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A quantitative genetic study on the proportion of males in tilapia

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Ås, October 2011

Carlos Lozano

SUMMARY

Early sexual maturation is one the main constraints in Tilapia farming since early breeding causes stunted growth and large size variability. To circumvent this problem all-male populations are used commercially, and production of all male fry with use of hormones is the industry standard for Nile tilapia. To evaluate alternatives for production of all male fry, variation of male proportion of different strains and among strains combinations of Nile tilapia were studied. Additionally, to evaluate the feasibility of selection for increased male proportion, genetic parameters for male proportion were studied in Nile tilapia and hybrids between Nile and blue tilapias.

None of the eight purebred Nile tilapia strain and strain crosses evaluated in **Paper I** showed a male proportion (MP) close to the desired commercial threshold (above 95% males). Additive genetic variation for male proportion was estimated within a synthetic population of Nile tilapia. Moderate to low heritabilities were obtained, but estimates may be biased upwards due to effects of the major genetic sex determination factors. Selection for increased male proportion will be very difficult to implement since it likely will result in an increased proportion of masculinized XX sires, which will counteract the response to selection. If selection is to be implemented, use of hormones will be needed to reproduce the population. Identification of genetic sex through the use of genetic markers could provide more reliable estimates of the genetic parameters for MP.

Genetic variation was also estimated among hybrids of Nile tilapia females and blue tilapia males. Heritability estimates were moderate to high. Since only one generation of data was evaluated there can still be some level of confounding between the additive genetic effects and the other effects common to full-sibs due to shallow pedigrees. Crossbreeding (hybrid production) may be a good way to increase male proportion in places where cold winters affect production since hybrids between these two species show high male proportion and increased low temperature tolerance as compared to pure Nile tilapia. To make the Nile x blue tilapia hybrid of interest also in a tropical environment the growth of the blue tilapia must be improved through selection.

SAMMENDRAG

Tidlig kjønnsmodning representerer en av de viktigste begrensningene i tilapiaoppdrett, siden tidlig reproduksjon medfører betydelig redusert vekst og stor variasjon i størrelse. For å omgå dette problemet er kommersiell produksjon som regel basert på bruk av "all male" populasjoner (dvs. kun hannfisk). I oppdrett av Nil tilapia (*Oreochromis niloticus*), som dominerer verdens tilapiaproduksjon, er kjønnsreversering av yngel ved hjelp av hormoner tilsatt i fóret i dag industristandarden. I dette arbeidet er alternative metoder for etablering av "all male" populasjoner basert på utnyttelse av naturlig variasjon i andel hannfisk mellom ulike stammer og stammekombinasjoner vurdert. I tillegg er det estimert genetiske parametre for andel hannfisk hos Nil tilapia og hos hybrider mellom Nil tilapia og blå tilapia (*O. aureus*).

Hos Nil tilapia undersøkt i et diallell krysningseksperiment gjennomført i GIFT prosjektet viste resultatene lave, men statistisk signifikante, additiv genetisk, heterosis og resiproke krysningseffekter for andel hannfisk. Av disse hadde de resiproke effektene størst betydning, og for å oppnå en økt andel av hanndyr bør derfor krysningene med høyest innslag av hanndyr benyttes. Basert på størrelsen på disse effektene synes det imidlertid klart at dette neppe vil være tilstrekkelig til å oppnå minimum 95% hanndyr, noe som kreves for at denne strategien kan være et reelt alternativ til konvensjonelle metoder som i dag benyttes for produksjon av "all male" populasjoner.

Genetisk variasjon for andel hanndyr ble estimert i en syntetisk populasjon av Nil tilapia. Den beregnede arvegraden for egenskapen var lav til moderat, men estimatet kan likevel være overestimert på grunn av samspill med kjønnskromosomer. Seleksjon for økt andel hanndyr vil være svært krevende, fordi det, mest sannsynlig, vil resultere i en økt andel maskuliniserte XX fedre, noe som vil motvirke den ønskede seleksjonsresponsen i neste generasjon. Dersom seleksjon for økt andel hanndyr gjennomføres, vil bruk av hormoner være nødvendig for å få reprodusert populasjonen, og YY hanndyr og XY hunndyr kan dermed selekteres. Genetiske markører for kjønn eller avkomsgranskning av foreldre vil kunne øke effektiviteten av en slik seleksjonsstrategi.

Genetisk variasjon for andel hanndyr ble også estimert for hybrider av Nil tilapia hunner og blå tilapia hanner. Arvegradsestimatene var moderate til høye. Siden det analyserte datasettet var begrenset til en enkelt årgang kan de additive genetiske effektene potensielt være sammenblandet med andre effekter felles for fullsøsken.

På grunn av høyere toleranse for lave temperaturer hos blå tilapia kan slik hybridproduksjon være en god strategi i områder der lave vintertemperaturer påvirker produksjonen. For at denne hybriden skal være kommersielt interessant i tropiske områder må tilveksten hos blå tilapia forbedres gjennom seleksjon.

LIST OF PAPERS

This thesis consists on the following three publications, which will be referred to through the thesis:

I. Lozano, C., Gjerde, B., Bentsen, H.B., Dionisio E.E., Rye, M., 2011. **Estimates of strain additive genetic, heterosis and reciprocal effects for male proportion in Nile tilapia, *Oreochromis niloticus L*.** Aquaculture, 312, 32-42.

II. Lozano, C., Gjerde, B., Ødegård, J., Bentsen, H.B., 2011**. Heritability estimates for male proportion in the GIFT Nile tilapia (*Oreochromis niloticus L*.)**. Submitted to Aquaculture (under revision).

III. Lozano, C., Gjerde, B., Ødegård, J., Rye, M., Luan, T.D. 2011. **Heritability estimates for male proportion in hybrids between Nile tilapia females (*Oreochromis niloticus*) and blue tilapia males (*Oreochromis aureus*).** Manuscript.

1. GENERAL INTRODUCTION

Aquaculture dates back several hundreds of years and has been practiced by different civilizations. For example, in China common carp has been farmed since 2000 B.C (Rabanal, 1998), and in Egypt Nile tilapia held in ponds are depicted in tomb sculptures dating back 4000 years (FAO, 2005). In the last four decades the increasing global demand for food coupled with the limited fishery stocks have motivated a continuous and fast development of this activity. In 2008 aquaculture supplied 37 percent of the total fisheries production[1] (142 million metric tons) and accounted for 46 percent of the total food fish supply (FAO, 2010). The large variety of species, environments and management procedures used in this industry reflect its widespread growth. Fish accounted for 15.7 percent of the global population intake of animal protein in 2007 (FAO, 2010), showing the importance that aquaculture plays in meeting the food requirements of a growing world population in a continuous and sustainable way.

1.1. Tilapia Aquaculture production

"Tilapia" is the common name given to some fresh warm-water fish from the *Cichlidae* family which inhabit the African continent, Israel and Jordan. Specifically, they belong to the genera *Oreochromis*, *Sarotherodon*, and *Tilapia* (McAndrew, 2000). They have been introduced to Asia, South East Asia, America and Europe for the purpose of aquaculture since 1965 (Philippart and Ruwet, 1982). As a result they are farmed in varied environments such as freshwater cages, earthen freshwater ponds, raceways, tanks, recirculation systems and brackish water ponds. Earthen freshwater ponds are their most common culture system. Polyculture of tilapia and other species such as carp or shrimp has recently proven to be beneficial (Fitzsimmons et al., 2011). Tilapia aquaculture accounted for 80**%** of the global tilapia production in 2009 (FAO, 2011).

Tilapias have become a favorite amongst fish farmers due to its rapid growth and resilience. Farmed tilapia production reached 3 million metric tons in 2009, making it the second most important aquaculture fish species after carps (FAO, 2011). Fitzsimmons et al. (2011) predict tilapia will become the most important aquaculture species in the future due to its wider distribution of production and consumption. Nile tilapia (*Oreochromis niloticus*) is the most common tilapia species farmed due to its excellent growth potential and general sturdiness. In 2009 Nile tilapia accounted for 82.1 percent of the total tilapia Aquaculture production (FAO, 2011). Blue tilapia (*Oreochromis aureus*) has also been favored due to its higher cold tolerance (0.2 percent of total tilapia Aquaculture production in 2009) and Mozambique tilapia (*Oreochromis mossambicus*) due to its salinity tolerance (1.1 percent of total tilapia Aquaculture production in 2009) (FAO, 2011). Hybrids between tilapia species are also commonly used. In 2009 hybrids and other tilapia species not elsewhere included were grouped[2] and accounted for 16.5 percent of the total tilapia Aquaculture production (FAO,

[1] Statistics do not include aquatic plants
[2] Common name used in FAO database is "Tilapias nei"

2011). The Longfin tilapia (*Oreochromis macrochir*), Mango tilapia (*Sarotherodon galilaeus*), Redbelly tilapia (*Tilapia zillii*), Redbreast tilapia (*Tilapia rendalli*), Sabaki tilapia (*Oreochromis spilurus*), and the Three spotted tilapia (*Oreochromis andersonii*) all together account for 0.2 percent of the total tilapia Aquaculture production in 2009 (FAO, 2011).

One of the hybrids most commonly used is the cross between blue and Nile tilapia, which is farmed in sub-tropical environments where cold temperatures restricts the growth period to summer (Hepher and Pruginin, 1982) since severe mortalities and decreased growth may occur in hard winters (Tave et al. 1990). These blue x Nile tilapia hybrids have better cold tolerance than pure Nile tilapia and also yield offspring with higher male proportion (see 1.3.4.), which is also beneficial since age is the most important factor affecting sexual maturity and older overwintered fingerlings reproduce during their grow-out period (Hepher and Pruginin, 1982) making all male populations more profitable to farm than mixed sex populations (see 1.2.2.1). The second most commonly used hybrids are those that produce attractive red coloration, since they command higher market values and in some domestic markets they are preferred (e.g. Colombia, Jamaica). Some of these red hybrids are also used since they tolerate higher salinities and may be farmed in brackish waters (Watanabe et al., 1988; Suresh and Kwei, 1992). Red tilapias are usually genetic mutants selected from *Oreochromis sp*. (Lovshin, 2000). Unfortunately the genetic makeup of many of the red hybrids used for farming is unknown since the original red tilapia strains commonly used (Table 1) have been crossed with other red tilapias of unknown origin and with wild *Oreochromis sp.* (Lovshin, 2000).

Table 1. Examples of some original red tilapia strains commonly used

Strain Name	Species crossed	Source
Taiwan red	Mutant red-orange female *O.mossambicus* with normal colored male *O. niloticus.*	Galman and Avtalion (1983)
Florida red	Normal colored female *O.hornorum* with male mutant red-gold *O.mossambicus.* Mated later with *O. niloticus* and *O. aureus* .	Behrends et al. (1982) Behrends and Smitherman (1984)
Israel red	Red colored *O.niloticus* with wild *O. aureus.*	Hulata et al. (1995)
Philippine red	*O. niloticus* and *O. mossambicus.*	Romana-Eguia and Eguia (1999)
Red stirling	Mutant Egyptian *O. niloticus.*	McAndrew et al. (1988)
Thai red	*O. niloticus* and *O. mossambicus.*	Pongthana et al. (2010)
Singapore red	Mutant *O. mossambicus.*	Romana-Eguia and Eguia (1999)

Tilapia production is reported in over 100 nations (Fitzsimmons et al., 2011), however it is most commonly farmed in Asia and Latin America. In 2009 China was by far the biggest producer, with a production of 1.2 million metric tons (41%), followed by Egypt (with 13%), Indonesia (with 12%), Philippines (with 8%), Thailand (with 7%), Brazil (with 4%), Vietnam (with 2%), Taiwan (with 2%), Colombia (with 1%) and Ecuador (with 1%), and all the remaining countries together account for 9% of the global aquaculture tilapia production in 2009 (FAO, 2011). As pointed out by Zimmerman (2005), of the five most human populated countries in the world, four are among the most important farmed tilapia producers (China, India, Indonesia and Brazil), and one (United States) is the largest tilapia importer. This means that tilapias are now very well known worldwide as popular source of protein for human consumption.

1.2. Constraints in Tilapia Aquaculture

Tilapias have excellent aquaculture potential because of their fast growth, herbivorous and omnivorous feeding habits, high food conversion efficiency, high tolerance to low water quality, ease of spawning, easy handling, good resistance to diseases and wide consumer acceptance (Chervinsky, 1982). Nile tilapia has excellent growth potential and harvest weight in tilapias is under additive genetic control and responds to selection. In Malaysia GIFT strains have been reported to grow from fry up to 600g in three months and selection response for live harvest weight has been estimated to be 14% (Ponzoni et al., 2011). However there are still some challenges to be encountered in tilapia farming.

1.2.1 Biological constraints

The tropical origin of tilapias is reflected in their ecological physiology, specially their temperature preference during reproduction (Chervisnky, 1982). The optimal water temperature range for most species is between 25°C and 28°C, reproduction stops at 22°C, feeding stops at 20°C and exposure to temperatures below 8-12°C for several days can be lethal (Wohlfarth and Hulata, 1981; Chervisnky, 1982). On the other hand, tilapias can tolerate temperatures up to 42°C (Wohlfarth and Hulata, 1981). Some species such as *T. sparmani*, *T. rendalli*, *T. zilli*, *S. galilaeus*, *O. aureus*, and *O. mossambicus* have higher cold tolerance (Chervisnky, 1982; Cnaani et al. 2000). Thus tilapia farming under ambient temperatures is limited to tropical and sub-tropical regions. Tilapias can be farmed in other regions only if water bodies are heated above ambient temperature by geothermal water sources or artificial heating (Chervisnky, 1982). When tilapias are farmed in sub-tropical regions grow-out is limited to summer and fry must be overwintered (Hepher and Pruginin, 1982). Cold tolerant tilapia species (or their hybrids) are recommended for sub-tropical regions. Hybrids have similar cold tolerance than the parental cold tolerant species (Lovshin, 1982; Lahav and Ra'anan, 1998).

It is assumed that tilapias evolved from a marine ancestor; hence some species like *O. mossambicus* are euryhaline and can reproduce and grow in fresh, brackish and seawater (32‰) (Wohlfarth and Hulata, 1981; Chervinsky, 1982). Certain hybrid strains of red tilapia are also salt water tolerant (32‰) and have good growth under these conditions (Lovshin, 2000). Some species such as *O. aureus* and *T. zilli* can withstand seawater, but they do not reproduce (Chervinsky, 1982). Other species such as *O. niloticus* and *S. galileus* do not tolerate sea water (Wohlfarth and Hulata, 1981; Lovshin, 2000), and not much divergence in the salt water tolerance has been found in different strains of *O. niloticus* (Cnaani et al., 2011). In Northern Vietnam Nile tilapia were evaluated for growth and survival in freshwater earthen ponds and brackish water ponds and the genetic correlations between the two environments for growth (r_g=0.45 ± 0.09) and survival (r_g=0.42 ± 0.05) were rather low, suggesting separate breeding programs should be considered Nile tilapias reared in fresh and brackish water (Luan, 2010). Tilapias have high tolerance to poor water quality and are able to live where most other fish could not survive. Tilapias seem to tolerate dissolved oxygen (DO) as low as 1ppm, and short term DO limit of 0.1 ppm has been recorded for *O. mossambicus* and *O. niloticus* (Chervinsky, 1982). Metabolism, growth and disease resistance may be depressed when DO are below 1ppm for prolonged periods

(Popma and Masser, 1999). Lethal acidic limit is approximately pH 4 and alkaline limit pH 11 (Swingle, 1961), but they perform best in a PH range from 6 to 9 (Popma and Masser, 1999). In *O. aureus* the un-ionized ammonia (NH_3) 48-hour median lethal concentration (LC50) was 2.4 ppm (Redner and Stickney, 1979). Prolonged exposure to un-ionized ammonia above 1ppm causes mortalities (Popma and Masser, 1999). With DO at 6 ppm and chloride concentrations at 22 ppm, the nitrite 4 day LC50 was 89 ppm (Popma and Masser, 1999).

