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Spatiotemporal expression patterns of maternal-zygotic gene *pou5f3* in hybrid fish embryos

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ABSTRACT

Maternal-zygotic genes play important regulatory roles in early embryonic development. Therefore, this study investigated the spatiotemporal expression patterns of *pou5f3* in goldfish (♀) × rare gudgeon (♂) [GFRG] and gudgeon (♀) × goldfish (♂) [RGGF] embryos to explore the role of maternal-zygotic genes in hybrid embryonic development. The distant hybridization of goldfish and rare gudgeon was carried out to monitor embryonic development and *pou5f3* expression. GFRG produced offspring, whereas RGGF embryos developed abnormally and eventually died. The development timing of the hybrid embryos was similar to that of their female parents. Gene sequence comparison showed that the two types of *pou5f3* in GFRG were similar to those of the female parent goldfish (GF) and showed chimeric characteristics. In contrast, RGGF had two types of *pou5f3* that were similar to GF and rare gudgeon (RG). Furthermore, real-time quantitative PCR and whole mount *in situ* hybridization showed that the spatiotemporal expression pattern of *pou5f3* in parental GF and RG were different at the starting point of zygotic gene expression. The spatiotemporal expression pattern of *pou5f3* in GFRG was similar to that of maternal GF, whereas the expression start and end of pou5f3 in RGGF were earlier than those of the parents and GFRG. These results indicate significant differences in the spatiotemporal expression patterns of *pou5f3* in RGGF embryos compared with those of the parents and GFRG. Thus, the abnormal spatiotemporal expression of maternal zygotic genes in hybrid embryos may be one reason for the death of hybrid embryos. These findings elucidate the possible mechanisms underlying reproductive isolation and provide a theoretical basis for effective crossbreeding.

1. Introduction

During embryonic development, zygotic gene transcription is only initiated after one–ten cell divisions in fertilized animal eggs (Schulz and Harrison, 2019). The initial expression of zygotic genes is usually accompanied by a sudden slowing of cell division, loss of synchronization, and changes in the cell phenotype in some species (Jukam et al., 2017). This phenomenon during embryonic development is called the midblastula transition (Jukam et al., 2017). Therefore, the regulatory factors required for embryonic development are supplied by the eggs when the zygotic genome is not activated for transcription (Xu and Meng, 2014). The maternal gene typically expresses products, such as proteins or RNA, in mature eggs. Moreover, its expression product is called the maternal factor. Genes that are transcribed only after fertilization are called zygotic genes and those that are transcribed during the ovum and zygotic stages are called maternal-zygotic genes (Xu and Meng, 2014).

Maternal zygotic genes function as both maternal and zygotic genes that are transcribed as maternal genes to produce maternal factors (RNA or proteins) during ovulation (Lee et al., 2014). Maternal factors are gradually consumed and zygotic genes are transcribed as the embryo develops after fertilization (Lee et al., 2014). Therefore, compared with pure maternal or homozygous genes, maternal-zygotic genes have a longer expression period and more extensive regulatory functions, which play an important role in the normal development of embryos (Yu et al., 2016). Moreover, correct temporal and spatial expression of maternal, maternal zygotic, and zygotic genes facilitate the normal development of embryos (Yu et al., 2016).

Pou5f3 belongs to the maternal-zygotic gene family. Its expression product, Pou5f3, is a member of the Class V family of POU transcription

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factors (Frankenberg and Renfree, 2013). These transcription factors are synthesized in both the egg and zygote and regulate key genes involved in cell determination and proliferation (Yang and Wu, 1999). The maternal expression product of *pou5f3*, a transcription factor, initiates the expression of the embryonic zygote genome. Additionally, its zygote expression product regulates embryonic development (Voronina and Pshennikova, 2016). In zebrafish, the maternal Pou5f3 factor and Sox family members synergistically activate the expression of early zygotic genes and control the temporal coordination of gene expression programs (Kehler et al., 2004). Pou5f3 binds to the promoter before the mid blastocyst transition to activate the expression of 7747 zygotic genes, including many important signaling pathway member genes, such as *bmp2b*, *dkk1*, *lefty1/2*, and *sdf2*. (Leichsenring et al., 2013). Moreover, Pou5f3 binds to its own promoter to initiate zygotic expression (Xu and Meng, 2014). The zygotic gene is not normally activated when Pou5f3, Soxb1, and Nanog are simultaneously absent from the embryo, resulting in delayed embryonic development and abnormal gastral movement (Lee et al., 2013). Similarly, various developmental defects at the time of embryo formation kill mutant embryos, usually within a few days of fertilization (Xu and Meng, 2014).

Goldfish (*Carassius auratus* var., GF, 2n = 100) and rare gudgeon (*Gobiocypris rarus*, RG, 2n = 50) belonging to different genera and with different chromosome numbers were used as parents for hybridization in this study. The first group comprised goldfish (♀) × rare gudgeon (♂) (GFRG) hybridization, whereas the second was rare gudgeon (♀) × goldfish (♂) (RGGF). The embryonic development of the two groups of hybrid offspring showed two different fates: survival or death. These hybrid embryos provide ideal experimental materials for studying the molecular mechanisms underlying the non-survival of distant hybrid embryos of fish.

This study explored the regulatory mechanisms of maternal zygote genes in the development of hybrid embryos to provide guidance for distant hybridization of fish. Our findings elucidate the possible mechanism underlying reproductive isolation and provide a theoretical basis for cross-breeding.

2. Results

2.1. Embryo development characteristics

The fertilized eggs of GF, RG, GFRG, and RGGF sequentially developed through the following stages: fertilized egg; the morula, early and late blastula, early and late gastrula, and segmentation stages; and hatching period under the water temperature of 20–22 °C. The developmental rate of the hybrid offspring was consistent with that of the female parents. However, the developmental rate of GF and GFRG embryos (hatching period: 72 h post-fertilization (hpf)) was faster than that of RG and RGGF embryos (hatching period: 84 hpf) (Table 1).

The embryonic morphology of GFRG was similar to that of the female GF parent, and its developmental process was normal. It developed to the segmentation stage at 25 h, and the normal fry hatched at 72 hpf. The embryonic morphology of RGGF was similar to that of the female RG parent. However, embryonic development was abnormal. Most (approximately 86 %) RGGF embryos had abnormal embryonic axes, and the yolk sac was not completely surrounded at 27 hpf. Many (approximately 80 %) RGGF embryos died during the segmentation stage. Finally, only a few (approximately 6 %) deformed fry with a curving dorsoventral axis or cardiocoelom enlargement hatched at 84 hpf. However, all died within 3 days after hatching (Fig. 1).

Table 1
Comparison of the developmental speed of goldfish, rare gudgeon, and hybrid embryos (20–22 °C).

Developmental stage	Developmental time (hpf: h post-fertilization)			
	GF	GFRG	RG	RGGF
zygophase	0.5	0.5	1	1
morula stage	2.5	2.5	3.5	3.5
early blastula stage	5	5	5.5	5.5
late blastula stage	6.5	7	9	9
early gastrula stage	7.5	7.5	10.5	10
late gastrula stage	11	11.5	16.5	15.5
segmentation stage	25	25	28	27
hatching period	72	72	84	84

2.2. Gene sequence alignment analysis

DNA and cDNA from GF, RG, GFRG, and RGGF embryos were used as templates for gene cloning (Table 5). One type of *pou5f3* DNA sequence (approximately 2700 bp) was cloned from GF and RG. Two *pou5f3* DNA sequences (approximately 2700 bp) were cloned into GFRG and RGGF: GFRG-I, GFRG-II, RGGF-I, and RGGF-II. All sequences had the same gene structure with five exons and four introns.

