CHAPTER – I

Intra- and inter-specific fertilization between fresh eggs of *D. rerio* (grey) / *D. frankei* (dotted) and fresh sperms or monosperms of *D. rerio* (albino).

1.1. Phenotypic markers

The results (Table 6) clearly show that the body colour and pigmentation of female *D. rerio* (grey), male *D. rerio* (albino) and female *D. frankei* (dotted) exhibit strain-specific distinctive features. Female *D. rerio* (grey), the dominant strain, exhibited silver grey body colour with brown pigmentation on the dorsal surface along with four blue longitudinal stripes on each lateral surface of the body; male *D. rerio* (albino), the recessive strain, exhibited golden yellow body colour with four silver longitudinal stripes on each lateral surface of the body without any pigmentation and the female *D. frankei* (dotted) showed dark brown spots dominating scanty blue spots on metallic gold coloured body without any bands on the lateral surfaces (Fig. 9a, b, c).

Earlier classical studies examining sex linkage of phenotypic markers in *Oryzias latipes*, *Poecilia reticulata*, *Poecilia nigrofasciata*, *Betta. splendens*, and in five species of *Xiphophorus* (*X. variatus*, *X. xiphiidium*, *X. couchianus*, *X. milleri* and *X. montezumae cortezi*) including some strains of *X. maculatus* revealed that sex was determined by an XY system (Aida, 1921; Wing, 1922; Yamamoto, 1969). More over, these early studies on sex-linked marker segregation in model species were the first to reveal the complex and plastic nature of sex determination systems that exist in fish. Specific phenotypic markers affecting skin pigmentation have been identified in several fish species where male phenotype is important for mate selection by females. Vorgelegt (2005) considered the skin color of *Maylandia zebra*, which shows a dimorphism, for the determination of sex. Similarly dark body coloration of spawning male *Pseudophoxinus alii* was taken into
consideration for morphometric assessment by Fahrettin (2007). Further Kucharczyk et al. (2008) used yellow-recessive colour and dark-dominant colour of golden Orfe / Ide, *Leuciscus idus* for the confirmation of purity and survival of androgenotes produced using UV irradiation. In the guppy *P. reticulata*, both the black caudal peduncle (Bcp) and red tail (Rdt) markers have been found to be dominant Y-linked traits (Fernando and Phang, 1989, 1990; Khoo et al., 1999).

Distinct colours and patterns that exist in female *D. rerio* (grey) and male *D. rerio* (albino) and female *D. frankei* (dotted) (Table 6) expressed as either dominant or recessive traits (Kavumpurath and Pandian, 1990; Unger et al., 1998) are expected to be the potential markers, useful in confirming the purity of putative androgenotes.

Bercsenyi (1998) used different recessive characters such as bubble eye and black telescopic eye of two different strains of male gold fish, *C. auratus* as phenotypic markers to assess the success of interspecific androgenesis with common carp. In monosex production of Nile Tilapia through androgenesis, Shelton (2000) also considered blond eye which appears as early as in 12 hr embryo even prior to hatching, as a marker to assess the success against the red eye strain. Further puffy eyes of *Xenopus tropicalis* have been used as a phenotypic marker to detect naturally occurring recessive mutations (Selina et al., 2005).

The eyes, found to be dark blue in colour in case of female *D. rerio* (grey), albino in case of male *D. rerio* (albino) and black in case of female *D. frankei* (dotted) indicate clear variation among the strains (Table 6). Since development takes as early as with 3 days after fertilization eye colour as a marker is expected to enable sex confirmation even at the hatchling stage and thus is considered as strain-specific markers.
Observations regarding other phenotypic characters presented in Table 7 show clear variation in length of different regions of the body of three different strains studied. For instance, significant variation in total body length was found among female *D. rerio* (grey), (34.2±1.25 mm), male *D. rerio* (albino) (31.6 ±1.1 mm) and female *D. frankei* (dotted) (38.8 ±1.04mm). Similarly standard length was found to be 29.2±1.21 mm in female *D. rerio* (grey), 25.4 ±0.87 mm in *D. rerio* (albino) and 32.1±1.03mm in female *D. frankei* (dotted), which were found to be significantly different from each other. While no significant difference was observed in head length between male *D. rerio* (albino) (6.36±0.15 mm) and female *D. rerio* (grey) (6.13±0.13 mm), that (4.30±0.31mm) of female *D. frankei* (dotted) was found to be significantly smaller. Further, body depth showed no significant difference between female *D. rerio* (grey) (9.10±0.72mm) and female *D. frankei* (dotted) (9.28±0.60mm); however body depth of male *D. rerio* (albino) was found to be significantly smaller (8.12±0.51mm) than the other two. The dorsal fin base was significantly longer in male *D. rerio* (albino) (4.13±0.15mm) than in either female *D. rerio* (grey) (3.51±0.12mm) or female *D. frankei* (dotted) (3.16±0.15mm).