1.2.2 Farming constraints

1.2.2.1 Early reproduction

Early sexual maturation of tilapias poses a major problem since fry produced by the stocked fish causes stunted growth and large size variability (Longalong et al., 1999; Little et al. 2003). Early reproduction in the culture ponds results in large amounts of fish of no commercial value. Traditional methods to counteract size variability such as partial harvesting are not effective with tilapia since the longer culture periods required increase natural spawning and make the proportion of market size fish at each partial harvest progressively smaller (Hepher and Pruginin, 1982). For Nile tilapia, Longalong et al. (1999) documented a possible negative correlation between growth rate and age at sexual maturation. Since most farmers and breeding companies select for increased harvest weight, a possible correlated selection response for decreased age at maturation may make it challenging to reach market size before maturation occurs. An alternative solution to the problem is polyculture with a predator species (e.g *Lates niloticus*) that preys on the tilapia fry produced in the pond, but this has not been used commercially due to the difficulty of obtaining reliable sources of predator fry (Guerrero, 1982; Hepher and Pruginin, 1982). Therefore early reproduction is considered the main disadvantage for tilapia culture.

Stunted growth caused by early reproduction can be dealt with by farming all-male populations (sex dimorphism favors males) or using fast growing mixed sex populations that are harvested before they reach sexual maturation (usually between 150-200g), but the latter depends on the acceptance the market has for small fish (Hepher and Pruginin, 1982). However, spawning may occur at 20 grams in slow growing Nile tilapias reared in sub-optimal conditions (Popma and Masser, 1999). Since main importing countries like the US prefer fish greater than 450g (Fitzsimmons 1999), the use of all male fry has become the norm.

All male populations may be obtained by manual sorting (Beardmore et al., 2001), inter-specific hybridization (Hickling, 1960; Pruginin et al., 1975; Wohlfarth and Hulata, 1981; Lovshin, 1982; Hulata et al., 1983; Hulata et al. 1993; Wohlfarth, 1994; Desprez et al., 2006), hormonal sex reversal (Guerrero, 1975; Phelps et al., 1992; Galvez et al., 1996), chromosome manipulation (Beardmore et al., 2001), and for Nile tilapia by the production of "YY" males through the Genetic Male Tilapia technology (GMT) (Mair et al., 1991a; Mair et al., 1997). Manual sorting is prone to human error and requires skilled labor (Guerrero, 1982; Hulata et al., 1983). Five different hybrid combinations using Nile tilapia females and males of other species (*O.aureus*, *O.machrochir*, *O.urolepsis hornorum, O. variabilis*, and *O.jipe*) have been shown to produce all male populations (Eknath and Hulata, 2009). But

inter-specific hybrid combinations that in theory should give 100% males (e.g. female Nile x male blue) show inconsistent results depending on the strains used (Pruginin et al., 1975; Garcia Pinto, 1982; Mair et al. 1991b). Additionally maintaining pure stocks over a long period has proven to be challenging since hybrids can easily contaminate the pure stock due to their physical resemblance (Lovshin, 1982; Hulata et al., 1983). Chromosome manipulations (androgenesis and gynogenesis) have been achieved experimentally, but it is difficult to use these technologies in a commercial scale (Beardmore et al., 2001). The production of "YY" Nile tilapia males through the use of GMT requires much labor, time (three generations of breeding and progeny testing) and is also dependent on the strains used (Mair et al., 1997; Tuan et al., 1999). Additionally production of "YY" males requires a laboratory with advanced facilities and generates a relationship of dependence between the hatchery and the laboratory; a situation that is not ideal especially in developing countries (Ponzoni et al, 2011). Thus, hormonal sex reversal of fingerlings using 17-α-methyltestosterone into the diet has become the common industry standard to produce all male fry (Phelps and Popma, 2000). Hormones are used in physiological doses, for short term treatment (for 21-28 days using 9–11 mm fry)(Phelps and Popma, 2000), and are eliminated before the fish reach market size; however the effect of methyltestosterone on the environment is not well studied and the commercial use is not always controlled (Piferrer et al., 2008). Nevertheless consumer resistance to the use of hormones may promote alternative methods to produce all male tilapia fry, and Best Aquaculture Management Practices (BAP) currently encourages methods other than hormone use (GAA, 2008).

1.2.2.2 Low fecundity

In general fecundity of mouthbrooding tilapias (*Oreochromis* and *Sarotherodon*) is lower than that of substrate breeders (*Tilapia*) (Wohlfarth and Hulata, 1981). Large variation in fecundity has been observed in hybrid crosses, and some particular hybridization attempts have been unsuccessful (Wohlfarth and Hulata, 1981). Low fry production has been a problem when performing interspecific spawns (Mires, 1982). For example, reduced fingerling production has been reported in hybrids between *O.niloticus* x *O.aureus* and *O. niloticus* x S. *hornorum* when compared to the pure species (Lovshin, 1982).

1.2.2.3 Diseases

When high water quality is not maintained diseases often appear. As stocking density intensifies and culture expands to places where proper conditions are difficult to maintain infectious diseases appear (Watanabe et al., 2002). Most mortalities in ponds are caused by bacteria, fungi and parasites. The most common diseases (with causative agent) that affect tilapia farming are: Motile Aeromonas Septicemia (MAS)(*Aeromonas spp*.), Bacterial hemorrhagic septicemia (Pseudomonas sp.), Vibriosis (*Vibrio spp*.), Columnaris (*Falvobacterium columnare*), Edwardsiellosis (*Edwardsiella tarda*), Streptococcossis (*Streptococcus sp.* and *Enterococcus sp.*), Saprolegniosis (*Saprolegnia parasitica*), Ciliates (e.g *Ichthyophthirius mutifiliis, Trichodina*), and Monogenetic trematodes (*Dactylogyrus spp*,

Gyrodactylus spp.)(Popma and Masser, 1999; FAO, 2005). Additionally a few viral diseases (Whirling viral disease and Iridiovirus) and a rickettsia like organism (RLO) have also been reported to cause mortalities (Popma and Masser, 1999; The Fish Site, 2006).

1.2.2.4 Off-flavor and fillet yield

A major problem for the tilapia industry has been the off-flavor caused by blue-green algae blooms in the production ponds (Fitzsimmons et al., 2011). However, management procedures to identify off-flavor and treatment of fish with off-flavor before processing are the common measures taken to prevent this problem (Fitzsimmons et al., 2011).

Low fillet yield in Nile tilapia, as compared to other species, is an additional constraint (Fitzsimmons et al., 2011). Reported genetic correlation between body weight at harvest and fillet yield varies from 0.74 to 0.44 (Rutten et al., 2005; Nguyen et al., 2010). Simultaneous selection for fillet yield and body weight at harvest has been implemented in China; and expected response for body weight was reduced 20% when compared with single-trait selection for only body weight (Thodesen et al., 2011). Nevertheless genetic correlations between body weight at harvest and fillet weight are very high (0.96 to 0.99)(Rutten et al., 2005; Nguyen et al., 2010), showing body was a better predictor for fillet weight compared to other body measurements (length, width, corrected length, head length)(Rutten et al., 2005). High fillet yield in small fillet is of little economic value, compared to large fillet with similar yield which is of good economic value; thus fillet weight is also of great importance and can be improved through indirect selection for body weight (Nguyen et al., 2010).

1.3. Sex determination in Tilapia

Sex determination is the process that directs the development of undifferentiated gonads into testes or ovaries (Stelkens and Wedekind, 2010). Genetic sex determination (GSD), environmental sex determination (ESD) or a combination of both can exist in fish species (Devlin and Nagahama, 2002). GSD and ESD represent opposite endpoints of a continuum rather than discrete categories (Baroiller et al., 2009; Stelkens and Wedekind, 2010), since an adaptive transition between GSD and TSD has been shown for other fish species (e.g *Menidia menidia;* Lagomarsino and Conover, 1993). GSD can be attributed to major genetic factors (e.g. sex chromosomes), minor autosomal influences or a combination of the two. Sexual differentiation is the physical process of gonad development after sex has been determined, and should not be confused with sex determination (Devlin and Nagahama, 2002). Species with true ESD do not have a primary sex fixed at conception and the first development difference between sexes is caused by the environment (Stelkens and Wedekind, 2010). In fish, temperature sex determination (TSD) is common (Devlin and Nagahama, 2002; Stelkens and Wedekind, 2010)**.** The process when environmental factors directly or indirectly override GSD is called environmental sex reversal (ESR) (Stelkens and Wedekind, 2010). Tilapias are a good example of ESR, since sex is determined by major genetic factors and minor autosomic influences (GSD), but high temperatures can override this effect and result in masculinized offspring (ESD)(Baroiller et al., 2009).

Different tilapia species have different kinds of sex determination systems. A system of male heterogamety (XX/XY) has been proposed for *O. mossambicus* and *O. niloticus* , and a system of female heterogamety (WZ/ZZ) has been proposed for *O. aureus* and *O. hornorum*. These systems have been proposed after examining the sex ratio of: progeny from masculinized females crossed with males, progeny from feminized males crossed to regular females, progeny from meiotic and mitotic gynogenetic progeny (e.g. Mair et al., 1991a,b) or progeny of hybrids between two species and examining both reciprocal crosses (e.g. Hickling, 1960).

1.3.1. Evidence of sex chromosomes and sex linked markers

Approximately 10% of the fish species have been found to have distinct sex chromosomes (Devlin and Nagahama, 2002). However, many fish species that do not possess visible sex chromosomes may utilize sex determination systems that are associated primarily with single chromosomes (Devlin and Nagahama, 2002). Cytogenetic evidence for sex chromosomes in tilapia has been found for *O. niloticus* (Carrasco et al. 1999) and *O. aureus* (Campos-Ramos et al, 2001) by examination of pairing in synaptonemal complex (SC) analysis. In *O. niloticus* one unpaired region in the longest bivalent (pair 1) was observed in the heterogametic male genotype (XY) (Carrasco et al, 1999), whereas for *O. aureus* two unpaired regions were found in two different bivalents (longest bivalent and one short bivalent) in the heterogametic female genotype (WZ), suggesting two pairs of sex chromosomes may be present (Campos-Ramos et al., 2001). For *O. mossambicus* no unpaired regions were observed, but one heterogametic (XY) individual (and several hybrids) showed diffuse lateral elements and staining that suggests chromosome pair 1 is also related to sex determination (Campos-Ramos et al, 2003). Homology of chromosome pair 1 found in *O. mossambicus* and *O. niloticus* was demonstrated with in situ hybridization (Campos-Ramos et al, 2003). Campos-Ramos et al. (2009) estimated synaptonemal complex total lengths (SCTL) for *O. niloticus*, *O. mossambicus* and *O. aureus* and found that SCTL were not influenced by the type of GSD system and did not correlate with sex-specific length differences in the *Oreochromis* linkage map, suggesting that the phenotypic sex (and not the genotype) determine the SCTL. “Sex chromosomes” seem to be in an early stage of differentiation in tilapia (Carrasco et al., 1999; Cnaani et al., 2008), and several theories regarding how “sex chromosomes” evolved have been proposed (Lande et al., 2001; Cnaani et al., 2008).

Sex linked markers were initially found in linkage group 1 (LG1) for *O. niloticus* (Lee et al., 2003) and in both LG1 and LG3 in *O. aureus* (Lee et. al 2004). Shirak et al. (2002) found an association between deleterious alleles and distorted sex ratios in *O. aureus*, and Cnaani et al. (2008) found evidence of sex linked lethal effects in LG1. Lee et al. (2005) constructed a linkage map for *O. niloticus* and *O. aureus*. Based on this map 6 tilapia species (*O. niloticus*, *O. aureus*, *O. mossambicus*, *O. karongae*, *T. mariae* and *T. zillii*) were tested for association with LG1 and LG3 (Cnaani et al., 2008). For *O. karongae* and *O. mariae* sex determining locus was on LG3 (WZ/ZZ system), for *O. niloticus* and *T. zillii* on LG1 (XX/XY system), and for

O. aureus and *O. mossambicus* in both LG1 and LG3 (Cnaani et al., 2008). The two loci found to be involved in sex determination are located in two different non-homologous chromosomes: LG1 locus is a dominant male determiner (XY) and LG3 is a female dominant determiner (WZ)(Cnaani et al., 2008). LG1 was linked with sex in two families, while in the third family there was not linkage between LG1 and sex (Lee et al., 2003). None of the markers explained the sex of every individual in the families tested by Cnaani et al. (2008). Eshel et al. (2010) found that both LG1 and LG23 had a strong association with sex in *O. niloticus*, but the strongest association was found with LG23 which explained sex in 97.4% of fish. A male-associated allele (MAA) was found in almost all males, and the mating of males with MAA and sex reversed females (with MAA) yielded 75% male offspring, whereas mating of sex reversed males without MAA and females (without MAA) gave 96%-100% females (Eshel et al., 2010). Further research is currently underway regarding LG23.

1.3.2. Evidence of polyfactorial sex determination

In some fish species, where crosses within the same species do not consistently produce 50% male proportion, Mendelian segregation of "sex chromosomes" is not responsible for sex determination (Devlin and Nagahama, 2002). In a strictly polyfactorial system sex is determined by the combinations of several genes (each with minor or epistatic effects), and the sex of the zygote depends on whether the sum of the effects of all genes surpasses a certain threshold value (Bulmer and Bull, 1982**;** Stelkens and Wedekind, 2010). Vandeputte et al. (2007) showed that sex in sea bass (*Dicentrarchus labrax*) is under polygenic control and obtained a heritability of 0.62 ± 0.12 for male proportion on the underlying scale.

In tilapia, deviations from the expected male proportion of 50% in crosses within the same species (e.g. Mair et al., 1991a), deviations from the expected male proportion of 100% in hybrid progeny produced from two species using homogametic breeders (i.e. XX females with ZZ males)(e.g. Pruginin et al., 1975) and deviations from the expected male proportion of 100% in crosses with two homozygous breeders of the same species (i.e. YY males with XX females)(e.g. Tuan et al., 1999) are attributed to the action of several minor autosomal genes. The presence of autosomal genes that influence sex ratio was proposed for tilapia by Hammerman and Avtalion (1979) (see 1.3.4). Two different loci which are not in the sex chromosome explained the presence of males in XX clonal lines of *O. niloticus* (Karayücel, et al., 2004), suggesting the existence autosomal masculinizing genes. Lester at al. (1989) reported a heritability estimate of 0.26 (confidence interval 0.13-0.48) on the underlying scale for male proportion in Nile tilapias kept under normal rearing temperatures (Lester et al., 1989); this is the only heritability reported so far in this species and more studies need to be performed either to confirm or refute these results.

