the tank where wild type females were crossed with transgenic males.

Another major distinction between the offspring was the presence of GFP in the yolk sac of larvae (until 72 hpf) from the tanks that had transgenic females (Figures 2a, 2b and 3). This presence of GFP was independent of the presence of the GFP in their bodies, meaning that even wild-type offspring whose maternal generation was transgenic , will have GFP in their yolk sac but not necessarily inherit the transgene. The presence of GFP in the yolk sac of larvae is the result of the presence of GFP in the oocytes of the transgenic females, in this case, from the parental generation. Expression of GFP in the yolk sac of wild-type offspring from transgenic females likely results from the maternal deposition of either GFP mRNA or protein, as described by Udvadia and Linney (2003). This early GFP expression occurs prior to the activation of zygotic transcription, which begins at the 1000-cell stage in zebrafish embryos. Therefore, the GFP observed in these embryos before this stage cannot be attributed to the embryo's own transcription, but rather to maternal loading, which is a well-documented phenomenon in zebrafish development (Udvadia & Linney, 2003).



a *b*

Fig. 2. Embryos that are offspring of the wild-type females have clear yolk sacs (a), whereas embryos derived from transgenic females have GFP in their yolk sac (b)

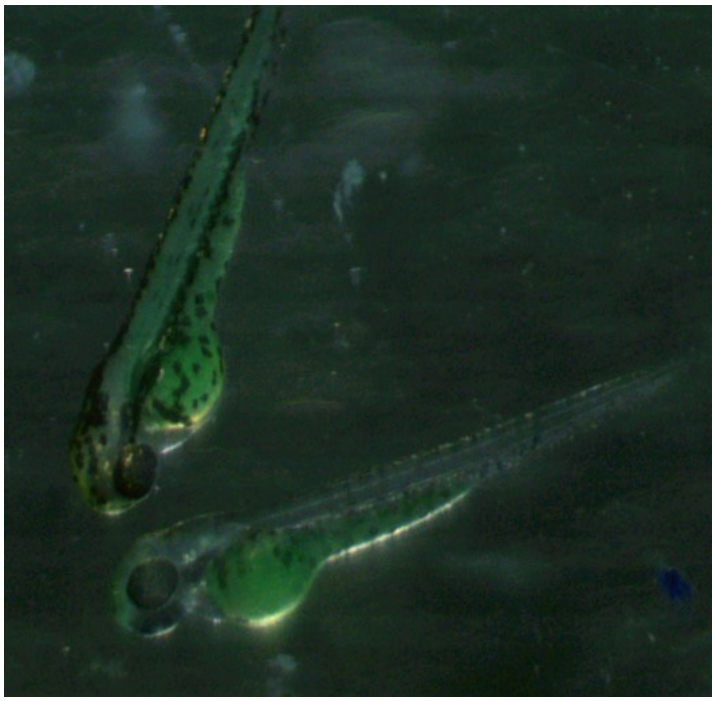


Fig. 3. Presence of GFP in the yolk sac was observed in larvae both with fluorescent pigmentation and in wild-type larvae lacking body fluorescence.

Some of the transgenic offspring exhibited variability among themselves, as it was found out that there is a difference in the intensity of the fluorescence (Figure 4). Even though there was a clear expression of GFP and fluorescent phenotype, some fish had bright green fluorescence, some had electric green, and some darker fluorescent green. At first it was suspected that there were some intermediate forms, but as other authors suggest, this might have been a consequence of the different number of copies of the transgene incorporated in the genome during transgenesis, and how many copies will the offspring inherit (Vick 2012).

Fig. 4. Difference in the intensity of fluorescence in two transgenic larvae from the offspring