The similarity in *pou5f3* DNA sequences between GF and RG was 95.02 %. GFRG-I and GFRG-II had higher similarity (GFRG-I: 99.03 %; GFRG-II: 98.03 %) with the *pou5f3* DNA sequence of GF but lower similarity (GFRG-I: 95.03 %; GFRG-II: 95.80 %) to RG. The similarity in *pou5f3* DNA sequences between RGGF-I and RG was high (99.18 %),

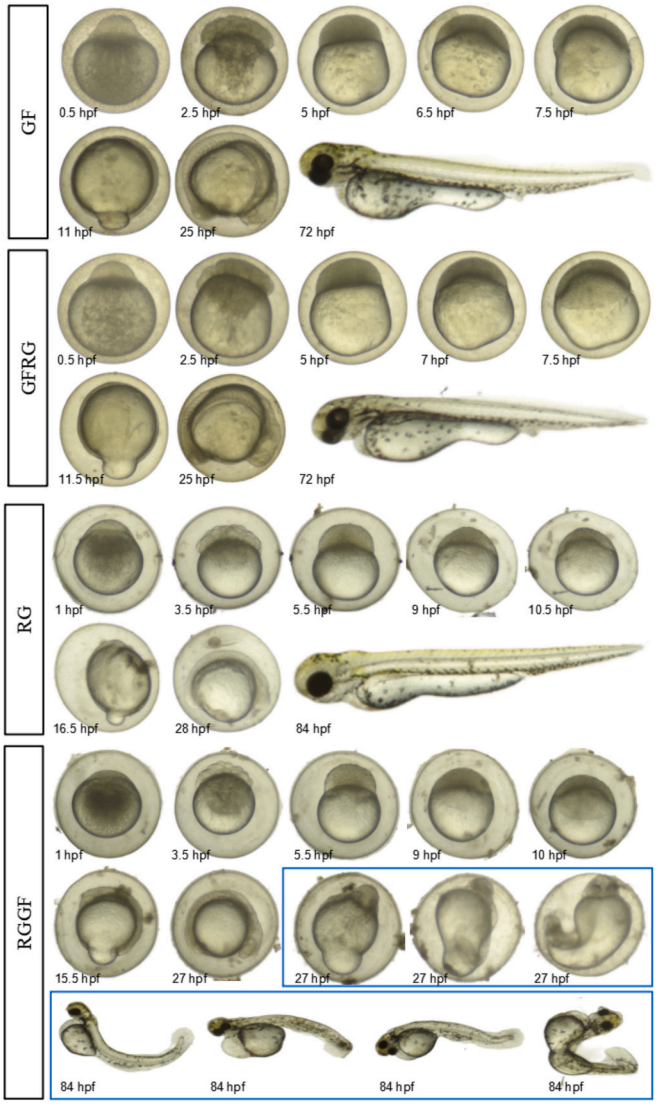

Fig. 1. Segmental and emergence stages of GF, RG, and reciprocal crosses. A–B: GF embryos; C–D: GFRG embryos; E–F: RG embryos; G–J: RGGF embryos. The blue box indicates abnormal development.

whereas that between RGGF-I and GF was lower (94.98 %). The *pou5f3* DNA sequence similarity between RGGF-II and GF was high (98.80 %), whereas that between RGGF-II and RG was low (95.55 %) (Table 2).

Similarity analysis of partial cDNA sequences further confirmed that both GFRG-I and GFRG-II were similar to the maternal GF. Contrastingly, RGGF-I and RGGF-II were similar to the maternal and paternal GF, respectively (Table 3).

Sequence alignment results were obtained using BioEdit 7.2.5 software (Fig. 2). Comparative sequencing revealed that the hybrid progeny inherited parent-specific nucleotides and contained base mutations. RGGF-I comprised 99 maternal-specific, two paternal-specific, and two mutant bases. There was one maternal-specific base, 99 paternal-specific bases, and 14 mutant bases in RGGF-II. This showed significant parental independence in genetic traits. In GFRG-I, there were 101 maternal-specific, zero paternal-specific, and two mutant bases. In contrast, there were 47 maternal- and 32 paternal-specific bases in GFRG-II. The mutant base in GFRG-II contained 46 sites, revealing extensive variation (Table 4).

2.3. The expression trend of pou5f3 in hybrid embryos

During GF and RG embryonic development, the expression levels of *pou5f3* decreased from the morula stage to the blastula stage, increased in the early gastrula stage, reached its maximum value, and then gradually decreased to zero expression in fry (Fig. 3). However, the starting point of *pou5f3* expression increased at the early blastula stage in GF embryos (Fig. 3A, C), whereas that in RG embryos increased at the late blastula stage (Fig. 3B, D).

As the hybrid progeny contained two types of parent-derived *pou5f3*, GF-specific, RG-specific, and universal primers were used to detect gene expression in the hybrids. Only GF-specific primers were detected in GFRG morula stage embryos, and their content was high. This indicates that *pou5f3* was only expressed maternally at this time. The expression of *pou5f3* in GF-specific primers gradually decreased from the morula to the late blastula stage, whereas that of RG-specific primers gradually increased. From the late blastula to early gastrula stages, *pou5f3* expression levels gradually increased and reached a peak upon detection of the two primers. Both primers showed a gradual decrease in expression from the early gastrula to the segmentation stage, and almost no transcription was detected at the segmentation stage (Fig. 4A, B, D, E). Universal primer detection showed a decreasing–increasing–decreasing trend in *pou5f3* expression in the GFRG embryos from the morula to the early blastula, early gastrula, and segmentation stages (Fig. 4C, F). This trend was consistent with that in GF embryos.

No expression was detected in RGGF embryos using GF-specific primers in the morula and early blastula stages. However, an increase in expression was detected using RG-specific primers. *Pou5f3* expression was revealed by GF-specific primers at the late blastula stage but decreased from the early to the late gastrula stages. Contrastingly, it was not detected at the segmentation stage. RG-specific primer testing revealed a gradual decrease in expression levels from the early blastula to the early gastrula stage, and almost no transcription was detected at the late gastrula stage (Fig. 4G, H, J, K). Universal primer detection showed an increasing–decreasing trend in *pou5f3* expression in RGGF embryos from the morula stage to the early blastula and segmentation stages (Fig. 4I, L), which showed significant differences compared to the parents and GFRG.

Table 2
Sequence similarity comparison of *pou5f3* DNA fragments.

pou5f3 DNA	GenBank accession No.	Sequence similarity with GF	Sequence similarity with RG
GF	OR916012	–	95.02 %
RG	OR916013	95.02 %	–
GFRG-I	OR916014	99.03 %	95.03 %
GFRG-II	OR916015	98.03 %	95.80 %
RGGF-I	OR916016	94.98 %	99.18 %
RGGF-II	OR916017	98.80 %	95.55 %

Table 3
Sequence similarity comparison of *pou5f3* cDNA fragments.

pou5f3 cDNA	GenBank accession No.	Sequence similarity with GF	Sequence similarity with RG
GF	OR711405	–	90.23 %
RG	OR791864	90.23 %	–
GFRG-I	OR801707	99.78 %	90.01 %
GFRG-II	OR801708	91.36 %	89.70 %
RGGF-I	OR801709	89.39 %	99.42 %
RGGF-II	OR801710	99.56 %	88.79 %

2.4. Spatiotemporal expression of pou5f3

Whole mount *in situ* hybridization (WISH) was performed on the hybrid offspring and parent embryos at each stage using an antisense probe, with a sense probe used as a control. The entire blastomere at the morula stage was lilac in GF embryos (Fig. 5G, control A). From the early blastula to the early gastrula stages, the entire blastomere was purple and gradually darkened (Fig. 5H-J, control B—D). The degree of embryo staining decreased sharply in the late gastrula stage, and only the median dorsal line was lilac (Fig. 5K, control E). The transcript of *pou5f3* was almost undetectable in segmentation-stage embryos (Fig. 5L, control F). In the RG, the entire blastomere was purple from the morula stage to the early gastrula stage compared to the control group (Fig. 5S-V, control M-P). Furthermore, the degree of embryo staining decreased sharply in the late gastrula stage, and only the median dorsal line was lilac (Fig. 5W, control Q). The *pou5f3* transcript was almost undetectable in segmentation-stage embryos (Fig. 5X, control R).