1.3.3. Temperature effects

Ospina-Alvarez and Piferrer (2008) propose that for a species to have TSD they should comply with the following two criteria: 1) Absence of sex chromosomes, 2) changes in sex ratio occur in temperatures ecologically relevant for the species (i.e. temperatures that may

be encounter in the wild). Thus, it is clear that tilapias do not exhibit solely TSD, but have GSD+ESR instead.

Baroiller et al. (1995) found that temperatures above 32°C applied during the period of gonad differentiation, from 10 until 20 days post fertilization (dpf), can masculinize all female XX Nile tilapia. Several studies confirmed the masculinizing effects of temperature treatment during the period of gonad differentiation in Nile (Tessema et al., 2006; Wessels and Hörstgen-Schwark, 2007, 2011) and blue (Deprez and Mélard, 1998) tilapia populations. Rougeot et al. (2008) showed that temperature treatment (35-36°C) before gonad differentiation, from 12 hours post fertilization (hpf) to 52 hpf, could also masculinize Nile tilapia. However, Wessels et al. (2011) did not find a masculinizing temperature effect in this period (temperature treatment of 34°C applied from 12 to 51 hpf, treatments of 35°C - 36°C showed total mortality). Constant high water temperatures are not likely to be found in natural tropical environments, since temperatures fluctuate during the course of the day. Nevertheless, Baras et al. (2000) found a masculinizing effect for *O. aureus* reared at fluctuating temperatures (from 27°C night to 35°C day) during 28 days after first feeding , which was of less magnitude than the masculinizing effect of fish maintained at a constant temperature of 35°C. Thus, results suggest that masculinization due to high temperature could occur in farmed tilapia.

In Nile tilapia not all progenies have the same sensitivity to temperature treatment during gonad differentiation; Baroiller and D'Cotta (2001) showed there was a parental effect and Tessema et al. (2006) showed that both the sire and the dam contributed to this parental effect. Wessels and Hörstgen-Schwark (2007, 2011) confirmed that temperature sensitivity was under additive genetic control and responded to selection**.** After three generations of sib selection carried out in two divergent lines (high-line>80% male proportion, low-line <60% male proportion), cumulated realized heritabilities were 0.63 in the high-line and 0.84 in the low line (Wessels and Hörstgen-Schwark, 2011). However it seems both masculinizing and feminizing genes exist in Nile tilapia, since high water temperature treatment during sexual differentiation has also been reported to cause a feminizing effect in progenies of YY males (Abucay et al., 1999) and in some progenies of normal XY males from the low-line (Wessels and Hörstgen-Schwark, 2011).

1.3.4. Hybridization

In some species the hybrid combination may determine the sex of the offspring**.** Hickling's (1960) pioneering work in Tilapia showed that hybrids between *O. mossambicus* and *O. urolepis hornorum* were not only fertile but also had high male proportion, and based on his results he proposed a male heterogametic XX/XY sex determination system for *O. mossambicus* and a female heterogametic WY/YY sex determination system for *O. urolepis hornorum*, as described by Gordon (1957) for platyfish. When O. *urolepis hornorum* males were mated with *O. mossambicus* females the offspring male proportion was nearly 100% as expected, however when *O. mossambicus* males mated with *O. urolepis hornorum* females the offspring did not give the expected male proportion of 50%, but instead gave

75% (Hickling, 1960). Chen (1969) made a series of hybrid crosses and backcrosses between *O. mossambicus* and *O. urolepis hornorum* obtaining similar results, and he explained his results with the four-gonosomal model (XX/XY and WZ/ZZ) suggested by Bellamy (1936) for platyfish. The four gonosomal model could explain the 75% male proportion obtained when mating *O. mossambicus* males with *O. urolepis hornorum* females, and most of the other crosses. Hammerman and Avtalion (1979) proposed an autosomal theory to explain Chen's (1969) results. In this theory the sum of the effects of three alleles (W, X and Z, where Y=Z) of a major sex determining locus and two alleles of autosomal locus (A, a) determines sex, and each alleles has relative value of maleness (e.g. A=5, a=0, W=-4, X=-7 and Y=3) (Hammerman and Avtalion, 1979). It is assumed that within each species autosomes are identical (AA or aa), but once F2 hybrids and backcrosses are performed a total of six gamete types (AY, AX, AW, aX, aY, aW) and 18 possible genotypes are theoretically possible predicting eight different possible male proportions (100%, 75%, 62.5%, 50%, 43.75%, 37.5%, 25% and 0%) (Hammerman and Avtalion, 1979). Nevertheless, deviations from the expected male proportions of 50% in pure species (e.g. from 30% to 80% in *O. niloticus*; Mair et al., 1991a,b) and deviations from 100% in hybrids between homogametic parents (e.g. from 34% to 100%, between female *O. niloticus* and male *O. aureus*; Pruginin et al., 1975; Garcia Pinto, 1982; Mair et al. 1991b) cannot be solely explained by any of these models.

1.4 Breeding Programs for tilapia

The growth of aquaculture in developing countries has been an incentive for the development of genetic improvement programs. In tilapia species animal breeding has mainly been used to increase growth, but other traits of economic importance such as cold tolerance, salinity tolerance, carcass quality, disease resistance and color have been included in different degrees in the breeding goals of several programs (Neira, 2010). For Nile tilapia the most recognized breeding programs are the GIFT program (Eknath et al., 1993, 2007; Bentsen et al., 1998, 2011), the Freshwater Aquaculture Center Selected Tilapia (FaST) (Abella et al., 1990), GET-EXCEL (Tayamen, 2004) and Genomar Supreme Tilapia (GST)(Zimmerman and Natividad, 2004). The GET-EXCEL and the GST strains are derived from GIFT material (Eknath and Hulata, 2009). Other 18 breeding programs (located in Egypt, Ghana, Bangladesh, China, Malaysia, Philippines, Sri Lanka, Thailand, Vietnam, Brazil, Colombia and Costa Rica) have been reported for Nile tilapia, 61% of them select only for growth and more than half operate with a public sources of funding and genetic material is for local use (Neira, 2010). Another two programs are reported for *O. aureus*, one for *O. shiranus*, and four for red tilapia hybrids (*O. sp.*) (Neira, 2010). Only one genetic improvement program in tilapia has been reported in a developed country in contrast with the 32 breeding programs reported for salmonid and trout species (Rye et al., 2010). However male proportion has not been included as a breeding goal in any tilapia selective breeding program. However, GMT tilapias developed by Fishgen offer YY males that produce all or nearly all male progeny (Fishgen Ltd., 2005), Aquaculture Production Technology (APT) Ltd. is a company from Israel that offers tilapia hybrids (female Nile tilapias x male blue tilapia) that produce approximately 98% males without hormone use (Aquaculture Production Technology Ltd., 2006), and Manit Farm offers all male Nile tilapia

fry (Manit Farm, 2007). Prior to the establishment of the GIFT program, most of tilapia production was performed with poorly maintained stocks (Macaranas et al., 1986). The dramatic increase in tilapia production in the last decade is due solely to aquaculture since capture production has been stable (Josupeit, 2010), and this success can partially be attributed to the establishment of genetic improvement programs.

2. AIMS OF THE STUDY

The main aim of this study was to obtain estimates of the genetic variation for male proportion in tilapia. This was studied in strains and strain crosses of the same species (*O.niloticus*), within a population of a single species (*O.niloticus*), and in hybrid families produced by crossing two species (*O.niloticus* females and *O.aureus* males).

Results are presented in three papers: **Paper I** reports estimates of the magnitude of the strain additive, reciprocal and heterosis effects in four Asian farmed strains and four African wild strains of Nile tilapia which were the genetic base of the GIFT population ; **Paper II** reports estimates of the genetic parameters for male proportion in six consecutive pedigreed generations of Nile tilapia; **Paper III** reports genetic variation for male proportion in families of hybrids between Nile tilapia females and blue tilapia males.

3. SUMMARY OF THE PAPERS

3.1. Paper I.

A complete diallel cross experiment with eight strains of Nile tilapia was performed and offspring were reared at seven different grow-out environments (Bentsen et al., 1998). The observed phenotypic sex of each animal expressed as a binary trait (male=1, female=0) was analyzed using two models: first a model to evaluate the significance of the interaction between each of the genetic effects (strain additive, reciprocal and total heterosis) and the test environment, and a second model to estimate the fixed (test environment, batch) and genetic effects (strain additive, reciprocal and total heterosis). Only the strain total heterosis by test environment interaction was found to be significantly different from zero ($P<0.05$). The test environment, batch, strain additive genetic effect, strain reciprocal and the strain total heterosis effects had a statistically significant effect ($P<0.05$) on male proportion, but low in magnitude.

3.2. Paper II.

The best performing individuals for harvest weight among the GIFT diallel crosses were selected to produce a synthetic base population (G0) for further selection of the GIFT genetic material (Eknath et al., 2007). Sex records (scored as male=1, female=0) from six consecutive pedigreed generations from the GIFT project (G0-G5; a total of 1077 full sib families) stocked in 2-7 different test environments within generation, were analyzed with two models both within and across generations: a univariate linear animal model (observed scale) and a univariate threshold animal model (underlying liability scale). Across all generations there was a low but significant additive genetic component for male proportion with heritability estimates of 0.12±0.02 (observed scale) and 0.22±0.04 (underlying liability scale). The within generation heritability estimates varied from 0.00±0.03 to 0.25±0.07 on the observed scale, and from 0.11±0.02 to 0.32±0.07 on the underlying liability scale. Across generations the environmental effect common to full-sibs as a proportion of the total phenotypic variance (c^2) was 0.04±0.00 on observed scale and 0.06±0.01 on the underlying liability scale.

To investigate whether variation in male proportion was solely attributed to polygenic inheritance, the association between the mid-parent estimated breeding values (EBV) obtained excluding offspring information and the mean male proportions of the fullsib families was studied. The regression coefficient of observed fullsib family male proportions on the associated mid-parent estimated EBV's was significantly different (0.64±0.12, $P<0.01$) from the expected value (1.0) if the trait had been under purely autosomal polygenic control. This suggests that the magnitude of the genetic variation in male proportion found in this study may be biased upwards by some parents having a phenotypic sex different from that determined by the major sex determining system (XX/XY).

3.3. Paper III.

Three different stocks of Nile tilapia females and three stocks of blue tilapia males were used to produce 83 crossbred (hybrid) full sib families in Vietnam. Sex records (scored as male=1, female=0) were analyzed using two models: a univariate linear sire-dam model (observed scale), and a univariate threshold sire-dam model (underlying liability scale). Both models included the fixed effects of batch and cross (combination of sire and dam origin) and were estimated assuming either equal ($\sigma_S^2 = \sigma_D^2$) or different ($\sigma_S^2 \neq \sigma_D^2$) sire and dam variances. Heritability estimated ranged from 0.38±0.07 $\left(\sigma_S^2 = \sigma_D^2\right)$ to 0.42±0.09 $\left(\sigma_S^2 \neq \sigma_D^2\right)$ on the observed scale, and from 0.79 ±0.11 $\left(\sigma_S^2 = \sigma_D^2\right)$ to 0.82± 0.13 $\left(\sigma_S^2 \neq \sigma_D^2\right)$ on the underlying liability scale. Effect common to full-sibs as a proportion of the total phenotypic variance (c^2) was marginal (0.04±0.01 on observed scale and 0.08±0.02 on the underlying liability) but significantly different from zero. For all models the effects of batch and cross (sire and origin combination) were statistically significant ($P < 0.001$ and $P < 0.05$, respectively).

4. GENERAL DISCUSSION

None of the eight purebred Nile tilapia strain and strain crosses evaluated in **Paper I** showed a male proportion (MP) close to the desired commercial threshold (above 95% males). The likelihood of finding Nile tilapia strain or strain crosses with an interesting commercial male proportion is therefore low. Other options to increase MP through genetic means are to perform selection within a purebred Nile tilapia population (**Paper II**), or through the systematic crossing of Nile tilapia females with blue tilapia males as the hybrid offspring of these species in this (**Paper III**) and other studies (e.g. Hulata et al., 1983, 1993) have been shown to produce high MP.

If sex in Nile tilapia was exclusively under the control of major genetic factors, and if there is no difference in fertilization rates of X- and Y-sperm and no sex-specific mortality, expected MP in all families would be 50% (only sampling variance) and both the genetic variance between families and the heritability for MP would thus be zero (e.g. as seen in pigs; Toro et al., 2006). Furthermore, in cases where MP differs from 50% as in most studies in Nile tilapia; e.g., due to different fertilization rate of sperm and/or sex-specific mortality, there will still be no genetic variation in MP unless the fertilization rates and sex-specific mortalities varies among the families. Substantial variation in sex-specific mortality among the families is not likely as no sex-specific mortality has been reported for any part of the tilapia lifecycle (Hickling et al, 1960; Tuan et al., 1999; Wessels and Hörstgen-Schwark, 2007, 2011; Wessels et al., 2011). As no information is available on the possible magnitude of the variation among families in fertilization rate of X- and Y- sperm, the possible effect of this factor cannot be quantified. Since phenotypic sex in **Paper I** and **II** was scored visually, and by more than one person per test environment, this may be an additional source of error in the data. Accuracy of visual scoring may range from 80% to 90% (Bardach et al., 1972, cited by Guerrero, 1982), but may be higher in **Paper I** and **II** as the GIFT staff were highly experienced. Moreover, as this error is likely distributed randomly over the families, the overall effect of this is a downward bias of the heritability estimates. In **Paper III** phenotypic sex recordings are more reliable since they were done using acetocarmine dye solution (Guerrero and Shelton, 1974).

Another possible source of error is the variable temperature in the critical phase for sex determination in all three papers. However, in **Paper I** and **II** the temperature was in general below the critical value (36°C) and in **Paper III** the effect of temperature on MP was found to be non-significant or very small. That phenotypic sex in **Papers I** and **II** was determined before the fish were stocked in the different test environments is supported by the high genetic correlation for MP between the different test environments found in **Paper II**.

In **Paper II** the heritability for MP in Nile tilapia was found to be significant (0.12±0.02 on the observed scale) and with a heritability on the underlying liability scale (h^2=0.22±0.04) similar to that reported in Nile tilapia by Lester et al. (1989) (h^2=0.26, CI=0.13-0.48). This suggests that MP (sex specific mortality, fertilization rate, or all three factors), in Nile tilapia is under polygenic autosomal control. .