Variations in the number of rays in different fins among female *D. rerio* (grey), male *D. rerio* (albino) and female *D. frankei* (dotted) were shown in Table 8 and Figure 10. Though not significantly higher, maximum number of fin rays was found in female *D. frankei* (dotted) (14±2.0) followed by male *D. rerio* (albino) (11±1.0) and female *D. rerio* (grey) (9±1.0). The number of pectoral fin rays of male *D. rerio* (albino) (12±1.0) was not significantly different from that of either female *D. frankei* (dotted) (10±2.0) or female *D. rerio* (grey) (9±1.0). Similarly the number of anal fin rays was not significantly different among female *D. rerio* (grey) (12±2.0), male *D. rerio* (albino) (13±2.0) and female *D. frankei* (dotted) (15±2.0). However
the number of caudal fin rays was maximum (21±2.0) and significantly higher in female *D. rerio* (grey) than in male *D. rerio* (albino) (16±2.0) or female *D. frankei* (dotted) (17±1.0) although the later two were not significantly different from each other.

Studies on variations in morphometric parameters such as total body length, head length, total body weight etc. to identify different strains have been initiated by Stanley and Jones as early as 1976 in *C. idella*. Gupta (1986) used morphometric analysis for confirmation of hybrids of common carp and mrigala, which showed similarity in the length of different regions of the body and number of different fin rays to both the parents. Male lumpfish, *Cyclopterm lumpus*, were identified based on stronger head, jaws and bigger suckers (both in length and width) compared to the females (Magdalena and Tomasz, 2000). Further biometric analysis done to assess differences between meristic characters of male and female snipefish, *Macroramphosus scolopax* showed significant differences in postocular head length (Barbara, 2005). Cervancia and Kottelat (2007) identified a new species of freshwater fish, *Cyclocheilichthyes* in Philippines by detecting unique coloration and number of rays in different fins.

Currently significant variations observed in total body length, standard length, base length of dorsal fin and number of caudal fin rays (Tables 7, 8) in male *D. rerio* (albino) compared to female *D. rerio* (grey) and female *D. frankei* (dotted) indicate that these parameters can be considered as markers in the assessment of purity of the progeny produced through chromosomal manipulations in general and androgenesis in particular. On the other hand the number of dorsal, pectoral, anal and caudal fin rays with no significant differences amongst the above strains were found not to be useful as selective markers in sex manipulation studies.
1.2. *Intra- and inter-specific monospermic fertilization using fresh monosperms*

Groups Considered

Intraspecific Fertilization – ♀ *D. rerio* (grey) Vs ♂ *D. rerio* (albino)

Interspecific Fertilization – ♀ *D. frankei* (dotted) Vs ♂ *D. rerio* (albino)

1.2.1. Fecundity

Results on fecundity, assessed as the total number of eggs per individual, clearly indicate that female *D. frankei* (dotted) produced significantly higher number of eggs (280±40) than female *D. rerio* (grey) (135±25) (Fig.11). Fresh unfertilized eggs of both *D. rerio* (grey) and *D. frankei* (dotted) showed no morphological demarcation between them and found to be yellow in colour with average diameter of 0.7±0.05mm.

Biswas *et al.* (1984) have shown high fecundity (90,000 eggs per individual) in *Labeo dero*, while Islam and Hossain (1990) recorded a mean fecundity of only 8635 eggs in *Puntius stigma* with a total body length of 86.0 mm and mean body weight 10.0 gm. According to Jonsson and Jonsson (1999), fecundity increases with body size because the amount of energy available for egg production and the body cavity accommodating the eggs increase with body size. *Labeo boga* showed a decrease in fecundity from 82,000 to 23,000 as an influence of decrease in body length from 290 mm to 227 mm (Parvin *et al.*, 2011). Further some species like *Garra rufa* exhibited abnormal variation in fecundity levels ranging from 283 to 3794 eggs, with an average of 1180 (Abedi *et al.*, 2011).