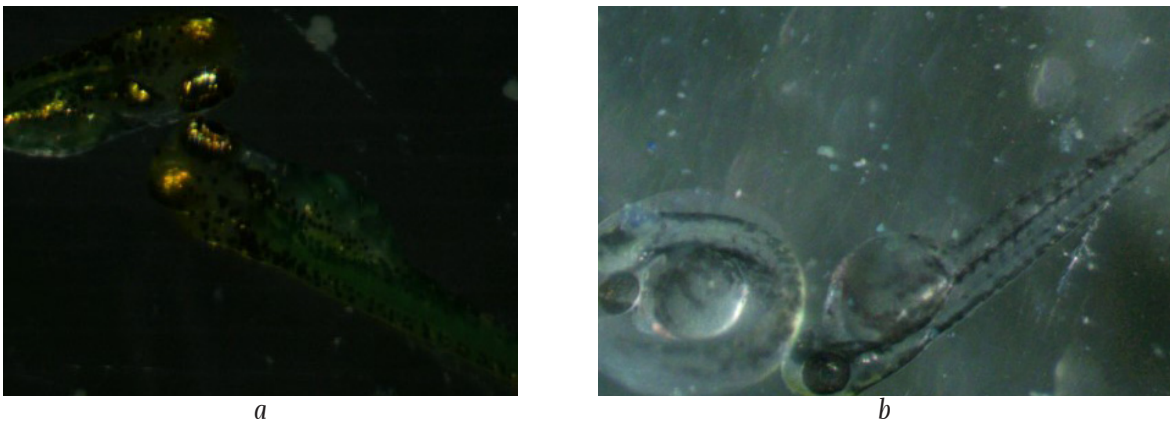
There was no form of mosaicism found, even though at first it was suspected due to the characteristic bright yellow fluorescence observed in the larvae. Upon observing this fluorescence even in the offspring of the wild-type control groups, it was concluded that this, in fact, was autofluorescence of the brain, visible in the larvae due to the transparency of their body (Figures 5a and 5b).

One of the most profound findings from this experiment was a wild-type offspring whose parental generation consisted of transgenic males and females. This proves the presence of heterozygotes among the transgenic population. With further molecular analysis it is possible to find out exactly how many are heterozygotes, but even with just phenotypic observation of their offspring, their presence is clear. This conclusion leads also to another one, that indeed there is need of only one copy of *EGFP* in the genome for the GFP to be expressed, since *EGFP* is dominant over the genes responsible for pigmentation in zebrafish.

Discussion

The primary objective for this experiment was to determine the inheritance pattern of the transgene, wheter it follows the Mendelian principles and patterns, or if there are other factors that influence the inheritance. Since the parental generation was with unknown genotype, phenotypic traits were the main focus of interest to gather data and results. The variability in the intensity of GFP fluorescence is in strong correlation with the number of transgene copies inserted in the genome (Vick 2012), and it represents the consistency of the expression of this transgene and its inheritance. That is why the difference in the intensity of fluorescence is actually in correlation with the variation of the expression of the transgene (Gibbs & Schmale 2000).

The frequency of inheritance of *EGFP* is statistically measured with chi-square test comparing the observed number of offspring with the expected Mendelian ratios This test is based upon the assumption that expected frequencies are different from zero and is used to evaluate the null hypothesis that no significant difference exists between observed and expected



a *b*

Fig. 5. Autofluorescence of the brain in transgenic (a) and wild-type larvae (b)

Table 2. Expected and observed results for the offspring from the control group with wild-type parental generation

Offspring phenotype	Expected ♂ WT x ♀WT	Observed ♂ WT x ♀WT
WT	100	100
GFP	0	0

outcomes. In this case the expected and actual results were in agreement (Table 2).

Accordingly, we start with the assumption that the inheritance pattern in question follows the rules of Mendelian monohybrid inheritance, and therefore the group with transgenic males and females would give 100% GFP offspring, under the condition that these zebrafish have more than one inserted copy of the transgene in their genome. Otherwise we can assume that they are not genetically authentic, pure lineage fish (Tables 3-5).

Assumption 1: The parental generation is homozygous dominant for *EGFP.*

P: ♀AA x ♂AA

F1: AA (100% probability for GFP positive)

Assumption 2: The parental generation is comprised of heterozygotes, and 75% of the offspring are expected to express GFP, while 25% are expected to be wild-type.

P: ♀Aa x ♂Aa

F1: AA, Aa, Aa, aa

Assumption 3: Only one of the parents is a heterozygote, and the expected outcome is 100% probability that the offspring will express GFP, even if they inherit one or more copies of the transgene.

P: ♀AA x ♂Aa

F1: AA, Aa (50%)

A number of research studies, such as the one from Gibbs & Schmale (2000), state that *EGFP* exibits incomplete dominance and variable expression. They report results of 100% GFP offspring from homozygous parental generation, and a 50% GFP offspring in crosses between heterozygotes for *EGFP*.