The estimated heritabilities for MP may be biased upwards due to several factors that were not closely monitored in the present study. In **Paper II** a likely upward bias of heritability of MP was confirmed by the regression coefficient of the mean male proportion of full-sib groups on their mid-parent estimated breeding value, which was lower than the expected value of unity for a trait under purely polygenic autosomal control. This bias may be caused by the use of naturally occurring sex reversed (or YY) parents, since major genetic factors necessarily influence sex (e.g. XX/XY, Cnaani et al., 2008) and naturally occurring XX sires (Mair et al., 1991a; Baroiller and D'Cotta, 2001; Bezault et al., 2007) and XY dams (Mair et al., 1991a; Bezault et al., 2007; Wessels and Hörstgen-Schwark, 2011) have been observed in Nile tilapia. However, only eight possible naturally sex reversed (or YY) parents were identified in **Paper II**, and when omitting these from the data the estimated heritabilities for MP did not change. Recently discovered markers associated with sex in linkage group LG23 (Eshel et al., 2010) could have helped confirm the major genetic sex of these fish as well as all parents and thus obtain more reliable heritability estimates for MP. However, as the data used in this study is nearly two decades old and no tissue samples were obtained for DNA extraction, this is not possible. Therefore, the results from **Paper II** indicate a substantial genetic variation in MP at normal temperature in Nile tilapia. Generally, such traits are expected to respond to directional selection. However, if selection was carried out for increased MP, more masculinized XX males will likely be produced and used as breeders, which will counteract the effect of selection. Additionally, if male proportion over generations of selection is successfully skewed toward males, the few appearing females in the population will necessarily be favored by natural selection, and thus counteract the selection program.

Within a Nile tilapia breeding nucleus population it will therefore be very hard to select efficiently for increased male proportion unless a proportion of the males can be feminized, e.g., with hormones. However, low response of YY-fry to functional feminization with hormones (Diethylstilbestrol) has been a bottleneck for the maintenance of some YY-lines (Müller-Belecke and Hörstgen-Schwark, 2007), and alternative protocols of feminization, such as temperature treatment (Abucay et al., 1999; Wessels and Hörstgen-Schwark, 2011) must be evaluated. If hormones are successfully used to produce feminized sires in Nile tilapia, there will be an increase of YY males in the population. If these YY males can be identified, by genetic markers or progeny testing, their use will increase the selection response and eventually drive the X chromosome towards extinction.

Significant genetic variation in MP is found at high (36°C) temperatures (Hörstgen-Schwark, 2007, 2011). However, the correlation between MP of families at normal (28°C) and high (36°C) temperatures was found to be not significantly different from zero (Wessels and Hörstgen-Schwark, 2011). This strongly indicates that selection for increased male proportion is feasible in temperature-treated fish, as there will always be sufficient females available in a population kept at normal temperature in the critical period for sex determination (10dpf to 20dpf). This implies that in the breeding nucleus selection of breeding candidates kept at normal temperature may be performed based on results of sibs at high temperature, and fry produced for grow-out should be reared using high temperatures in the critical period of sex determination. The magnitude of the genetic variation in MP of the GIFT Nile tilapia population should therefore be evaluated at both

high and normal temperature, as well as the genetic correlation between MP at these two temperatures. In addition, the genetic correlation of MP at high temperature and other traits of economic importance (e.g. growth) must be determined to evaluate the true potential and implications of selection for increased male proportion in Nile tilapia.

That MP in tilapia show significant genetic variation under normal temperatures is supported by the high estimated heritability for MP of Nile females x blue males hybrids on both the observed (0.38) and underlying liability scales (0.79) (equal sire and dam variances, **Paper III**). However, as purebred families of the two species were not produced in **Paper III**, the genetic correlation between MP in hybrids and MP in the purebreds of each species could not be estimated. The magnitude of this correlation is of importance for the feasibility of implementing Reciprocal Recurrent Selection (RRS) for increased MP in hybrids of the two species. If this correlation is medium to high this will most likely result in an increased MP also in the purebred species, and consequently in a lack of Nile tilapia females, making it difficult or impossible in the long run to reproduce the Nile tilapia (without use of hormones). Furthermore, the necessary use of Nile tilapia females to reproduce the pure Nile tilapia population will counteract the selection.

In a tropical environment the advantage of the increased MP of the Nile tilapia x blue tilapia hybrid is impaired by the lower growth potential of the blue tilapia. However, in an environment where low temperature is a constraint to the culture of tilapias, the hybrid has an additional value due to the better cold tolerance of the blue tilapia. To make the Nile x blue tilapia hybrid of interest also in a tropical environment the growth of the blue tilapia must be improved through selection.

If selection is to be performed for increased MP, the sign and magnitude of the genetic correlation between MP and other traits of economic importance, such as growth until harvest, is of interest. However, no such estimates are available for Nile tilapia. In European sea bass (*Dicentrarchus labrax*), Vandeputte et al. (2007) reported a positive genetic correlation of 0.52±0.13 between harvest weight and female proportion. Using the GIFT data in **Paper II,** the genetic correlation between male proportion and harvest weight was -0.60±0.04 (not reported in **Paper II**), and thus of the same magnitude as that reported for sea bass (Vandeputte et al., 2007). Thus, if selection is performed for increased harvest weight a negative correlated response in MP should theoretically be expected. Figure 1 shows the mean estimated breeding values for MP and harvest body weights over six generations in the GIFT population. As expected, the selection performed for increased harvest weight over six generation resulted in an estimated favorable genetic trend for this trait (Figure 1b). However, the mean estimated breeding value for MP (Figure 1a) showed no negative correlated response. The reason for this may be improper adjustment of the observed body weights for sex effects (as only the phenotypic sex was known, but genetic sex may also have an effect). Furthermore, unintentional natural selection for stable 1:1 sex proportions may occur. In this population, naturally occurring parents of both sexes were used, and both sexes thus contribute equally to the next generation.

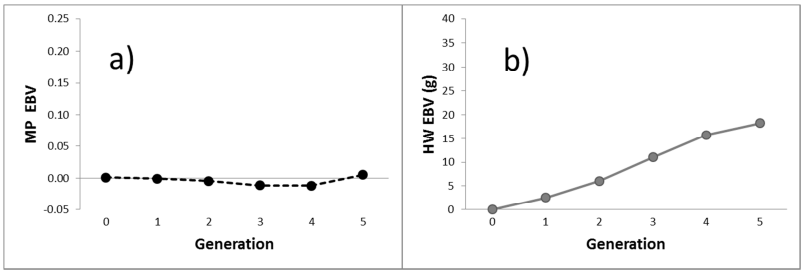

Figure 1. Mean estimated breeding values per generation (as deviation from the overall mean in generation zero) obtained from a univariate animal models* for male proportion (MP, 1a), and harvest weight (HW, 1b) using Nile tilapia data from six generations of the GIFT population selected for increased harvest weight (preliminary unpublished results).

5. CONCLUSIONS AND FUTURE PERSPECTIVES

Genetic variation for MP was found in pure Nile tilapia and hybrids between Nile females x blue tilapia males; however estimates were affected by the major genetic sex determination systems (i.e XX/XY or WZ/ZZ). Thus, reliable estimates of genetic variance due to polygenic factors can only be estimated when the true genetic sex of the parents is known and accounted for in the statistical models. Markers associated to sex (e.g. Eshel et al., 2010) could serve to determine genetic sex of the parents, but selection for MP will still be difficult to implement since it will result in a decreased number of females in the population, which will most likely have strong feminizing genes or lack masculinizing genes, that will necessarily be selected and contribute half of the genes to the next generation counteracting the effect of selection (unless females are produced with the use of hormones, and only mating of XX females and XY males identified through genetic markers are performed). If genetic sex of parents is accounted for by the use of genetic markers, future studies could also obtain more reliable estimates of the genetic correlations between MP and other traits of economic importance and this will provide the means to assess possible favorable or unfavorable correlated responses to selection for increased MP in Nile tilapia. However, as mentioned above, selection for MP would still be difficult to implement.

Genetic markers associated with sex could aide in the production of YY populations of Nile tilapia (i.e. progeny testing not needed to identify YY sires and YY dams). Nevertheless the interaction of other minor genetic factors with the XX/XY genetic determination system may create deviations from the expected male proportion of 100%, as seen currently in some YY populations. Thus production of YY sires and dams must be coupled with selection to increase masculinizing minor genetic factors in the population, to produce YY sires which give consistent all male offspring. Alternatively, selection for increased MP could be achieved by testing families at high temperature during the sex differentiation period, since temperature sensitivity is under additive genetic control, while keeping the breeding candidates at normal temperatures; given that the genetic correlation between MP at normal and high temperatures is zero, as suggested by the non-significant phenotypic correlations found between MP of families at 28°C and 36°C (Wessels and Hörstgen-Schwark, 2011).

Selection of Nile females and blue sires based on male proportion performance of hybrids should be advantageous in sub-tropical countries due the higher cold tolerance of hybrids when compared with pure Nile tilapia, and could only be advantageous in tropical countries given substantial improvement of growth in blue tilapia. Genetic correlations between male proportion of pure (Nile or blue) and hybrids should be assessed, since this strategy is only feasible if the genetic correlation between MP of the purebred and hybrids is low.

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Paper I

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Estimates of strain additive genetic, heterosis and reciprocal effects for male proportion in Nile tilapia, *Oreochromis niloticus* L.

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Strain reciprocal effect

ABSTRACT

Data from the GIFT complete diallel cross with eight strains of Nile tilapia reared at seven different grow out environments were analyzed to evaluate the feasibility of using breeders from the best performing strains or strain combinations to increase male proportion in the population. Of the three strain genetic by test environment interaction effects evaluated, i.e., strain additive, strain reciprocal and strain heterosis, only the latter was found to be significantly different from zero ($P<0.05$). However, it was difficult to see any trend with respect to the ranking of the strain total heterosis estimates across different environments, a prerequisite for a commercial utilization of these heterosis effects in a breeding program. Of those effects found to have a significant effect ($P<0.05$) on male proportion, the environment effect accounted for 0.12%, the batch effect for 0.23%, the strain additive genetic effect for 0.06%, the strain reciprocal effect for 0.25% and the strain total heterosis effect for 0.42% of the total variation in male proportion. The largest difference between two strains was 13.3 ± 4.6 percentage points ($P<0.001$) for the additive genetic effect, 12.0 ± 2.2 percentage points ($P<0.001$) for the reciprocal effect and 5.7 ± 2.2 percentage points ($P<0.01$) for the general heterosis effect. Average heterosis for male proportion was not significantly different from zero (1.8 ± 1.2 percentage points; $P>0.05$), however for some strain crosses the total strain heterosis effect was substantial with 19.7 ± 4.4 percentage points ($P<0.001$) as the largest difference between two strain crosses. The strain additive effect explained only 3%, the strain reciprocal effect 25% and the strain total heterosis effect 59% of the variation in total performance (strain additive + strain reciprocal + strain total heterosis) in male proportion. However, most of the variation in total heterosis was due to the specific heterosis effect which explained a large proportion (53%) of the variation in total performance in male proportion among the crosses, and thus is more important than any of the other studied strain genetic effects; i.e. the strain additive, reciprocal and general heterosis effects. Therefore to maximize gain in male proportion the strain cross or crosses with highest total performance should be chosen. It can be concluded that genetic improvement of male proportion through the use of breeders from the strain and strain crosses with highest male proportion in this study will have an insufficient impact for the immediate commercial applications under the tested farming conditions in the Philippines.

1. Introduction

Fishes are the top aquaculture organisms by quantity and value, and within this group tilapias are ranked third (FAO Fisheries Department, 2006). Nile tilapia (*Orechromis niloticus*) accounted for 85% of the total farmed tilapia production in 2007, reaching two million tonnes (FAO, 2009). This tropical species has been introduced in many countries due to its plasticity and resilience. It is currently reared in a wide array of systems including cages, tanks and ponds.

Sexual dimorphism in this species results in larger males. Females sexually mature and reproduce at an early age causing stunted growth, overcrowding and variable harvest sizes (Longalong et al., 1999). In pond environments fish may even lose weight due to mating, brooding and aggressive territorial behavior (Eknath et al., 2007). This is a major problem in tilapia farming, thus all-male tilapia production has been widely used to profit from sex dimorphism and to prevent stunting. All-male populations are usually obtained either by manual sorting (Beardmore et al., 2001), inter-specific hybridization (Pruginin et al., 1975; Hulata et al., 1983, 1993; Wohlfarth, 1994; Desprez et al., 2006), sex reversal using hormones (Longalong et al.,

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1999; Beardmore et al., 2001) or chromosome manipulation (Mair et al., 1991, 1997; Tuan et al., 1998, 1999). Manual sorting is a simple but time consuming method that requires skilled manpower, thus it is subject to human error (Hulata et al., 1983; Tuan et al., 1998; Beardmore et al. 2001). Reliable inter-specific hybridization is difficult to practice in a commercial scale because brood stock ponds are easily contaminated with hybrids due to inadequate care separating the ponds by species and sex (Wohlfarth, 1994; Beardmore et al., 2001). Hybrids also may have lower growth performance compared to pure *O. niloticus* (Beardmore et al., 2001). Genetic manipulation methods such as production of triploids and all male population by the use of androgenesis are not easily applied to commercial production. Despite the fact that ploidy is expected to influence sex ratios in polygenic systems (Devlin and Nagahama, 2002), triploids from normal parents in *O. niloticus* (XY system) tend to have 1:1 sex ratios (Mair et al., 1991). The production of YY males is an effective method, but it is laborious and time consuming due to the considerable amount of progeny testing required (Beardmore et al., 2001). YY male production also seems to be species or strain specific (Tuan et al., 1999; Wessels and Hörstgen-Schwark, 2007). Therefore the most widely used method for sex reversal of fry in *O. niloticus* is oral administration of hormones. The environmental impact of this hormonal treatment has not yet been clearly demonstrated or refuted, and some food safety oriented consumers may not find it desirable. Thus, environmentally friendly alternatives to increase the male proportion in commercial Nile tilapia stocks are needed.

Nile tilapia have a complex system of sex determination, which combines both genetic and environmental factors (Baroiller et al., 1995a; Baroiller and D'Cotta, 2001; Bezault et al., 2007). Studies suggest that the genetic component of sex is determined by a combination of major sex chromosomes (XX/XY system) and autosomal polyfactorial influences (Devlin and Nagahama, 2002). Deviations from sex ratios predicted by chromosomal sex determination seem to be attributed to polyfactorial, to environmental, or to both kinds of influences (Ezaz et al., 2004). Temperature has been demonstrated to override the sexual determination, with masculinization of the female genotype at high temperatures (Baroiller et al., 1995a; Baras et al., 2001; Tessema et al., 2006; Rougeot et al., 2008). Temperature has also been shown to affect the sex ratio of both domestic and wild populations of Nile tilapia (Baroiller et al., 2009). In summary, sex determination appears to be largely controlled by the interaction of three components: a major determinant locus, a minor polygenic component and temperature during early fry phase (Baroiller et al., 2009).