In GFRG embryos, the blastomere from the morula to the late blastula stage was lilac (Fig. 6G-I, control A-C). The cell color was dark purple from the late blastula to the early gastrula stage (Fig. 6I-J, control C—D). At the late gastrula stage, the degree of embryo staining became extremely shallow and converged at the midline of the back (Fig. 6K, control E). The transcript of *pou5f3* was almost undetectable in embryos at the segmentation stage (Fig. 6L, control F).

From the morula stage to the early blastula stage, the entire blastomere was lilac and gradually darkened in RGGF embryos (Fig. 6S-T, control M-N). All blastoderm cells were purple from the late blastula to the early gastrula stages (Fig. 6U-V, control O—P). At the late gastrula stage, the embryos were almost unstained (Fig. 6W-X, control Q-R). This was consistent with the qPCR results showing that *pou5f3* was only undetected in RGGF at this stage.

2.5. Defective forms of pou5f3

To elucidate the function of *pou5f3* in embryonic development, antisense morpholino oligonucleotides were administered to interfere with the early development of GF and GFRG. Microinjection of *pou5f3* morpholinos effectively interfered with *pou5f3* function. Similar to RGGF embryos, *pou5f3* inhibition embryos showed morphological abnormalities, including curvature of the spine and enlargement of the pericardial cavity (Fig. 7C, D, G, H). In contrast, the embryos were not significantly abnormal in the control group (Fig. 7A, B, E, and F).

3. Discussion

3.1. The fate of hybrid embryos

Only a few hybridization groups produce normal embryos that develop into fry during distant hybridizations in fish (Chen et al., 2018). Most hybridizations, especially above-specific hybridization, are unsuccessful in obtaining offspring, with low hybridization compatibility observed (Wang et al., 2019). Most embryos develop abnormally, and

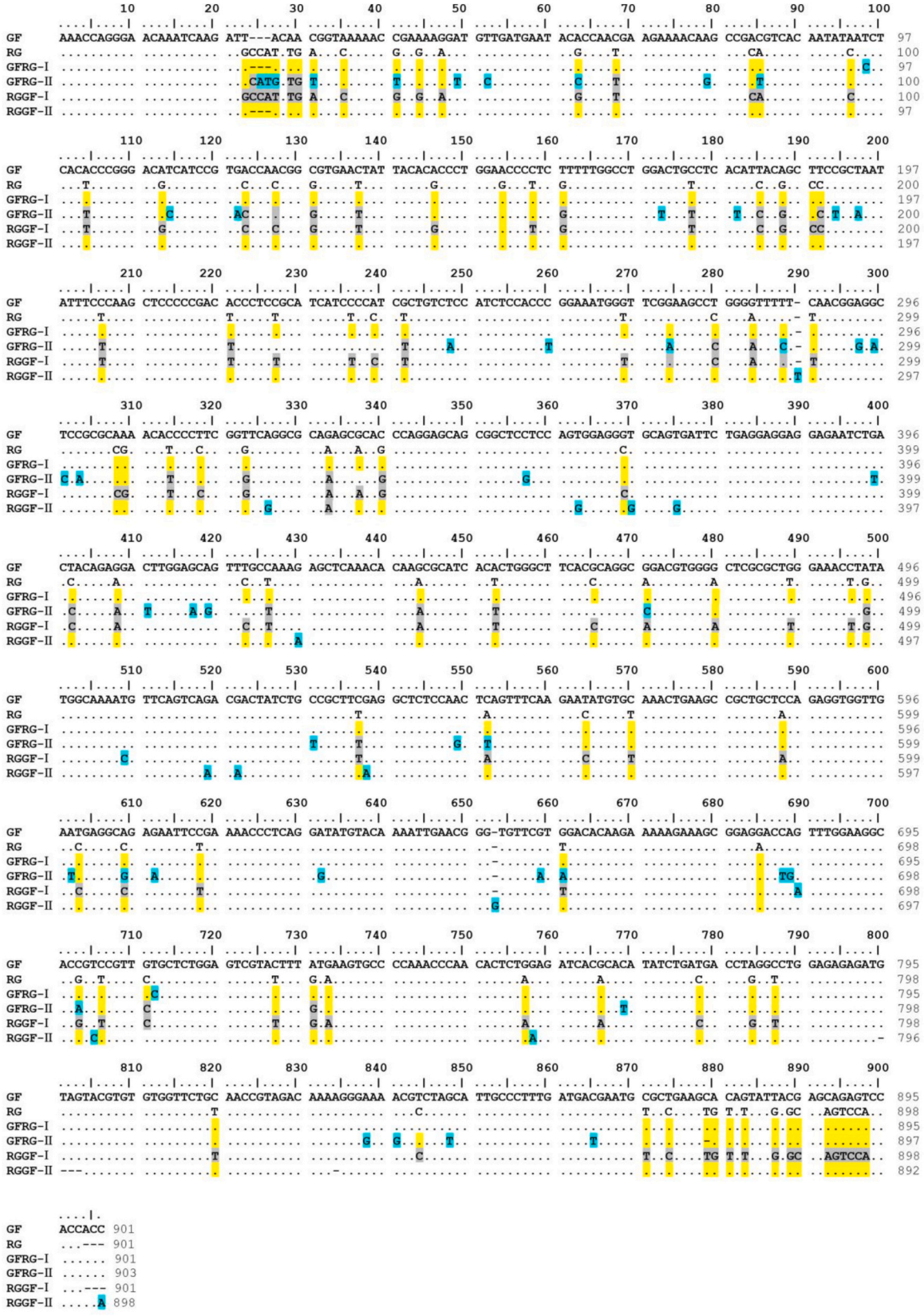

Fig. 2. Comparison of *pou5f3* cDNA sequences.
Yellow, grey, and blue rectangles indicate the GF-specific, RG-specific, and mutant bases, respectively.

Table 4
Base changes of *pou5f3* cDNA fragments in hybrid progeny.

cDNA	Sequence length	Total number of differential bases	Number of maternal-specific bases	Number of paternal-specific bases	Number of mutant bases
GFRG-I	901 bp	103	101	0	2
GFRG-II	903 bp	125	47	32	46
RGGF-I	901 bp	103	99	2	2
RGGF-II	898 bp	114	1	99	14

many die before hatching. Although a small number of hybridizations can produce fry, they show abnormalities in later larval development (Zhang et al., 2014).

Hybrid offspring are more likely to be obtained when the number of maternal chromosomes exceeds that of paternal chromosomes. However, hybrids do not survive if the number of maternal chromosomes is less than that of paternal chromosomes (Liu, 2010; Song et al., 2012; Wang et al., 2019). In this study, the surviving hybrid offspring were obtained in the orthogonal GFRG group wherein the maternal chromosomes exceeded the paternal chromosomes. In contrast, the hybrid offspring did not survive in the anticrossed RGGF group, wherein the maternal chromosomes were fewer than those of the male parent.