The analyzed groups with mixed ratios of wild-type and transgenic zebrafish exhibited differing offspring distributions (Tables 6 and 7). When transgenic males (assumed Aa) were crossed with wild-type females (aa), a significant deviation from the expected Mendelian 1:1 ratio was observed ($\chi^2 = 9.93$, $p < 0.05$). In contrast, the reciprocal cross–transgenic females (assumed Aa) with wild-type males (aa)–did not significantly deviate from the expected ratio ($\chi^2 = 1.01$, $p > 0.05$). These results support the hypothesis that maternal inheritance of GFP may be more stable or efficient in some populations, possibly due to maternal loading of mRNA or proteins, or varying zygosity and transgene copy numbers between sexes. The results also highlight the need to verify zygosity using molecular methods to better interpret phenotypic ratios. One of the possible reasons for the higher frequency of inheritance of EGFP in the tank with transgenic females and wild type males could be the selection of a mate with better traits (Vargas et al. 2018). On the other hand, this could as well be a result of more copies inserted in the genome of the transgenic females than in transgenic males. Krøvel & Olsen (2002) explain about conserved *vas* trancripts and VAS protein

Table 3. Expected and observed number of offspring from the transgenic crossbred group (assumed homozygous dominant individuals)

Offspring phenotype	Expected ♂GFP x ♀ GFP	Observed ♂GFP x ♀ GFP
WT	0	85
GFP	229	144

Table 4. Expected and observed number of offspring from the transgenic crossbred group (heterozygotes)

Offspring phenotype	Expected ♂GFP x ♀ GFP	Observed ♂GFP x ♀ GFP
WT	57.25	85
GFP	171.75	144

Table 5. Expected and observed number of offspring from the transgenic crossbred group (one heterozygous parent)

Offspring phenotype	Expected ♂GFP x ♀ GFP	Observed ♂GFP x ♀ GFP
WT	0	85
GFP	229	144

Table 6. Results from the analyzed group with transgenic males and wild-type females

Offspring phenotype	Expected ♂GFP x ♀WT	Observed ♂GFP x ♀WT
WT	29	**41**
GFP	29	**17**

Table 7. Results from the analyzed group with transgenic females and wild-type males

Offspring phenotype	Expected ♂WT x ♀GFP	Observed ♂WT x ♀GFP
WT	97	90
GFP	97	104

closely connected with a conserved signaling pathway that controls the development of gametes. The Vas::EGFP transcripts inherited through the maternal line are segregated in the primordial gametes during early embryogenesis and are stabilized after 50hpf. In contrast, this transcript is not found in individuals that are expected to inherit it from the paternal line, likely due to absence of some of the regulatory elements in those pathways.

The chi-square test was used to evaluate the null hypothesis that GFP and wild-type phenotypes in the offspring would segregate in a 1:1 ratio, as expected for an Aa × aa cross.

Chi-square(χ^2)=9.93
Since $\chi^2 > 3.841$ ($p < 0.05$), this indicates a statistically significant deviation from the expected 1:1 ratio. The null hypothesis is rejected.

Chi-square(χ^2)=1.01
Since $\chi^2 < 3.841$ ($p > 0.05$), the observed values do not significantly deviate from the expected 1:1 ratio. The null hypothesis is not rejected.

These results suggest that in the cross involving transgenic males, the inheritance of GFP significantly deviates from a simple Mendelian expectation, while in the reciprocal cross with transgenic females, it does not.

Conclusion

This study highlights the effectiveness of using transgenic and wild-type zebrafish crosses to investigate the inheritance dynamics of the EGFP transgene. The results demonstrated a clear maternal bias in transgene transmission, with significantly higher frequencies of fluorescent phenotypes observed when the transgene was maternally inherited. Additionally, the presence of wild-type phenotypes in the first filial generation from fluorescent parents strongly suggests heterozygosity in the parental generation. These findings emphasize the importance of considering both parental origin and genotype when interpreting phenotypic outcomes in transgenic models. Future studies should apply molecular techniques such as genomic PCR or transcriptome analysis to verify the zygosity of transgenic individuals and explore the mechanisms behind maternal deposition of fluorescent proteins in early-stage embryos.

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