Some inter-specific tilapia hybrids produce predominantly all-male progeny (Pruginin et al., 1975; Hulata et al., 1983; Wohlfarth, 1994). This could be explained because "between closely related species, striking differences in sex-factor number, strength and location can exist, and when such genomes are placed together in inter-specific hybrids, abnormal sex ratios may result" (Devlin and Nagahama, 2002). A summary of tilapia species crosses for all-male hybrid production is provided by Beardmore et al. (2001). The *O. niloticus* x *O. aureus* is the only hybrid used commercially due its advantageous cold tolerance (Hulata, 2001), but male percentages are highly inconsistent and can vary between 50% and 100% (Beardmore et al., 2001; Devlin and Nagahama, 2002). Studies of the sex ratio of a complex *Oreochromis* hybrid suggested that sex determination has a polygenic component (Desprez et al., 2006). Within Nile tilapia, Tuan et al. (1999) found that the Thai-Chitralada strain showed higher evidence of a polygenic sex determination component than in the Egypt-Swansea strain (Mair et al., 1997).

The "Genetic Improvement of Farmed Tilapias" (GIFT) collaborative research project, conducted in the Philippines from 1988 to 1997, compared the growth performance and survival of four Asian farmed strains and four African wild strains of Nile tilapia (Eknath et al., 1993). Subsequently, a complete diallel cross experiment with eight strains was produced and the additive, reciprocal and heterosis strain effects were estimated for growth performance (Bentsen et al., 1998). A first analysis of the sex ratio of this data was reported by Dionisio et al. (1995).

The main goal of this study was to estimate the magnitude of the strain additive, reciprocal and heterosis effects and the interaction between each of these strain genetic effects and the test environment effect for the trait male proportion (i.e. number of males/total number of males and females) from the complete diallel cross of four Asian farmed strains and four African wild strains of Nile tilapia produced in the GIFT project (Eknath et al., 1993; Bentsen et al., 1998) when using a more robust analysis of the trait than applied by Dionisio et al. (1995).

2. Material and methods

2.1. Strains

Eight strains of Nile tilapia were used. Four were wild strains imported from Egypt (E2), Ghana (Gh), Kenya (Ke) and Senegal (Se), and four were previously introduced strains commonly known in the Philippines as Israel (Is), Singapore (Si), Taiwan (Tw) and Thailand (Th). Details of origins and characterization of the strains were given by Eknath et al. (1993) and Macaranas et al. (1995).

2.2. Mating design, rearing of fry and tagging

A total of 64 possible purebred and crossbred strain combinations were produced from 27 December1989 to 16 March 1990. Purebred female and male brood stock were previously conditioned in separate hapas for two weeks and then distributed into breeding hapas where they mated and spawned. Two females and one male were placed per hapa (Bentsen et al., 1998). On average, seven sires and nine dams contributed progeny per strain combination; however, there were large differences in the number of contributing breeders, ranging from 2-19 for sires and 2-24 for dams (Bentsen et al., 1998). The genetic sex of the sires and dams (according to the XX/XY system) was unknown.

Six to thirteen breeding hapas were used per strain combination and collection of swim-up fry was done at five different occasions at intervals of 12 to 25 days, referred to as batches (Bentsen et al., 1998). All hapas were in the same pond at a water temperature ranging from 26 to 32 °C. Thus in this study the batch effect represents an overall combined effect of age and hapa environment of the swim-up fry. In each batch, fry from the same strain combination were pooled and reared together in fine mesh hapas for 21 days and then transferred to larger mesh hapas until reaching 3 to 6 grams, at which size they were individually tagged with Floy Tag fingerling tags (Longalong et al., 1999) to trace their strain combination origin.

2.3. Test environments

Equal numbers of fingerlings from each strain combination were pooled before being stocked in earthen ponds/cages at eight different test environments (Table 1) described in detail by Eknath et al. (1993). In summary, environments P1, P2, S1 and S2 were fertilized ponds under standard management (P2 had supplementary feeding). Environment C2 was cage culture in reservoir (without fertilization) and environments W2 and W4 were ponds fertilized with agricultural residues (*Leucena sp.* leaves for W2 and buffalo manure for W4). After approximately 90 days animals were harvested and their individual body weights and sex were recorded. Sex was recorded based on external secondary sexual characteristics (phenotypic sex) by experienced personnel (Longalong et al., 1999).

An additional pond environment (S3) was included in Eknath et al. (1993). However, S3 had very high mortality (85%) and very low

Table 1
Male proportion, tag loss, mortality and mean harvest body weight with standard deviations (sd) of males and females for each environment.

Test environment	N (harvest)	Male proportion mean	Tag loss[a] (%)	Mortality[b] (%)	Harvest body weight (g)			
					females		males	
					mean	sd	mean	sd
C2	897	0.54	0.0	43.8	15.7	4.8	21.4	7.2
P1	4383	0.57	1.8	12.4	33.2	10.0	55.0	14.0
P2	4016	0.58	6.8	14.8	50.4	16.0	82.2	19.0
S1	2149	0.58	2.0	13.8	30.0	9.4	39.8	11.0
S2	2229	0.53	1.0	11.9	20.9	6.2	31.3	6.6
W2	1183	0.55	10.4	29.9	55.5	15.0	83.5	17.0
W4	1415	0.55	10.8	18.3	36.3	10.0	57.3	12.0
All	16272 [c]	0.56[d]	4.0	22.2[d]	36.0[d]		57.2[d]	

[a] Information from Table 3 in Bentsen et al. (1998).
[b] Mortality does not include tag loss.
[c] Total.
[d] Mean of all data.

harvest weights (5.7 g), possibly caused by restricted food supply due to low temperatures and inadequate fertilizer application (Eknath et al., 1993). The data from S3 were not included in this study since sexing of small animals is less accurate and also due the high mortality.

2.4. Data analysis

The recorded phenotypic sex of the fish is a binary trait (males = 1, females = 0). For such a trait the binomial distribution can be used as an approximation to the normal distribution when N*θ is greater than 5 (Freund and Walpole, 1987), where N is the number of observations in the smallest sub-cell and θ is the incidence of the trait. In this study the average male proportion was 0.56 (across environments) and there were on average 12.8 records (range from 1 to 73) in the smallest sub-cell (strain cross/environment/batch). In 23% of the sub-cells the number of fish (N) was less than 5. Additionally the recorded phenotypic sex is a rough measure of many assumed underlying genetic and environmental variables that together determine the phenotypic sex. Furthermore sex was subjectively scored and prone to human error. Therefore, when modeling male proportion as a linear trait it is to be expected that the studied effects will explain a low proportion of the variance in the trait.

For a binary trait as sex in this study the variance of a single sex observation is Var(1 or 0) = θ (1- θ) (Freund and Walpole, 1987). For θ = 0.56 the standard deviation of θ, SD(θ) = 0.496, very close to the maximum of 0.5 for a binary trait. Thus, although the studied effects were expected to explain a low proportion of the variation in male proportion the differences between levels of the studied effects (e.g. strains and their crosses) may become substantial.

An overview of the male proportion data across the seven test environments was obtained with a general linear model fitting the fixed effects of test environment, batch and strain combination (SAS Institute Inc., 2004). Least square means for male proportion across environments for the 64 strain combinations are presented in Appendix A and their distribution in Fig. 1.

To reduce the problem of low number of breeders contributing offspring to each strain combination, the effects of each pure strain and strain combination were decomposed into strain additive, strain reciprocal and strain total heterosis effects as shown in Models 1 and 2 following the model of Fimland (1983).

2.4.1. Model 1

Model 1 was used to evaluate the significance of the interaction between each of the genetic effects (strain additive, reciprocal and total heterosis) and test environment and included the following effects:

$$y_{klijm} = \mu + E_k + B_l + \sum_i a_i t_i + \sum_i r_i w_i + \sum_{ij} h_{ij} t_{ij} + \sum_i a_i t_i * E_k + \sum_i r_i w_i * E_k + \sum_{ij} h_{ij} t_{ij} * E_k + e_{klijm} \quad (1)$$

where y_{klijm} is the recorded sex (1 for males and 0 for females) of the mth offspring of the ith and jth strain combination ($i = 1, 2,...8$; $j = 1, 2, \ldots 8$); μ is a constant; E_k is the fixed effect of the kth test environment ($k = 1, 2 \ldots 7$); B_l is the fixed effect of the lth batch ($l = 1, 2, \ldots 5$); a_i is the regression coefficient of the additive genetic effect of the ith strain on the proportion of genes of the strain ($i = 1, 2, \ldots 8$); t_i is the proportion of genes in the mth offspring originating from the ith strain ($t_i = 0.0, 0.5$ or 1.0 and $\sum t_i = 1.0$); r_i is the regression coefficient of the reciprocal effect of the ith strain on the proportion of genes of the strain; w_i is the proportion of genes of the mth offspring of the ith strain ($w_i = 0.0$ for purebreds, $w_i = -0.5$ for sire strain origin, $w_i = 0.5$ for dam strain origin and $\sum w_i = 0.0$; h_{ij} is the regression coefficient of the total heterosis effect for the ith and jth strain combination on the proportion of genes originating from both reciprocals of the ith and jth

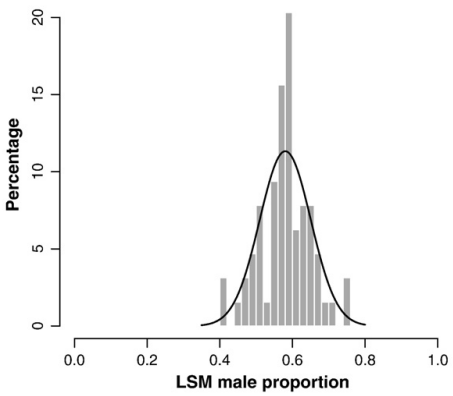

Fig. 1. Distribution of Least square means (LSM) for male proportion at harvest for all 64 strain combinations across test environments (obtained from a general linear model fitting the fixed effects of test environment, batch and strain combination).

strains ($i \neq j$ and $ij = ji$; thus $ij = 1, 2, \ldots 28$, $\sum h_{ij} = 0.0$); t_{ij} is the proportion of genes in the mth offspring of the ith and jth strain combination ($t_{ij} = 0.0$ for $i = j$, $t_{ij} = t_{ji} = 1.0$ for $i \neq j$, $\sum t_{ij} = 0.0$ for purebreds and $\sum t_{ij} = 1.0$ for crossbreds); $a_i t_i {}^* E_k$ is the interaction effect between the ith strain additive genetic effect and the kth test environment; $r_i w_i {}^* E_k$ is the interaction effect between the ith strain reciprocal effect and the kth test environment; and $h_{ij} t_{ij} {}^* E_k$ is the interaction effect between the total heterosis effect for the ith and jth strain combination and the kth test environment; and e_{klijm} is a random error for the mth offspring. The strain additive and strain reciprocal effects were restricted so that $\sum_i a_i = \sum_i r_i = 0$. The choice of the above coding of the proportion of genes of the reciprocal effect (w_i) is discussed in Section 2.4.3.

Of the three genetic strains by test environment effects, only the strain heterosis by test environment effect was found to be significantly different from zero (Table 3). However, the standard errors of the different $h_{ij} t_{ij} {}^* E_k$ estimates were all large, and it was difficult to see any trend with respect to the ranking of the total heterosis estimates across different type of test environments; e.g. between ponds or between ponds versus cages, a prerequisite for a commercial utilization of a genetic effect in a selective breeding program. Consequently, a simplified version of Model 1 excluding the interaction terms (Model 2) was used to obtain estimates of a_i, r_i, h_{ij} across test environments, and also within each test environment by also excluding the test environment effect (E_k) from the Model 1.

2.4.2. Model 2

Model 2 across test environments was:

$$y_{klijm} = \mu_{PB} + E_k + B_l + \sum_i a_i t_i + \sum_i r_i w_i + \sum_{ij} h_{ij} t_{ij} + e_{klijm} \qquad (2)$$

where y_{klijm}, E_k, B_l, a_i, t_i, r_i, w_i, h_{ij}, t_{ij}, e_{klijm} are as explained in Model 1. The strain additive and strain reciprocal effects were restricted so that $\sum_i a_i = \sum_i r_i = 0$. Similarly, E_k and B_l were restricted so that $\sum E_k = 0$ and $\sum B_l = 0$. Under these restrictions μ_{PB} is the mean male proportion of the eight purebred strains. Thus the strain additive genetic (a_i), reciprocal (r_i) and total heterosis (h_{ij}) effects are expressed as a deviation from μ_{PB}.

Estimates of the strain additive, reciprocal and total heterosis effects within test environment (Model 2 without the E_k effect) were in general not significantly different from zero (see Appendix B). Therefore, only parameter estimates obtained across test environments will be presented.

2.4.3. Interpretation and coding of the strain reciprocal effect

The presence of a significant reciprocal effect in diallel cross experiments can be due to a true effect (i.e. a benefit or a loss when fish of a particular strain are used as dams or sires). A significant effect can also be due to a sampling effect due to the use of non-random samples of breeders. Nevertheless, the presence of a significant reciprocal effect in a diallel cross will bias the estimates of additive-genetic effects (Crusio, 1987) and should therefore be accounted for. This can be accomplished using different coding strategies (Table 2). The choice of coding strategy will influence the magnitude and possibly also the ranking of the estimated strain additive genetic effects, while the magnitude of reciprocal effects and heterosis effects will remain unchanged. The choice of coding strategy must therefore be based on sound biological assumptions.

Bentsen et al. (1998) defined reciprocal effects of harvest body weight of Nile tilapia strains as the difference in growth of the progeny when a given strain is used as a dam strain as compared to when used as a sire strain; i.e. as a maternal effect (Table 2). This is a sound biological assumption for growth bearing in mind that Nile tilapia is a mouth brooder (Bentsen et al., 1998). However, in our study we have no evidence to support that maternal ability in Nile tilapia would influence male proportion. For Nile tilapia, Wohlfarth and Wedekind (1991) suggested that only the males were responsible for the response of sex ratio to selection, but Baroiller and D'Cotta (2001) later demonstrated paternal as well as maternal effects influencing sex ratio at basal temperature. Therefore, we considered the cause of the reciprocal effects unknown and of the same magnitude, but with opposite signs, for sires and dam of the same strain ("Unknown" in Table 2). The same coding strategy was used in diallel cross studies of harvest body weight of strains of rohu carp (Gjerde et al., 2002) and tilapia (Maluwa and Gjerde, 2006), but in those cases primarily to account for possible effects of non-random sampling of breeders within strain thus providing more reliable estimates of the strain additive genetic effects.

Table 2
Alternative coding for the strain reciprocal effect and the coding of the strain additive and strain total heterosis effects.

Strain cross	Reciprocal effects (w_i)						Strain additive effect (t_i)		Strain heterosis effect (t_{ij})	
	maternal *		paternal		unknown **					
♂ X ♀	w_A	w_B	w_A	w_B	w_A	w_B	t_A	t_B	t_{AB}	t_{AC}
A x A	1.0	0.0	1.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0
A x B	0.0	1.0	1.0	0.0	-0.5	0.5	0.5	0.5	1.0	0.0
B x A	1.0	0.0	0.0	1.0	0.5	-0.5	0.5	0.5	1.0	0.0
B x B	0.0	1.0	0.0	1.0	0.0	0.0	0.0	1.0	0.0	0.0

* As in Bentsen et al. (1998).
** As in Gjerde et al. (2002) and Maluwa and Gjerde (2006).