Our findings showed that hybrid embryonic development is consistent with maternal embryonic development. Furthermore, the developmental fate of embryos in the cross-back group was significantly different in this study. The early stages of embryonic development are mainly regulated by maternal genetic materials, including maternal factors and maternal-zygotic gene expression products (Schulz and Harrison, 2019). This results in a hybrid embryonic development rate similar to that of the female parent. However, embryonic development is mainly regulated by the zygotic genome during the middle and late stages (Xu and Meng, 2014). If the parent embryo develops at different rates, temporal differences appear in the expression of genes critical for embryonic development. Therefore, the asynchronous expression of zygotic genomes derived from heterologous parents is a barrier to embryonic development in hybrid embryos. This suggests that the survival of hybrids may not only be related to the genome size of parents but may also be associated with different developmental rates of hybrid parents.

3.2. Maternal-zygotic gene

Embryonic development is first regulated by maternal factors carried by the egg and then by zygotic genes (Schulz and Harrison, 2019). Maternal zygotic genes have two common sources: maternal and synzygotic (Xu and Meng, 2014). The expression pattern of maternal-zygotic genes is very special because these genes function as both maternal and zygotic genes. As maternal genes, they are transcribed and

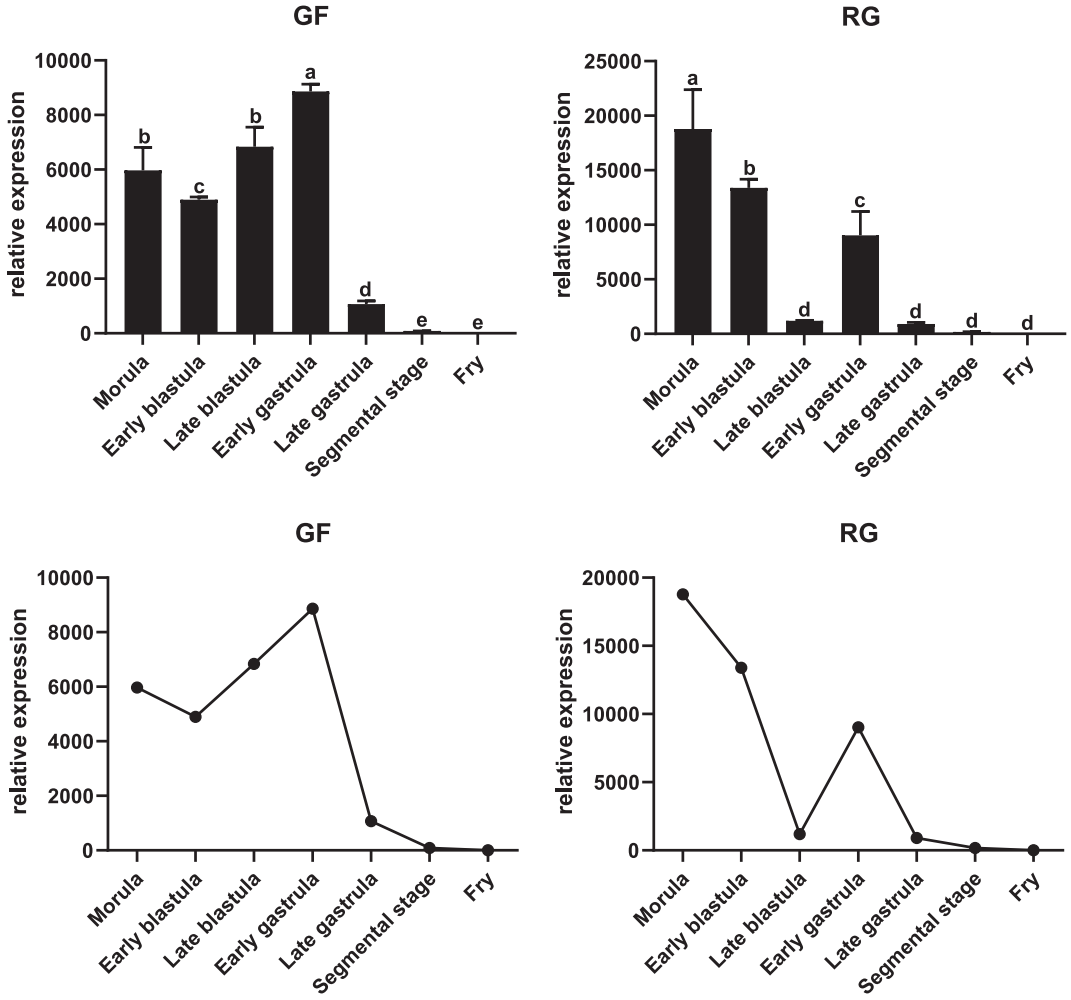

Fig. 3. Real-time quantitative PCR results of *pou5f3* in GF and RG embryos.
Histograms showing *pou5f3* expression in GF (A) and RG (B) embryos; Line charts showing *pou5f3* expression in GF (C) and RG (D) embryos.