Zebrafish starts laying eggs as early as 90 days after birth which continues throughout the year (Nekoubin *et al.*, 2012). Species specific variation in fecundity between *D. rerio* (grey) and *D. frankei* (dotted) can be
attributed to the fact that fecundity of teleostean fishes is affected by many environmental and genetic factors (Thorpe et al., 1984; Fleming and Gross, 1990; Morita and Takashima, 1998).

1.2.2. Assessment of Sperms, Sperm count, Viability, Motility and Fertilization

Danio rerio (albino) sperms were found to have a distinct head measuring about 2.4±0.2 µm and a long thread-like motile tail measuring about 27.0±0.5 µm. (Fig.12).

Sperm count in a fresh male D. rerio (albino) was found to be 4.11±0.03x10^6 (4.11±0.03 million) per ml of semen (Table 9) of which 97 % sperms were found to be viable upon staining with 1% trypan blue.

Fish sperm remain immotile in the testis and the initiation of motility can be controlled by changing the ion concentration of the external medium (Alavi and Cosson, 2006). Upon induction of motility, accomplished by suspending sperms in hypotonic solution, following the procedure demonstrated by Morisawa and Suzuki (1980), 83% sperms of D. rerio (albino) were found to exhibit forward and zigzag movements showing their potential to fertilize the eggs. Upon insemination using these sperms, 96% eggs of D. rerio (grey) and 91% eggs of D. frankei (dotted) exhibited successful fertilization (Fig. 13) although the same was not significantly different between the two groups (♀ D. rerio (grey) x ♂ D. rerio (albino); ♀ D. frankei (dotted) x ♂ D. rerio (albino)) (student’s t-test). A perivitelline membrane was found to form outside the plasma membrane of eggs, 5 to 10 min after fertilization (Fig. 14) followed by a germ ring formation (Fig. 15) confirming successful fertilization followed by onset of cleavage (Kimmel et al., 1995, www.zfin.org).
Motility and fertilization ability of sperms are species-specific and hence usually tested to assess their compatibility with other species. Plaice, *Pleuronectes platessa*, sperms were reported to exhibit 41% motility but fertilizing only 25% of eggs (Gavin et al., 1999). Vilok and Vuren (1988) observed 8.4±2.6 x 10^6/ml sperm count, 92.5% sperm viability and 65% motility in small mouth yellowfish, *Barbus aeneus*. Though *Labeo ruddi* and *Labeo rosae* were found to have sperm count of 5.3 x 10^6/ml and 5.4 x 10^6/ml respectively, the sperm motility levels in both species were found to be >80% (Viljoen and Vuren, 1991). Recently wide variations in the sperm count (1.9 to 3.2 x 10^9/ml) of silver carp, *Hypophthalmichthys molitrix* was found to result in variations in fertilization success (54 to 90.3%), irrespective of high rate of sperm motility (95%) (Rahman et al., 2011). Studies made on zebrafish species, *D. rerio* inhabiting Canadian waters showed 72% intraspecific fertilization (Corley-Smith et al., 1996). Further *D. rerio* inhabiting Chinese waters though reported to exhibit 1 to 6 x 10^8 sperms/ml (Yang et al., 2007) and 5 x 10^7 sperms/ml (Rongyan et al., 2009), showed over 90% sperm motility but only 65% fertilization success.

Among studies of interspecific fertilization made in teleosts Bercsenyi (1998) reported only 50% fertilization success between eggs of common carp and sperms of gold fish. But 98% fertilization success was noticed by Kirankumar and Pandian (2004) between the eggs of tiger barb and sperms of rosy barb. **In the present study no significant variations were observed in the rate of fertilization between the eggs of *D. rerio* (grey) and sperms of *D. rerio* (albino) and eggs of *D. frankei* (dotted) and sperms of *D. rerio* (albino)** (Fig. 13) indicating the compatibility of these two species/strains for interspecific progeny production.

* * * *