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2.4.4. Importance of effects

The relative importance of the interaction effects in Model 1 and the strain additive genetic, strain reciprocal and total heterosis effects in Model 2 were obtained as the marginal increase in the mean square error when excluding each effect from the full model and tested for significance using a partial F-test (Myers and Well, 2003):

$$F_{\alpha, df_1, df_2} = \frac{(1/df_1) \times (SS_{FM} - SS_{RM})}{MSE_{FM}}$$

where α is the test level significance (0.05, 0.01, or 0.001); df_1 is difference between the model degrees of freedom of the full and the reduced models; df_2 is the error degrees of freedom of the full model; SS_{FM} is the sums of squares of the full model; SS_{RM} is the sums of squares of the reduced model; MSE_{FM} is the mean square error of the full model (error variance full model).

As none of the three interaction effects in Model 1 was significant ($P > 0.05$), a final Model 1 was obtained by using a stepwise backward selection procedure by excluding the least significant interaction effect at a time and testing each of the remaining effects using the above partial F-test.

2.4.5. Components of the total heterosis (Model 2)

Total heterosis $h_{ij} = \bar{h} + h_i + h_j + s_{ij}$ (Gardner and Eberhart, 1966) of a particular strain cross was partitioned into average heterosis ($\bar{h}$), general strain heterosis ($h_i$and h_j) and specific heterosis (s_{ij}). Total heterosis (h_{ij}) pools records of both reciprocals, therefore average heterosis was calculated as the mean of the total heterosis estimates $\left(\bar{h} = \frac{1}{28}\sum_1^{28} h_{ij}\right)$. The general heterosis effect of ith strain was calculated as the mean of the total heterosis estimates of all crosses involving the ith strain expressed as a deviation from the average heterosis i.e., $h_i = \frac{1}{7}\sum_{j=1}^{7} h_{ij} - \bar{h}$, and with $\sum_i h_i = 0$ and $\sum_i s_{ij} = \sum_i s_{ji} = 0$ as required restrictions (Gardner and Eberhart, 1966). Specific strain heterosis was calculated as $s_{ij} = h_{ij} - \left(\bar{h} + h_i + h_j\right)$.

2.4.6. Calculation of total strain cross, strain additive and strain reciprocal performances (Model 2)

The total performance for male proportion of the 56 crosses (excluding the purebreds) was calculated as:

$$TP_{ij} = \mu_{PB} + {}^1/_2 a_i + {}^1/_2 a_j - {}^1/_2 r_i + {}^1/_2 r_j + h_{ij}$$

where h_{ij} is the estimate of the total heterosis effect from Model 2 ($h_{ij} = h_{ji}$). The pure strain performance for male proportion was $TP_{ii} = \mu_{PB} + a_i$, since $h_{ij} = 0$ and $i = j$ for the eight pure strains.

The strain additive performance (AP_{ij}) and the strain reciprocal performance (RP_{ij}) for male proportion for the 56 crosses as a deviation from the mean value of the purebred strains were calculated as:

$$AP_{ij} = {}^1/_2 a_i + {}^1/_2 a_j$$

$$RP_{ij} = {}^1/_2 r_j - {}^1/_2 r_i$$

The strain performance contribution for male proportion (additive and reciprocal effects included) of the *i*th strain when used as a sire or a dam strain was calculated as:

$$SP_{sire_i} = {}^1/_2 \mu_{PB} + {}^1/_2 a_i - {}^1/_2 r_i$$

$$SP_{dam_i} = {}^1/_2 \mu_{PB} + {}^1/_2 a_i + {}^1/_2 r_i$$

where a_i and r_i are the estimates obtained from Model 2.

For total performance (TP_{ii} and TP_{ij}) and total heterosis (h_{ij}) estimates within test environment were also obtained. Pearson correlation coefficients were calculated between the total performance of the 64 strain combinations in the different test environments and between the total heterosis estimates (h_{ij}) of the 28 crosses in the different test environments.

2.4.7. Contribution of each genetic effect to variation in total performance (Model 2)

A measure of the relative contribution of each genetic effect in Model 2 to the variation in total performance (TP_{ij}) for male proportion was obtained as (a) the regression of (TP_{ij}) on the performance of each effect (AP_{ij}, RP_{ij} and h_{ij}) and (b) the Pearson correlation coefficient of TP_{ij} with each genetic effect. This was done including only the 56 strain crosses (purebreds excluded) since for all purebreds $h_{ii} = RP_{ii} = 0$ and $TP_{ii} = AP_{ii} = \mu_{PB} + a_i$.

3. Results

3.1. Descriptive statistics

Descriptive statistics of the data from the seven test environments are given in Table 1. The male proportion across all environments was 56% and small differences were observed between the test environments. The test environments with highest harvest body weights (P2, W2 and W4) showed the highest tag losses while the test environment with lowest harvest weight (C2) had no tag loss. This suggests an increasing loss with increased fish size. Mortality varied from 11.9% (S2) to 43.8 % (C2) and showed a positive association with tag loss (excluding environment C2). As expected, sexual dimorphism was observed in all environments. Males were on average 59% heavier than females, ranging from 66% heavier in P1 to 33% heavier in S1 (Table 1). Size dependent tag loss will then imply higher tag loss for males than for females. However, for each test environment the correlation coefficient between male proportion and the raw unadjusted means for harvest body weight of males or females of the 64 strain combinations were not significantly different from zero ($P > 0.05$), with the exception of the P1 females where the correlation between harvest weight and male proportion was positive and significantly different from zero ($r = 0.37$, $P < 0.01$).

Male proportion least square means (LSM) for the 64 strain combinations across test environments (corrected for environment and batch) ranged from 0.41 (Is sires x Se dams) to 0.76 (Th sires x Tw dams)(Appendix A). The Th strain had the highest male proportion both when used as sire and dam (average 0.64) while crosses involving the Gh strain had the lowest male proportion (average 0.55).

3.2. Importance of the effects in Model 1

The analyses of variance results of Model 1 are given in Table 3. Model 1 explained 3.42% of the variation in male proportion. The total heterosis by test environment interaction effect accounted for the largest part of the variation in male proportion (1.18%), while the strain additive by the test environment interaction effect (0.29%) and the strain reciprocal by test environment interaction effect (0.15%) explained much less. The stepwise backward selection procedure (Section 2.4.4) revealed that the strain total heterosis by test environment interaction effect was the only significant interaction effect (Step3), and with only this interaction effect included Model 1 explained 2.96% of the total variation in male proportion. In this final Model 1 the effect of batch explained a larger amount (0.23%) of the variation in male proportion than the test environment effect (0.03%). Of the genetic effects, the strain total heterosis effect (0.33%) explained more of the variation in male proportion than the strain reciprocal effect (0.23%) and roughly five times as much as the strain additive genetic effect (0.06%).

The existence of a significant total heterosis by test environment effect found in Model 1 (Table 3) is confirmed by correlation coefficients not significantly different from zero (in 19 of 21 cases, Appendix C) between the total heterosis estimates in the different test environments (obtained from Model 2).

3.3. Importance of the effects in Model 2

Within test environments Model 2 explained from 1.9% (P1) to 5.8% (W4) of the variation in male proportion (Appendix B), and all effects were significant only for environment S1. In the other test environments the strain additive, reciprocal and total heterosis effects were not statistically significantly different from zero, except for total heterosis effect which was significant for environments P2 and W4 (Appendix B). However, within all test environments, except for C2, the total heterosis effect was the effect that showed the highest marginal increase in R^2. This general lack of significance within test environment may be due to the relatively low number of records.

As expected, Model 2 across test environments explained a lower proportion of the variation in male proportion than the final Model 1 (1.74%, Table 4); however, all the effects in Model 2 were significantly different from zero ($P < 0.05$). Of the genetic effects, the strain additive effect accounted for the smallest proportion of the variation (0.06%), while the strain reciprocal (0.25%) and the strain heterosis (0.42%) effect each accounted for a larger but also low proportion of the total variation in male proportion (Table 4).

3.3.1. Test environments and batches

As expected from the minor effect of test environment on male proportion in Model 2 (Table 4), the differences between test environments were small for male proportion (Fig. 2A). The largest difference was found between test environment S1 and S2 (5 percentage points). Despite the small but statistically significant effect of batch on male proportion seen in Model 2 (Table 4), a higher male proportion was observed for the early batches; i.e. batch 1 had 14 percentage points higher male proportion than batch 5 (Fig. 2B).

Table 3
Analysis of variance for male proportion for Model 1 and final Model 1.

Effect	Model 1			*P* value of F-test[a]			Final Model 1 (step 3)		
	Degrees of freedom	Mean square	Marginal R^2 increase X100	Step1	Step2	Step3	Degrees of freedom	Mean square	Marginal R^2 increase X100
Test environment (Env)	6	0.22	0.03				6	0.21	0.03
Batch	4	2.17	0.22				4	2.30	0.23
Strain additive genetic ($\sum a_i$)	7	3.38	0.08				7	2.47	0.06
Strain reciprocal ($\sum r_i$)	7	7.03	0.18				7	9.38	0.23
Total heterosis ($\sum h_{ij}$)	28	15.65	0.39				28	13.24	0.33
Strain add.x Env ($\sum a_i$xEnv.)	42	1.97	0.29	0.18	0.17	-	-	-	-
Strain rec.x Env ($\sum r_i$xEnv)	42	0.98	0.15	0.98	-	-	-	-	-
Strain total het.x Env ($\sum h_{ij}$xEnv)	168	7.85	1.18	0.12	0.12	0.03	168	7.70	1.15
Error	15967						16051		
Model	304		3.42				220		2.96

[a] Step1 represents the significance levels of the three interaction effects in Model 1, while Step2 and 3 represents the significance levels of these effects for sub-models of Model 1 (see 2.4.4).

3.3.2. Strain additive genetic effect

The largest difference in strain additive genetic effects was that between Th and Gh (Table 5); a difference of 13.3 ± 4.6 percentage points ($P<0.001$). Th, Is, E2 and Ke had estimates of strain additive effect above the mean of the eight purebred strains (μ_{PB}) (Table 5), from which Th and Is were the highest. The other strains had estimates of strain additive effects which were below the mean of the purebred strains (μ_{PB}) (Table 5). However, only the estimate of the Th strain was significantly different from the mean of the purebred strains ($P<0.05$). As mentioned in 2.4.3, estimates of the strain additive genetic effect may change according to how the reciprocal effect is coded. Nevertheless, the additive estimate of Th was the highest for all three codings (w_i) of the reciprocal effect shown in Table 2.

3.3.3. Strain reciprocal effect

The coding of the reciprocal effect shows the increase (or decrease) in male proportion when using a strain as a dam strain as compared to as a sire strain. The strain reciprocal effect (r_i) ranged from -5.8 (Se) to 6.2 percentage points (Is) (Table 5), a difference of 12.0 ± 2.2 percentage points ($P<0.001$). The estimates of Se, Is, Si and Th were significantly different from the mean of the purebred strains ($P<0.05$), while the estimate for E2, Gh, Ke and Tw were not ($P>0.05$). The estimated reciprocal effect for the Is and Si strains were positive, while those for Se and Th were negative (Table 5).

Fig. 3 shows the strain performance contribution for male proportion when used as sire (SP_{sire_i}) or dam (SP_{dam_i}) strains. The Is (6.2 percentage points) and Si (3.3 percentage points) strains have an advantage if used as dam strains, whereas Se (5.8 percentage points) and Th (3.0 percentage points) have an advantage when used as sire strains. For the other strains the differences between a strain used as a sire or a dam strain were marginal (ranging from 0.4 to 1.8 percentage points).

3.3.4. Strain total heterosis, average heterosis and general heterosis

Of the 28 possible strain combinations only six had total heterosis effects that were statistically different from the mean of the purebred strains (Table 6). The largest difference (19.7 ± 4.4 percentage points, $P<0.001$) was that between (Se x Is) and (Si x Th). Of the six significant strain combinations the Se x Is (-6.7 percentage points) cross had negative total heterosis and thus of no practical commercial significance, while three crosses (Gh x Si, Ke x Tw and Se x Si) had positive total heterosis between 7 and 8 percentage points and two

Table 4
Analysis of variance for male proportion (Model 2).

Effect	Degrees of freedom	Mean square	Marginal R^2 increase X100
Test environment	6	0.8**	0.12
Batch	4	2.4***	0.23
Strain additive genetic ($\sum a_i$)	7	2.5[a, *]	0.06
Strain reciprocal ($\sum r_i$)	7	10.0[a, ***]	0.25
Strain total heterosis ($\sum h_{ij}$)	28	16.7[a, ***]	0.42
Error	16219	0.243	
Model 2	52		1.74

[a] Significance tested with F-test (see 2.4.4).
* $P<0.05$.
** $P<0.01$.
*** $P<0.001$.

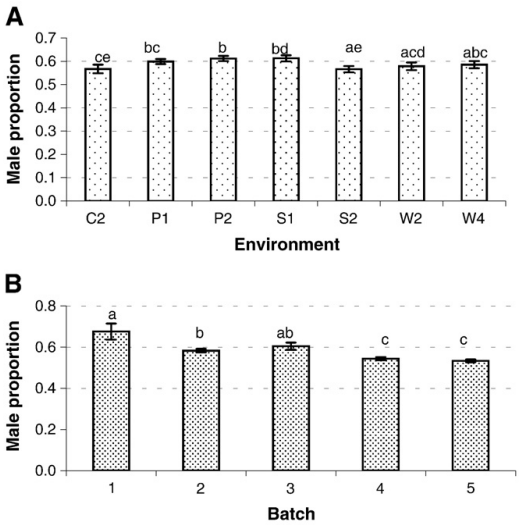

Fig. 2. Least square means (± se) for male proportion at harvest of the different test environments (A) and batches (B) according to Model 2. Columns with different letters are significantly different.

Table 5
Estimates (percentage points) of strain additive genetic (a_i), strain reciprocal (r_i) and strain general heterosis (h_i) effects for male proportion recorded at harvest (Model 2 estimates x 100) and expressed as a deviation from the mean value of the purebred strains (μ_{PB}).

Strain	Additive genetic effect	Reciprocal effect[a]	General heterosis
E2	1.2	-0.8	-1.1
Gh	-6.0	0.4	-0.6
Ke	0.1	1.4	-0.9
Se	-5.0	-5.8	0.1
Is	5.2	6.2	-3.5
Si	-1.4	3.3	1.9
Tw	-1.5	-1.8	2.0
Th	7.3	-3.0	2.2

Range of standard errors 2.9-3.1 percentage points for the strain additive effect, 1.4-1.5 percentage points for the strain reciprocal effect and 1.4-1.5 percentage points for the strain general heterosis effect.
[a] The difference in male proportion of the strain when used as a dam strain as compared to used as a sire strain.

Table 6
Estimates (percentage points) of strain total (h_{ij}) and strain average ($\bar{h}$) heterosis for male proportion (Model 2 estimate x 100) expressed as deviation from the mean value of the purebred strains (μ_{PB}).