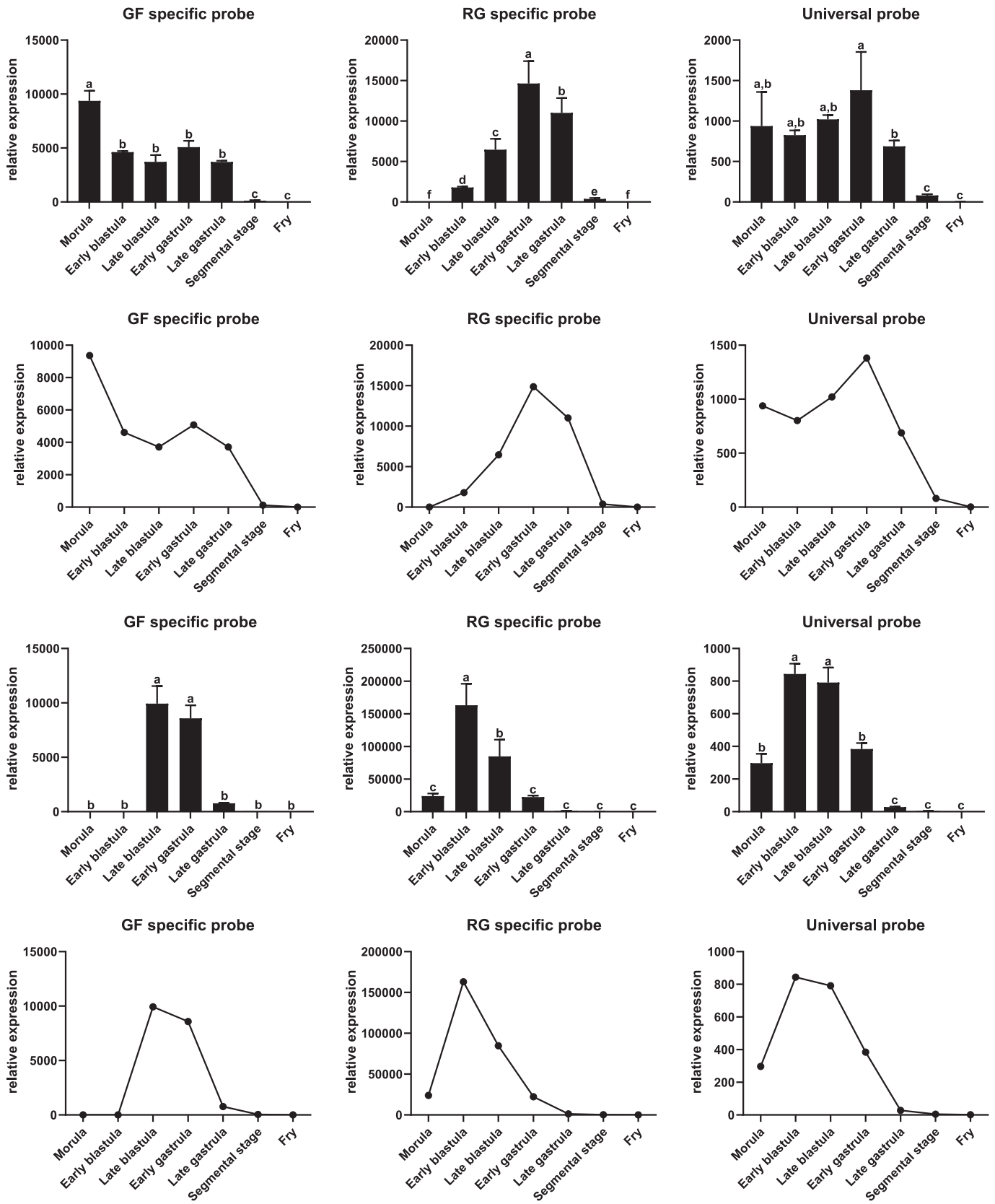

Fig. 4. Real-time quantitative PCR results of *pou5f3* expression in GFRG and RGGF embryos.
A-C: Histograms of *pou5f3* expression in GFRG embryos; D-F: Line charts of *pou5f3* expression in GFRG embryos; G-I: Histograms of *pou5f3* expression in RGGF embryos; J-L: Line charts of *pou5f3* expression in RGGF embryos.

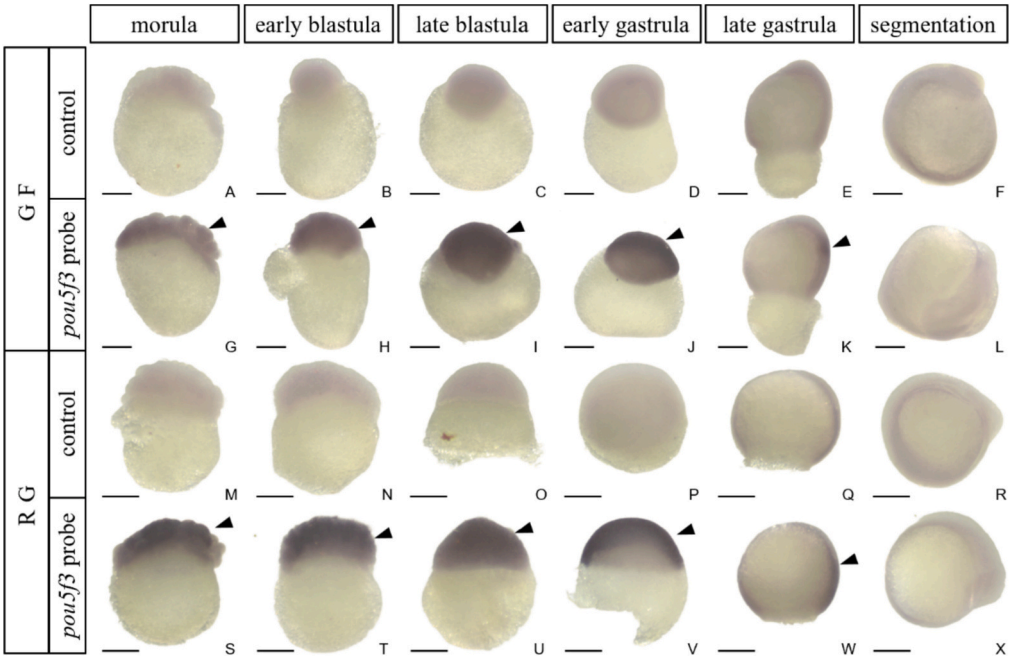

Fig. 5. Whole-embryo *in situ* hybridization results of GF and RG embryos.
A-F: GF control group; G-L: GF experimental group; M-R: RG control group; S-X: RG experimental group. Black arrows indicate the *pou5f3* expression positions. The scale means 0.5 mm.

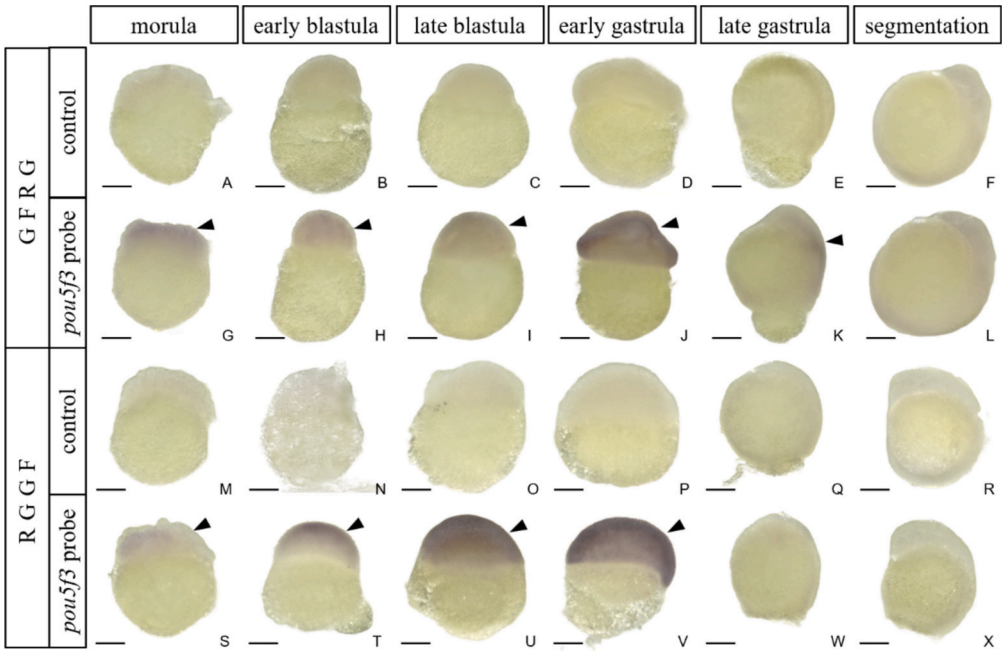

Fig. 6. Whole-embryo *in situ* hybridization results of GFRG and RGGF embryos.
A-F: GFRG control group; G-L: GFRG experimental group; M-R: RGGF control group; S-X: RGGF experimental group. Black arrows indicate the *pou5f3* expression positions. The scale means 0.5 mm.

accumulated in the cytoplasm of oocytes during oogenesis (Tora and Vincent, 2021). After fertilization, maternal products are rapidly degraded with the continuous division of early embryonic cells. This promotes the transformation of the embryonic regulatory mechanism from maternal to zygotic genome regulation mechanisms (Schulz and Harrison, 2019). As a zygotic gene, it is widely expressed in the middle and late stages of blastocyst development and regulates the subsequent embryonic development (Lee et al., 2014).