	Strain (j)						
Strain (i)	Gh	Ke	Se	Is	Si	Tw	Th
E2	-0.6	0.2	1.0	-2.2	-1.8	5.3	3.0
Gh		-1.4	3.2	-3.0	7.4*	-1.8	4.6
Ke			3.2	3.4	-4.2	7.2*	-2.3
Se				-6.7**	8.1**	2.4	2.4
Is					1.1	-0.7	-3.8
Si						2.6	13.1***
Tw							11.4***
Average heterosis ($\bar{h}$)	1.8 ± 1.2						

Standard errors for total heterosis ranged from 3.0 to 3.3 percentage points.
Estimates with no subscripts are not significantly different from zero.
* $P<0.05$.
** $P<0.01$.
*** $P<0.001$.

crosses (Si x Th and Tw x Th) had total heterosis between 11 percentage points and 13 percentage points (Table 6).

Average heterosis was 1.83 ± 1.19 percentage points and thus not significantly different from zero. General strain heterosis was highest for the Th (2.2 percentage points), Tw (2.0 percentage points) and Si (1.9 percentage points) strains and lowest for the Is strain (-3.5 percentage points) (Table 5). All the other strains had low general heterosis estimates (range from -1.1 to 0.1). The largest difference in general strain heterosis was that between the Th and Is strains (5.7 ± 2.2 percentage points, $P<0.01$).

3.3.5. Total performance for male proportion

The total pure strain performance for male proportion (TP_{ii}) was highest for the Is (65%) and Th (62%) strains, and between 51 to 58% for all the other strains (Fig. 4). Each strain may confer an additional increase (or decrease) in male proportion when crossed with the other strains due to the sum of its average and general heterosis ($\bar{h} + h_i$) effects. Such increase in male proportion was highest for the Th, Tw and Si strains (range from 3.8 to 4.0 percentage points) and less than 2 percentage points for the other strains, with the exception of the Is strain which showed a decrease of 1.7 percentage points (Fig. 4). When considering both TP_{ii} and $\bar{h} + h_i$ simultaneously, the Th strain had the highest male proportion (Fig. 4).

The regressions of the total performance for male proportion of the 56 crosses (TP_{ij}) on the strain additive performance (AP_{ij}), strain reciprocal performance (RP_{ij}) and strain total heterosis (h_{ij}) are shown in Fig. 5. The correlation between TP_{ij} and AP_{ij} was low ($r = 0.17$) and not significantly different than zero ($P>0.05$; Fig. 5A), whereas the correlations of TP_{ij} with RP_{ij} ($r = 0.60$, Fig. 5B) and h_{ij} ($r = 0.77$, Fig. 5 C) were much higher and significantly different from zero ($P<0.001$). Thus among the 56 strain crosses the strain additive, reciprocal and total heterosis effects each explained 3%, 25% and 59%, respectively, of the variation in total performance for male proportion (as defined by TP_{ij}). The remaining 13% of the variation in total performance was due to interactions between the additive, reciprocal and total heterosis effects. Therefore, both the strain reciprocal and the strain total heterosis effects seem to be more important than the strain additive genetic effect in determining the total performance in male proportion of the strain crosses.

The correlation coefficient of the total performance for male proportion with the sum of average and general heterosis ($\bar{h} + h_i + h_j$) effects of the 56 crosses was low ($r = 0.35$, $P<0.01$) whereas that with the specific heterosis effect was much higher ($r = 0.73$, $P<0.001$). Thus, the specific heterosis effect explained the largest proportion (53%) of the variation in total performance in male proportion among the strain crosses than any of the other studied effects; i.e., the strain additive, reciprocal, average and general heterosis effects. However, as the specific heterosis effect for a given strain cross is calculated as the difference between the total heterosis effect and the sum of the average and general heterosis effects for the respective strains, it may be subjected to large sampling effects.

The Pearson correlation coefficients between the total performance for male proportion of the 64 strain crosses (both TP_{ij} and TP_{ii}) in the different test environments are given in Table 7. The correlations of C2 with the other test environments were all not significantly different from zero (Table 7), as were three of the six

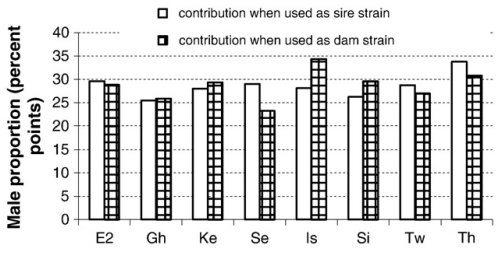

Fig. 3. Strain performance contribution for male proportion when a strain is used as a sire (SP_{sire_i}) or a dam (SP_{dam_i}) (Model 2 estimates).

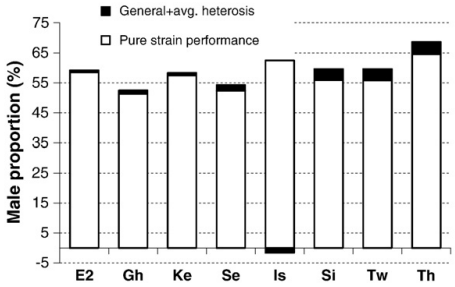

Fig. 4. Pure strain performance for male proportion (TP_{ii}) (open bars) and the sum of the average and general heterosis ($\bar{h} + h_i$) of the strain when crossed with the seven other strains (colored bars) (Model 2 estimates).

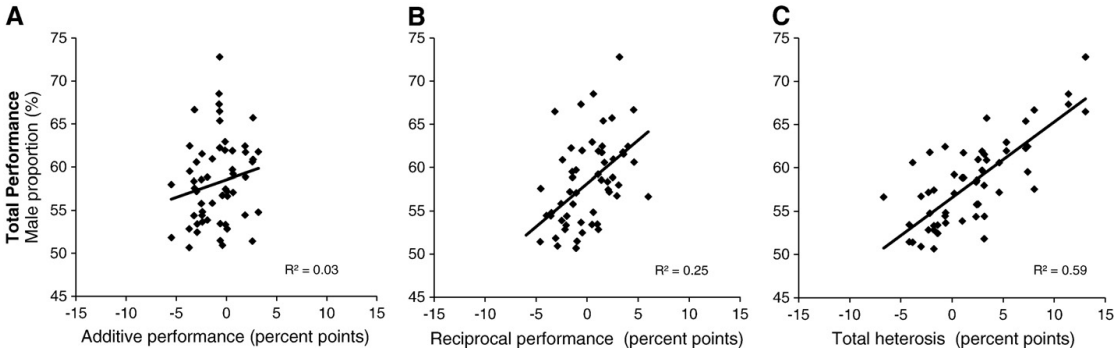

Fig. 5. The regression (with plots) of the total performance for male proportion of the 56 strain crosses (TP_{ij}) on: A) the strain additive genetic performance (AP_{ij}) for male proportion, B) the strain reciprocal performance (RP_{ij}) for male proportion, and C) the strain total heterosis for male proportion (h_{ij}). Estimates from Model 2 across test environments were used.

correlation coefficients of W2 with the other test environments. The correlation coefficients between the total performances of all the other test environments were all positive, but of relatively low magnitude (average r = 0.40) and significant ($P<0.05$), thus indicating substantial genetic by test environment interaction for male proportion.

4. Discussion

4.1. Sex dependent mortality and tag loss

The highest tag loss was observed in the three test environments with highest harvest body weight (P2, W2, W4). Higher tag loss in larger fish can be due to dislodgement of the Floy tag anchor caused by pressure from additional muscle tissue. Larger fish may also be more active due to more pronounced mating behavior (nest building, territory establishment and courtship) which may result in tag loss. Since Nile tilapia males are larger than females, the results may suggest that there is sex dependent tag loss. But given the fact that the correlations between mean harvest body weight (within environment and sex) and male proportion of the 64 strain combinations were not significantly different from zero, sex dependent tag loss is not likely. Moreover, the test environments with the highest male proportion (0.58) showed low (S1, 2%) and intermediate (P2, 6.8%) tag loss. Similarly, environments that experienced mortality between 12% and 16% covered the whole range of male proportions (0.53 to 0.58), and environments with higher mortality or tag loss had male proportion in the mid range (Table 1). Thus there seems to be no strong evidence of sex dependent tag loss or sex dependent mortality in this study.

Table 7
Pearson correlation coefficients between total performance for male proportion of the 64 strain combinations (TP_{ij} and TP_{ii}) in the different test environments (Model 2).

	S2	P1	P2	C2	W2	W4
S1	0.45***	0.29*	0.50***	0.05ns	0.18ns	0.53***
S2		0.28*	0.33**	0.02ns	0.24ns	0.41***
P1			0.43***	0.03ns	0.40**	0.44***
P2				-0.16ns	0.34**	0.34**
C2					0.10ns	0.18ns
W2						0.39**

ns Not significantly different from zero.
* $P<0.05$.
** $P<0.01$.
*** $P<0.001$.

According to Tuan et al. (1999) no sex ratio study in tilapia has confirmed differential mortality at any stage of their cycle. However, a study in which there is high natural mortality or tag loss, or both, may not be the best to examine male proportion or sex ratio. In future experiments data quality could be improved by reducing mortalities and using tags with higher retention rates. Furthermore, fish with lost tags and dead fish should ideally be sexed and their body weights recorded, and sexing of fish below 15 grams should be avoided because it is inaccurate. In C2 a total of 301 fish (34%) were below 15 grams, which may explain the lack of correlation between C2 and the other environments for male proportion (Table 7). In future experiments sexing could be done by gonad squash inspection (Guerrero and Shelton, 1974).

4.2. Strain genetic effects

Only the strain total heterosis by test environment effect was found to be significantly different from zero. With the same Nile tilapia data, Bentsen et al. (1998) reported that the non-additive genetic component (i.e. strain total heterosis) for growth was more sensitive to the genotype by environment interaction than the additive genetic growth component.

However, the standard errors of the total heterosis estimates within test environment were large, and it was difficult to see any trend with respect to the ranking of the total heterosis estimates across test environments, which is a prerequisite for a commercial utilization of a genetic effect in a selective breeding program. Consequently, only estimates of the Model 2 effects across test environments were presented and are further discussed.

The investigated strain additive, reciprocal and heterosis effects were statistically significant but explained altogether less than 1% of the total variation in male proportion, and thus a large error (within strain cross) variance in male proportion. The structure of the current study does not allow for examination of the magnitude of the additive genetic variation in male proportion between and within full and half sib families of importance in a quantitative genetic study. This will be the main objective of a following up study.

Less than 0.5% of the variation was explained by the test environment and batch effects. Consequently, a large proportion of the variation in male proportion in the current study is due to other non observed effects (e.g. temperature differences not accounted by the batch effect, animal additive and non-additive genetic effects), confirming the complexity of sex determination in tilapia.

The largest estimated difference between the strains or strain combinations for the investigated strain genetic effects were

substantial and statistically significant; i.e., 13.3 ± 4.6 percentage points for the additive effect, 12.0 ± 2.2 percentage points for the reciprocal effect and 19.7 ± 4.4 percentage points for the total heterosis effect. The Th strain ($a_i + \bar{h} + h_i$) showed a deviation of approximately 11 percentage points from the mean of all the purebred strains (μ_{PB}) (Fig. 4). Both the Th and Is strains had the highest additive genetic effect. The Is strain had a positive reciprocal effect, favoring its use as a dam strain while the Th strain is the best if used as a sire strain (Fig. 3). The Th strain had a favorable heterosis effect, but only the Th x Tw and Th x Si crosses (including both reciprocals) had LSM means (corrected for test environment and batch) which were higher than LSM of any particular pure strain (Appendix A). Bentsen et al. (1998) analyzed the same data for growth and showed that for mean body weight (including additive, reciprocal and heterosis) the Th strain ranked 3 rd and the Is strain ranked 6th of the eight strains, implying that the best performing strains for male proportion were not the best for growth. It is important to note that Th was mislabeled as Tw in Bentsen et al. (1998), the rankings referred to above have taken this correction into account.

Strain total heterosis was an important effect in this study and explained 59% of the variation in total performance for male proportion, and thus much more important than the strain additive and the reciprocal effects which explain 3 and 25% of the variation in total performance, respectively. However, most of the variation explained by the total heterosis effect was due to the specific heterosis effect which strongly indicates that not much is to be gained in increased male proportion due to heterosis by performing a systematic crossing of a number of Nile tilapia strains, and therefore the choice of specific crosses has to be performed based on their male proportion. In this context, selection for specific combining ability (reciprocal recurrent selection) may be of use; however, this procedure requires large number of crosses to be tested and practical evidence of this method is conflicting (Falconer and Mackay, 1996). The choice of the strain combination with the best total heterosis (Si x Th) would increase the male proportion by 13.1 percentage points (Table 6), i.e., for example from 57 to 70 %, which is below the usually desired commercial proportion of at least 97% males. Furthermore, the five strain crosses with the highest total heterosis estimates for male proportion (mean h_{ij}= 9.4 percentage points; $\bar{h}$ = 1.83 percentage points; Table 6) had low total heterosis estimates for growth (mean b_d= 0.05 g; $\bar{b}_d$= 1.5 g; Bentsen et al., 1998). Thus, to reach high male proportion levels of commercial interest through repeated testing and selection of strains while at the same time improving the growth rate of the strains would take many generations, require the use and maintenance of pure sire and dam lines, and thus involve additional costs as compared to keeping a single strain or population. Since total heterosis explained a large proportion of the variation in total male proportion and the interaction between total heterosis and test environment was significant, results found in this study imply that to fully exploit the genetic improvement for male proportion the end users must have uniform farm environments and apply similar management procedures.

Nevertheless, the finding of a statistically significant and considerable strain additive genetic, reciprocal and heterosis effects for male proportion in Nile tilapia is important and an encouragement for a study of the magnitude of both the additive an non-additive genetic effects of male proportion within strains. The existence of a significant strain reciprocal effect (maternal, paternal or both) for male proportion, may be analogous to the parental thermo-sensitivity of sex ratio documented in Nile tilapia by Baroiller and D'Cotta (2001) and Tessema et al. (2006).

The choice among the investigated strains will have insufficient effect for increasing male proportion in Nile tilapia and the cost/benefit ratio of implementing crossbreeding strategies to produce all male populations will most likely not be favorable. However, this does not exclude the possibility that strain additive, strain reciprocal and strain heterosis effects for male proportion of higher magnitude could be found under other environmental conditions, or in other Nile tilapia populations. If so, this may facilitate the use of within- species crossbreeding strategies for commercial all male production in Nile tilapia.