Recent studies have elucidated the function of maternal zygotic genes. However, the regulatory mechanism of their expression pattern in hybrid embryos remains unclear. Some scholars attribute the low survival rate of distant hybrid embryos to the large differences in chromosome type and number between parents, resulting in incoordination and combination disorder of certain alleles, delayed or accelerated gene expression in hybrid embryos, impeded embryo development, and death (Lou and Li, 2006). When a heterozygote is formed after fertilization, the

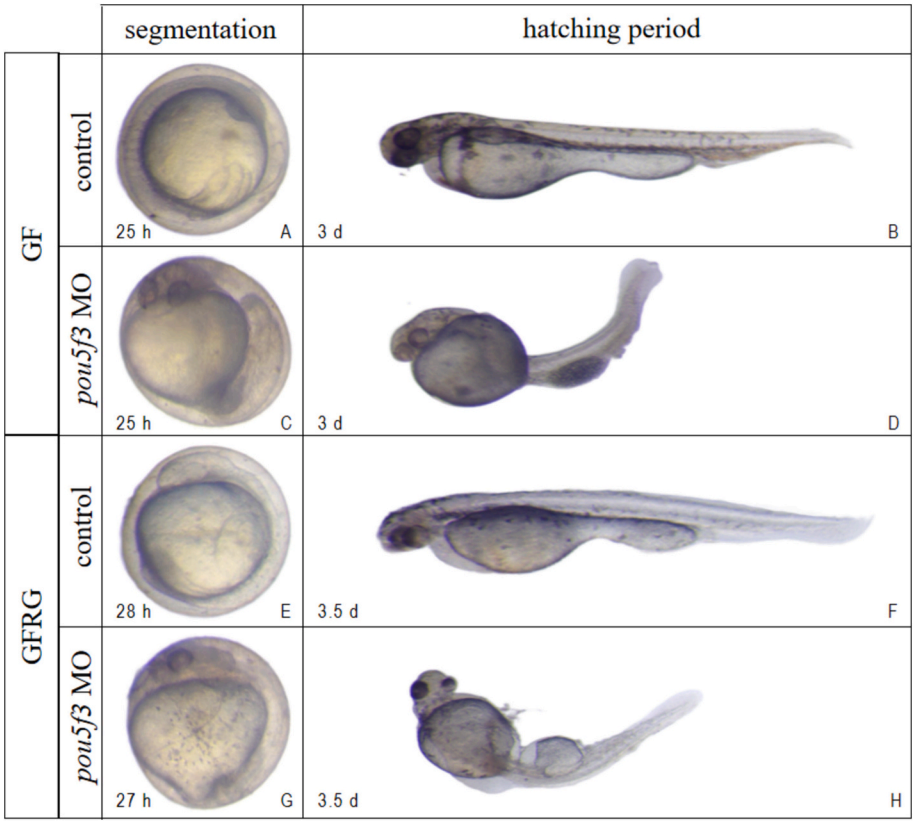

Fig. 7. GF and GFRG embryonic development after *pou5f3* morpholinos (MO) injection.
A-B: GF embryos as a control; C-D: GF embryos injected with *pou5f3* MO; *E*-F: GFRG embryos as a control; G-J: GFRG embryos injected with *pou5f3* MO.

fused genome is first expressed as a maternal-zygotic gene, which affects embryonic development (Eckersley-Maslin et al., 2018). Therefore, studying the expression characteristics of maternal zygotic genes in hybrid embryos can facilitate further understanding of the mechanisms underlying embryonic development in hybrid fish embryos with different fates.

OCT4, SOX2, and NANOG are transcriptional regulatory centers that maintain the pluripotency of embryonic stem cells. Together with other transcription factors, they constitute a transcriptional regulatory network that collaboratively controls cell development and differentiation (Lee et al., 2013). In zebrafish, the maternal Pou5f3 factor and Sox family activate the expression of early zygotic genes and control the temporal coordination of gene expression programs (Kehler et al., 2004). Analysis of loss-of-function mutants has implicated *pou5f3* in dorsoventral (DV) patterning, endoderm formation, and epiboly during early development (Belting et al., 2011) as well as in isthmus formation at the MHB and hindbrain segmentation at later stages (Kobayashi et al., 2018). In zebrafish, exogenous expression of *pou5f3* at the single-cell stage improves somatic cell nuclear transfer embryo development from the blastula to the gastrula stage (Reim and Brand, 2006; Wang et al., 2021). Of these, the expression product of the maternal zygotic gene *pou5f3* is the most important regulatory factor in embryonic development (Lee et al., 2014). However, the effects of the altered expression pattern of *pou5f3* on completely different developmental destinies of hybrid fish are still unknown.

3.3. Types of pou5f3

The important role of *pou5f3* in early embryonic development has been successively studied in zebrafish (Ho and Kimmel, 1993), medaka (Sánchez-Sánchez et al., 2010), Chinese sturgeon (Ye et al., 2012), and goldfish (Marandel et al., 2012). The *pou5f3* structure is highly consistent across different species. The *pou5f3* sequence contains five exons and four introns (Sukparangsi et al., 2022), and the POU domain has almost the same position across different fish species (Onichtchouk, 2012). In addition, multiple sequence alignments have shown that the POU domain is highly conserved among species (Verrijzer and Van der Vliet, 1993).

The structures of *pou5f3* in GF and RG were the same in this study, indicating that *pou5f3* in these two species has high homology. However, different base sites that may be related to species specificity were observed. The genotype in hybrid offspring contains two types derived from parents. In RGGF, one *pou5f3* genotype was more similar to maternal genotype (RG), and the other was more similar to paternal genotype (GF). Two *pou5f3* genotypes were observed in GFRG, one of which was more similar to that in GF. However, the other was not similar to RG, which has several mutation sites and obvious hybridization mosaicism. Thus, the sequence similarity with GF was higher than that with RG. In survival GFRG, the gene is classified as chimeric or mutated to reduce hybridization incompatibility. Therefore, simple genome fusion can easily lead to the death of hybrid offspring. In contrast, extensive recombination and mutation reduce genome incompatibility, leading to a completely different developmental fate for RGGF than for GFRG.

3.4. Expression of pou5f3

The affinity for distant hybridization in fish is directly affected by the genome size of the parent species (Liu, 2010). When the number of chromosomes in the parents is different, the number and nature of the genome also differs. This leads to the incoordination of alleles from the chromosomes of the parents during zygote formation, resulting in a disorder of gene regulation, failure of the embryo to develop normally, and ultimately, the death of the embryo (Chen et al., 2018; Wang et al.,

2019; Zhang et al., 2014). This may explain why most distant hybridizations fail to emerge successfully or have a very low emergence rate (Wang et al., 2019).

In fish, *pou5f3* may be involved in cellular decision-making and play a regulatory role in early embryonic development (Onichtchouk et al., 2010). Moreover, *pou5f3* transcripts exist in all blastoderm cells from the multicellular stage to the early gastrula stage. Blastoderm cells at this stage have stem cell omnipotency, suggesting that *pou5f3* may be involved in maintaining cell pluripotency. In addition, this suggests that high expression in the early gastrula may be related to dermal differentiation during the gastrula stage (Onichtchouk, 2012). At the late gastrula stage, *pou5f3* transcripts in parental self-crossed and hybrid embryos were significantly reduced. Moreover, they aggregated towards the median line of the back of the embryo, which are related to the development of the neural plate and the formation of the mesocerebrum. This suggests that *pou5f3* may be involved in the regulation of the developmental fate of the neural plate and mesocerebrum (Inomata et al., 2020). The expression of *pou5f3* has strict temporal and spatial specificities during embryonic development (Bakhmet and Tomilin, 2022).

There was no significant difference in the spatial expression of *pou5f3* in GF and RG embryos. However, there was a difference in its temporal expression. In RGGF, the expression trends of the two types of *pou5f3* genes were consistent with those of the parent GF. Contrastingly, the expression trends of the two types of *pou5f3* genes in RGGF were different, such as at the highest-level and end stages. The overall expression period was extended and significantly different from that of both parents and the GFRG. The spatiotemporal expression of *pou5f3* originating from different parents in hybrid embryos is not synchronized. This may cause gene regulation disorders, such as tissue induction and organ formation regulation disorders, during the development of RGGF embryos. Thus, deformed embryos or direct death in the middle of development were observed in this study, resulting in completely different developmental destinies for RGGF embryos.

4. Materials and methods

4.1. Animals

GF and RG were obtained from the State Key Laboratory of Developmental Biology of Freshwater Fish (Changsha, China), and artificially fed.

4.2. Obtainment of hybrid embryos

During the breeding season (April to June), GF (2n = 100, ♀) × RG (2n = 50, ♂) and RG (2n = 50, ♀) × GF (2n = 100, ♂) were hybridized, respectively. Two self-crossings, GF and RG, were conducted. The fertilized eggs were cultured in glass dishes (φ = 10 cm) at a water temperature of 20–22 °C. Embryonic development was observed and photographed using a stereoscope (MZ16FA; Leica, Wetzlar, Germany).

4.3. Pou5f3 sequencing

Total genomic DNA and RNA were isolated from GF, RG, GFRG, and RGGF embryos using a DNA extraction kit (Sangon, Shanghai, China) and the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), respectively, according to the manufacturers' instructions. Total RNA was reverse transcribed to complementary DNA (cDNA) using a cDNA Synthesis Kit (Thermo Fisher Scientific, Carlsbad, CA, USA) with random primers. The reaction mixture (25 μL) comprised 20 ng cDNA, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer, 1× PCR buffer, and 1.25 U Taq polymerase (Takara, Dalian, China). The amplification process started with denaturation at 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. The final extension step was performed at 72 °C for 10 min. The PCR products were sequentially separated on 1.2 % agarose gel, purified using a gel extraction kit (Sangon, Shanghai, China), ligated into a pMD18-T vector, and transferred into *E. coli* DH5α. Finally, twenty positive clones per PCR band were Sanger sequenced bidirectionally using an automated DNA sequencer (ABI PRISM 3730; Applied Biosystems, Carlsbad, CA, USA). Primers used for *pou5f3* DNA amplification are listed in Table 5. The sequences were analyzed using the BioEdit 7.2.5 software and submitted to GenBank.

4.4. Real-time quantitative PCR (qPCR)

Here, qPCR was performed on a QuantStudio5 instrument (Applied Biosystems) using PowerUp SYBR Green Master Mix (Applied Biosystems). The reaction mixture (10 μL) contained 2.5 μL cDNA (200 ng/μL), 5 μL PowerUp SYBR Green Master Mix, 0.5 μL specific forward primers, 0.5 μL reverse primers, and 1.5 μL water. The primers used are listed in Table 5. The amplification conditions were as follows: 50 °C for 5 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 45 s. The average threshold cycle (Ct) was calculated for each sample using the $2^{-\Delta\Delta Ct}$ method and normalized to β-actin. Melting curve analysis was performed to validate the specific amplification of the expected products.

4.5. Whole mount in situ hybridization

WISHwas performed to detect the temporal and spatial expression of *pou5f3* mRNA. The probes were synthesized using T7 RNA polymerase (EP0111; Thermo Fisher Scientific) and cloned from a cDNA library template. The forward and reverse primer sequences were 5′-GCCGCAGTCACAATATCATC-3′ and 5′-CTCCAGAGCAGAACGAACC-3′, respectively. The embryos were collected at different stages, including the morula, early blastula, late blastula, early gastrula, late gastrula, and segmental stages. They were subsequently fixed in 4 % paraformaldehyde in phosphate-buffered saline (PBS) at 4 °C overnight and stored in 100 % methanol at −20 °C. WISH was performed according to standard protocols (Thisse and Thisse, 2008). Probe hybridization was then performed overnight at 62 °C with 3 ng/mL probe concentration. The anti-digoxigenin-AP Fab fragments (11,093,274,910; Roche, Basel, Switzerland) were diluted to 1:2500 with 2 mg/mL bovine serum albumin and 2 % sheep serum in PBS containing 0.1 % Tween-20. The embryos were photographed using a stereoscope (MZ16FA; Leica).

Table 5
Primers for PCR and real-time quantitative PCR.

Primer	Sequence	Template
1	**F:** CTATCAAACCAACAGCCTCA **R:** CAGCGACTTTCATCTATCACC	GF, GFRG, and RGGF DNA
2	**F:** CAGAAGTGAGATTGGGAAGA **R:** AGCGAATGTCATCTATCACC	RG, GFRG, and RGGF DNA
3	**F:** GAGTTCACTGGGCAGGTTG **R:** GGTGGTGGACTCTGCTCGTA	GF, GFRG, and RGGF cDNA
4	**F:** AACATCAGCCCGCCTATC **R:** TGGTCCTCCGCTTTCTTT	RG, GFRG, and RGGF cDNA
5	**F:** GGGACATCATCCGTGACCAA **R:**AAAACCCCAGGCTTCCGAAC	GF, GFRG, and RGGF cDNA
6	**F:** GGCTTCACGCAGGCAGACG **R:**CGACTCCAGAGCAGAACGAACC	RG, GFRG, and RGGF cDNA
7	**F:** CATCTCCCTCTCTGTCTCCATCTC **R:**CGTCTGCCTGCGTGAAGC	GF, RG, GFRG, and RGGF cDNA
8	**F:** GAGTGATGGTTGGCATGGGA **R:**CAGTGAGCAGGACAGGGTG	β-actin

Data are expressed as the mean ± standard error of the mean (S.E.M., $n = 3$). Independent-sample Student's *t*-tests were performed using GraphPad Prism 7.04 (San Diego, CA, USA) to identify significant differences between samples. Statistical significance was set at $P < 0.05$.

4.6. Specific inhibition of pou5f3

The GF *pou5f3*-specific antisense morpholino oligonucleotides (*pou5f3* MO 5′-TTGATAGTCAATACACGGCGATCAT-3′) and standard control morpholino oligonucleotides (MO-Vivo 5′-CCTCTTACCTCAGTTACAATTTATA-3′) were designed and synthesized by Gene Tools (Philomath, OR, USA) and dissolved to 1 mM in nuclease-free water. Approximately 200 one-cell stage embryos from each group were injected with *pou5f3* MO (13.975 ng *pou5f3* MO per embryo) to generate *pou5f3*-deficient GF and GFRG embryos. The injected embryos were raised in culture dishes (φ = 10 cm) at 22 °C until approximately 7 days post-fertilization.

Compliance and ethics

The author(s) declare that they have no conflict of interest.

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Ethics approval statement

Fish treatments were conducted according to the regulations for protected wildlife and the Administration of Affairs Concerning Animal Experimentation and approved by the Science and Technology Bureau of China, and the Department of Wildlife Administration.

CRediT authorship contribution statement

Jing Wang: Writing – original draft, Funding acquisition, Conceptualization. **Yirui Zhang:** Methodology, Investigation, Data curation. **Wen Wang:** Methodology, Investigation. **Chang Wang:** Methodology, Investigation. **Jiahao Wu:** Data curation. **Yan Wang:** Data curation. **Zhonghua Peng:** Methodology, Investigation. **Ting Liu:** Methodology, Investigation. **Shengwei Wang:** Methodology, Investigation. **Chengxi Liu:** Methodology, Investigation. **Kaikun Luo:** Resources, Conceptualization. **Yinjun Jiang:** Methodology, Investigation. **Yu Deng:** Writing – review & editing, Conceptualization. **Shaojun Liu:** Writing – review & editing, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflicts of interest concerning this article.

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Data availability

The data are available from the corresponding author based on reasonable requests.

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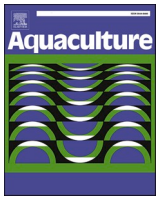

Corrigendum to "Spatiotemporal expression patterns of maternal-zygotic gene pou5f3 in hybrid fish embryos" [Aquaculture, Volume 598, 15 March 2025, 741954]

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1. The authors regret an omission in the funding information and would like to revise it as follows:

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2. The authors regret an omission of letter number in Figs. 3–4.

The revised Figs. 3–4 are as follows:

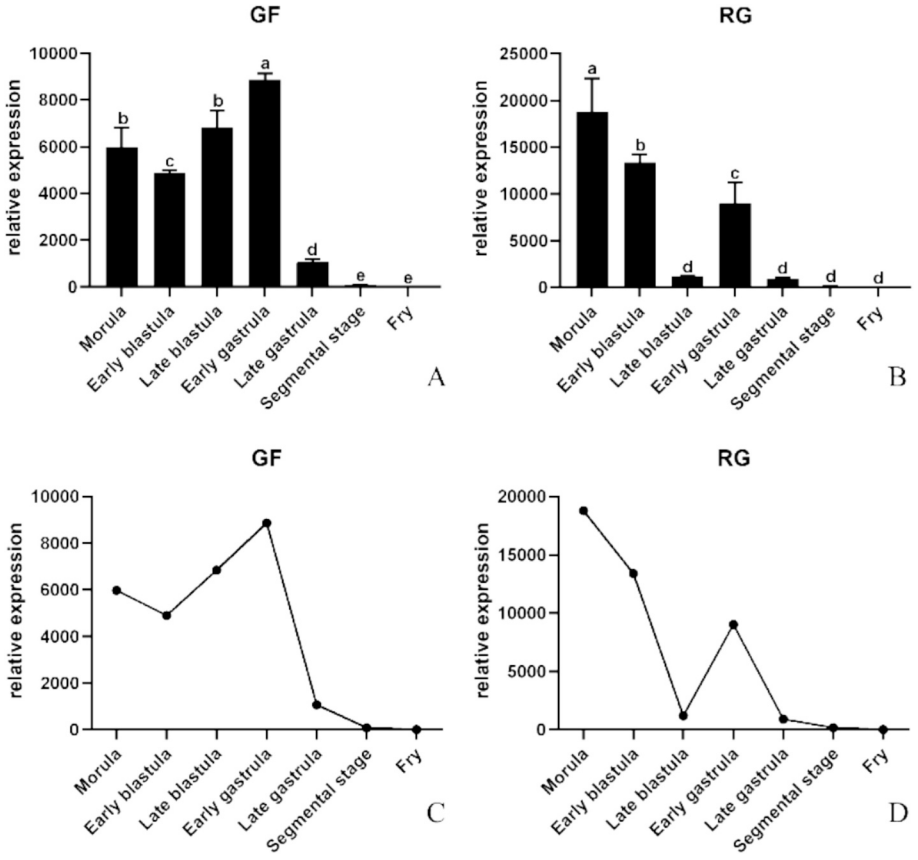

Fig. 3 Real-time quantitative PCR results of pou5f3 in GF and RG embryos.

Histograms showing pou5f3 expression in GF (A) and RG (B) embryos; Line charts showing pou5f3 expression in GF (C) and RG (D) embryos.

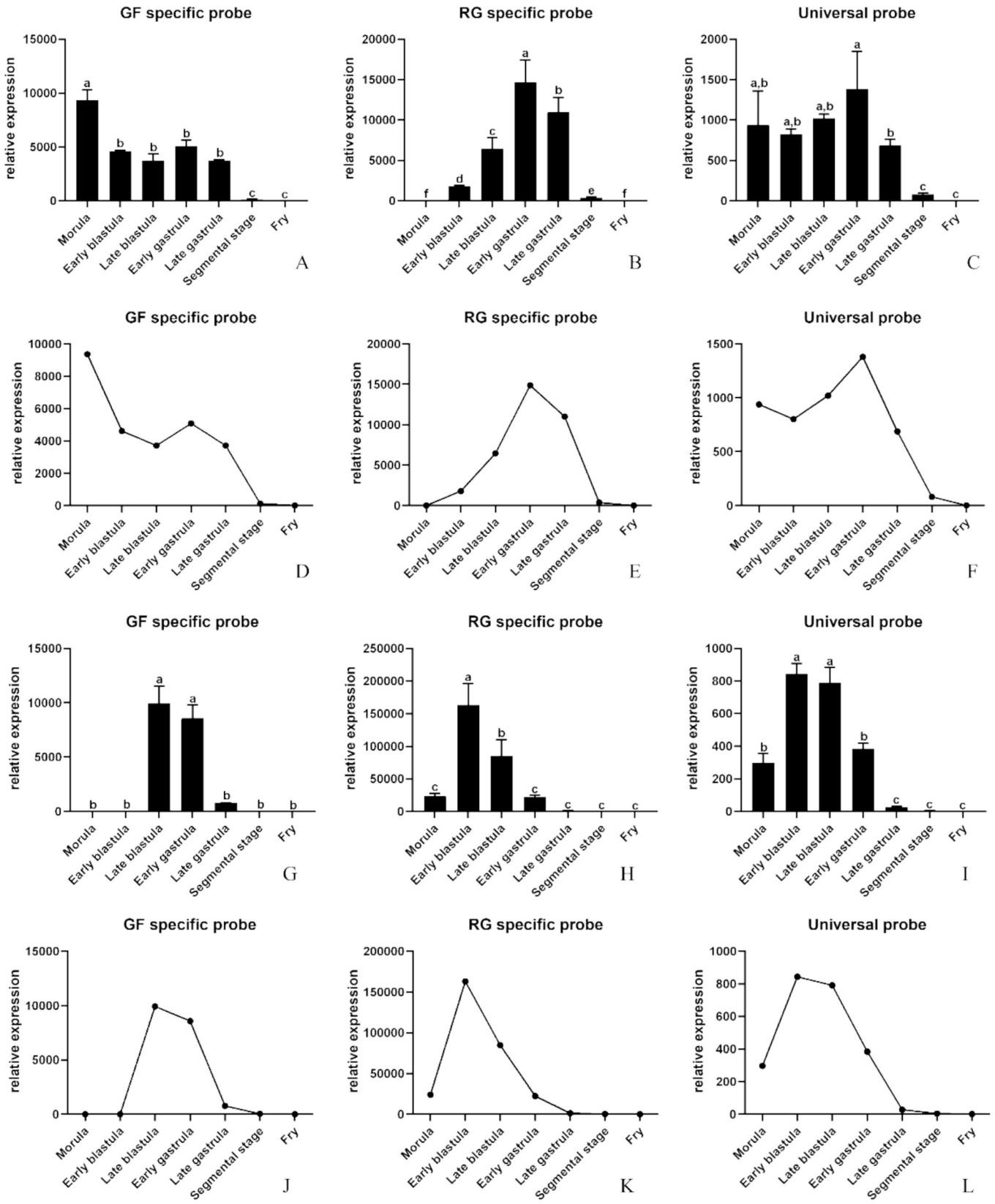

Fig. 4 Real-time quantitative PCR results of pou5f3 expression in GFRG and RGGF embryos.

A-C: Histograms of pou5f3 expression in GFRG embryos; D-F: Line charts of pou5f3 expression in GFRG embryos; G-I: Histograms of pou5f3 expression in RGGF embryos; J-L: Line charts of pou5f3 expression in RGGF embryos.

The authors would like to apologise for any inconvenience caused.