4.3. Influence of temperature and genetic parameters for male proportion

In Nile tilapia high temperatures (≥ 35 °C) (Baroiller et al., 1995b) and hormone treatments have been demonstrated to increase male proportion (Baroiller and D'Cotta, 2001). However, these treatments need to be applied just before or during histological differentiation of the primitive gonad (Devlin and Nagahama, 2002). In general terms, the critical sensitive period for this species is between 14-24 days after fertilization (Baroiller and D'Cotta, 2001), but an additional sensitive period (between 12-52 hours post fertilization) for precocious high temperature treatment has been reported by Rougeot et al. (2008). The fry in this study were kept in hatching and nursery hapas attached to poles in the same earthen pond at water temperatures in the range of 26-32 Cº. Fry remained in the nursery hapas until tagging, at an age ranging from 66 to 81 days (depending on batch and test environment) and before they were subsequently stocked in the communal grow-out test environments. Thus, in the present study this critical period mostly took place shortly after the swim up fry were collected and placed in separate rearing hapas in a common pond. All fish therefore were exposed to similar environmental conditions in the critical period for sexual differentiation, and reared at a lower water temperature than required to obtain higher male proportion. The studied strains and strain combinations were therefore not expected to fully express their potential genetic differences for male proportion in the current experiment. Even if masculinization may occur in the wild when tilapia encounter high temperatures of 34-35 °C (Baroiller and D'Cotta, 2001), tilapias kept in the hapas will not be exposed to these temperatures since they will not be able to reach the warm shallows. Differences in male proportion between batch 1 and 5 may be due to the environmental differences in the nursing hapas during the different collection periods. The above production and management procedures represent what was commonly applied in commercial production in the Philippines in the 1990 s.

Thermal sensitivity in the critical 14-24 days period after fertilization (Baroiller and D'Cotta, 2001) may be different for diverse strains and strain combinations. Tessema et al. (2006) reported difference in male proportion of two Nile tilapia populations in Lake Manzala (78% males) and Lake Rudolph (61% males) reared at 36 °C from day 10-20 post-fertilization. However, when reared at 18 °C both populations had similar male proportion (54% Lake Manzala and 53% Lake Rudolph).

Moreover, the variation in male proportion within strains may be larger than the variation observed between strains and strain crosses. Strong evidence of a substantial additive genetic variation within a population of Nile tilapia (h^2 = 0.26, CI(95%) 0.13-0.48) is reported by Lester et al. (1989). Beardmore et al. (2001) reports difference for male proportion between single-pair of matings within the AIT strain (Tuan et al., 1999) and Auburn strain (Mair et al., 1991) of Nile tilapia. Wessels and Hörstgen-Schwark (2007) found evidence that sensitivity to temperature treatments used to increase male proportion were under additive genetic control. In a two way selection experiment carried out for two generations, realized heritability estimated from cumulated realized response and cumulated selection differential was in average 0.78 (0.69 in the high response line and 0.86 in the low response line) (Wessels and Hörstgen-Schwark, 2007).

The best performing fish with respect to growth, survival, and readiness to spawn from most of the strain combinations in the diallel cross experiment were selected to produce 123 maternal full-sib families within 50 paternal half-sib families forming a diverse base population for further selection (Eknath et al., 2007). Variation of male proportion among and within these families and successive generations produced by the GIFT project should be studied to assess if the variation of male proportion in Nile tilapia can be exploited to produce higher male proportions.

The above discussion on differences in male proportion between strain and strain combination is based on the assumption that all male and female breeders used to produce the diallel were in fact genetically males or females and also on the assumption that they were all pure Nile tilapia strains. Macaranas et al. (1986) showed a degree of introgression of *O. mossambicus* genes in some of the previously introduced strains of Nile tilapia farmed in the Philippines. If this was true for some of the farmed strains used in this experiment the within and between strain variance for male proportion could be inflated. Furthermore, if some of the breeders used were naturally sex reversed individuals [i.e. reversal of genetic females (XX) to phenotypic males, or genetic males (XY) to neofemales or supermales (YY)] and this proportion varied among the different strains, this may have also had a significant effect on the estimated strain additive, strain reciprocal and strain heterosis effects. However, genetic sex of the parents determined by the XX-XY sex determination system (Jalabert et al., 1974; Mair et al., 1991; Cnaani et al., 2008) are not known. Moreover, departure from the sex ratio predicted by the XX-XY chromosomal system can be caused by unidentified genetic factors, which appear to be autosomal and to have partial penetrance (Mair et al., 1991; Hussain et al., 1994; Sarder et al., 1999), and being heritable, polymorphic and able to influence sex ratio in both directions (Ezaz et al., 2004). Without knowledge of the genetic sex (XX or XY) of the parents, evidence of the inclusion of sex reversed parents would require information of the male proportion of full sib family groups. This issue will be discussed in a following paper.

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Appendix A

Least square means (LSM) across test environments for male proportion at harvest for all 64 strain combinations (obtained from a general linear model fitting the fixed effects of test environment, batch and strain combination).

Sire strain	Dam strain								
	E2	Gh	Ke	Se	Is	Si	Tw	Th	mean
E2	0.57	0.49	0.58	0.53	0.66	0.61	0.56	0.63	0.58
Gh	0.58	0.50	0.41	0.51	0.56	0.62	0.58	0.60	0.55
Ke	0.56	0.64	0.56	0.56	0.59	0.47	0.58	0.55	0.56
Se	0.59	0.57	0.58	0.52	0.59	0.63	0.59	0.63	0.59
Is	0.49	0.50	0.66	0.41	0.62	0.59	0.58	0.54	0.55
Si	0.48	0.58	0.55	0.59	0.61	0.55	0.47	0.75	0.57
Tw	0.66	0.44	0.67	0.51	0.59	0.68	0.55	0.66	0.60
Th	0.65	0.64	0.60	0.58	0.64	0.71	0.76	0.64	0.65
mean	0.57	0.55	0.58	0.53	0.61	0.61	0.58	0.62	
Overall mean	*0.56*								

Standard errors of the LSM ranged from 0.031 to 0.036.

Appendix B

Percent contribution of each effect and R^2 from the analysis of variance (model 2) for male proportion within each test environment.

Effect	Marginal R^2 increase X100 (within test environment)						
	S1	S2	P1	P2	C2	W2	W4
Batch	0.13^{ns}	0.43^{*}	0.14^{ns}	0.25^{*}	1.76^{**}	1.21^{**}	0.26^{ns}
Strain additive genetic ($\sum a_i$)	0.80^{**}	0.20^{ns}	0.18^{ns}	0.27^{ns}	0.26^{ns}	0.41^{ns}	0.97^{ns}
Strain reciprocal ($\sum r_i$)	0.62^{*}	0.42^{ns}	0.21^{ns}	0.31^{ns}	0.10^{ns}	0.21^{ns}	0.99^{ns}
Total heterosis ($\sum h_{ij}$)	3.70^{***}	1.15^{ns}	0.32^{ns}	1.12^{***}	1.28^{ns}	2.93^{ns}	3.58^{***}
Error variance	0.24	0.25	0.24	0.24	0.25	0.24	0.24
Degrees of freedom error	2102	2182	4336	3969	850	1136	1368
Model 2 R^2 (x100)	4.63	2.54	1.93	3.40	4.55	5.53	5.78

^{ns} $P>0.05$, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. The significance of the strain additive, reciprocal and total heterosis effects were tested with a partial *F*-test (see 2.4.4).

Appendix C

Pearson correlation coefficients between total heterosis estimates for male proportion (h_{ij}) in the different test environments (Model 2).

Test environments	S2	P1	P2	C2	W2	W4
S1	0.34^{ns}	0.11^{ns}	-0.06^{ns}	0.04^{ns}	-0.20^{ns}	0.54^{**}
S2		0.22^{ns}	0.11^{ns}	-0.03^{ns}	0.24^{ns}	0.49^{**}
P1			0.15^{ns}	-0.02^{ns}	0.18^{ns}	0.37^{ns}
P2				-0.37^{ns}	0.21^{ns}	-0.04^{ns}
C2					0.34^{ns}	0.19^{ns}
W2						0.35^{ns}
W4						

^{ns} Not significantly different form zero. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

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Paper II

Paper II

Heritability estimates for male proportion in the GIFT Nile tilapia (*Oreochromis niloticus* L.)

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ABSTRACT

The main goal of this study was to estimate the heritability of the proportion of phenotypic males in progeny of Nile tilapia broodstock based on data from six consecutive pedigreed generations from the GIFT project. Across all generations there was a low but significant "additive" genetic component for male proportion with heritability estimates of 0.12±0.02 (observed scale) and 0.22±0.04 (underlying liability scale). The within generation heritability estimates varied from 0.00±0.03 to 0.25±0.07 on the observed scale, and from 0.11±0.02 to 0.32±0.07 on the underlying liability scale. As expected, genetic correlations between male proportion in the different test environments were not significantly different from unity (P>0.05) in 16 out of 17 cases since sex was determined before fish were stocked in the different test environments, indicating no or the same degree of sex-specific mortality among the families in the different environments.

The regression coefficient of observed full-sib family male proportions on the associated mid-parent estimated breeding values (estimated without offspring information) was significantly different (P<0.01) from unity (0.64±0.12), which would have been the expected regression coefficient if sex determination had been a purely autosomal polygenic trait. This suggests that the magnitude of the genetic variation in male proportion found in this study may be biased upwards by some parents having a phenotypic sex different from that determined by the major sex determining system (XX/XY).

Selection for increased male proportion based on family means may lead to an increased fraction of masculinized XX males, which will counteract the response to selection if used as sires. Furthermore, phenotypic males and females will naturally contribute equally to the genetics of the subsequent generation. For this reason, genetic selection for increased male proportion in a purebred population (without use of hormones or heat-shocking) is expected to be difficult, if at all possible.

1. Introduction

Early reproduction of tilapia in aquaculture causes stunted growth and large size variability (Longalong et al., 1999; Little et al., 2003). Unwanted reproduction in mixed sex populations may cause up to 70% of the total harvest weight to be small fish of no commercial value (FAO, 2010). The use of hormonal sex inverted male fry has been the industry standard for mono-sex culture in Nile tilapia as well as for other tilapia species. However, best aquaculture management practices (BAP) for tilapia encourages methods other than the use of hormones for the production of all-male fry (GAA, 2008), such as manual sorting (Beardmore et al., 2001), inter-specific hybridization (Pruginin et al., 1975; Hulata et al., 1983, 1993; Wohlfarth, 1994; Desprez et al., 2006), use of "super-males" (YY) (Mair et al., 1991a, 1997; Tuan et al., 1998, 1999) and selection for increased male proportion (MP) under temperature treatment (Wessels and Hörstgen-Schwark, 2011). However, manual sorting has proven to be labor intensive and subject to human error (Beardmore et al., 2001), the inter-specific hybridization has shown breakdowns due to contamination of the broodstock species with misidentified hybrids (Beardmore et al., 2001; Hulata, 2001) and YY-male technology requires several generations of progeny testing and has shown deviations from the expected male ratio of 1.0 based on a simple XX/XY sex determination system (Mair et al., 1997; Tuan et al, 1999). Production of all-male Nile tilapia through androgenesis and gynogenesis has been achieved on an experimental scale, but these techniques are not easily applied for commercial production (Beardmore et al., 2001). The use of mixed sex sterile triploid populations of Nile tilapia (Hussain et al., 1996) to control unwanted reproduction has been constrained by the ability to produce large amount of triploids at a reasonable cost.

Nile tilapia has a complex sex determination system where the phenotypic sex is determined by major genetic factors (i.e. XX/XY), several autosomal genetic factors, as

well as rearing water temperature during early development stages (Baroiller et al., 2009). The hypothesized "sex chromosome" (XX/XY) exhibits male heterogamety (Jalabert et al., 1971; Mair et al., 1991a). Using synaptonemal complex analysis, Carrasco et al. (1999) observed an incompletely paired segment in the XY genotype providing cytological evidence for a XX/XY sex determination system, and recently sex-linked markers have been identified for Nile tilapia in linkage group 1 (LG1) (Lee et al., 2003; Cnaani et al., 2008). Markers found by Lee et al. (2003) predicted sex correctly for 95% of individuals in two of the three families studied; however in the third family there was no association between LG1 and sex of its members. Cnaani et al. (2008) suggested that two different linkage groups (LG1 and LG3) may contribute to sex determination in some families, explaining the results obtained by Lee et al. (2003). Eshel et al. (2010) found that indeed two linkage groups (LG1 and LG23) had an association with sex, but it was LG23 that showed the strongest association. Nevertheless "sex chromosomes" in Nile tilapia appear to be at an early evolutionary stage of differentiation (Lee et al., 2003; Cnaani et al., 2008). The occurrence of females in progeny of YY males has been attributed to the action of several autosomal sex modifying genes (Mair et al., 1997). Large between-family variance for sex ratio among progeny of Nile tilapia (Tuan et al., 1999) and large variation in crosses between Nile and blue tilapias (Mair et al, 1991b) also suggest an autosomal polygenic mechanism for sex determination. Autosomal genes in the Thai-Chitralada strain seem to have a greater influence on sex determination than in the Egypt-Swansea strain of Nile tilapia (Tuan et al., 1999).

Temperatures above 32°C applied during the period of sex differentiation (from 10 to 20 days post fertilization) can masculinize progeny overriding the influence of both major genetic factors and autosomal sex determining genes (Baroiller et al., 1995, 2009;

Tessema et al., 2006; Wessels and Hörstgen-Schwark, 2007, 2011). Both a significant parental sire and dam effect has been found for temperature sensitivity during sex differentiation (Baroiller and D'Cotta, 2001; Tessema et al., 2006). High realized heritability for temperature sensitivity with respect to sex determination was observed in a selection experiment with two divergent lines selected for high (h^2=0.63) or low (h^2=0.84) response to temperature treatment over three generations (Wessels and Hörstgen-Schwark, 2011). Temperature sensitivity with respect to sex determination also responds to selection, since after three generations of selection the temperature treated group (36°C) selected for high response increased from 65% males in the base population to 93% males, whereas at normal temperatures (28°C) MP remained almost unchanged (52% males in the base population to 54% males in G3) (Wessels and Hörstgen-Schwark, 2011).

Selective breeding experiments and programs have been carried out with Nile tilapia to improve traits such as growth, carcass quality, fillet yield, cold tolerance and early sexual maturation (Longalong et al., 1999; Bolivar and Newkirk, 2002; Charo-Karisa et al., 2005, 2006; Ponzoni et al., 2005; Rutten, 2005; Neira, 2010; Rye et al., 2010). One of these programs is the widely recognized "Genetic Improvement of Farmed Tilapias" (GIFT), a collaborative research project which started in the Philippines in 1988 (Eknath et al., 1993, 2007; Bentsen et al., 1998). Using data from the GIFT diallel cross experiment, Lozano et al. (2011) found significant strain additive genetic, strain reciprocal and strain total heterosis effects for MP, suggesting that strain genetic effects are present in the determination of the phenotypic sex in Nile tilapia. However, the genetic variation in MP between families within this population has not been assessed. Lester et al. (1989) reported a heritability of medium magnitude (h^2= 0.26) for MP in

Nile tilapia on the underlying liability scale, using half-sib families produced with 18 sires and 37 dams. So far, this is the only reported heritability estimate for MP in tilapia under normal rearing temperatures.

In general, estimates of additive genetic variance are inferred from the (co)variance among full- and half-sibs. Preferably, also the genetic relationships among the parents should be taken into account. For species with a sex determination system completely controlled by a single major genetic sex determination factor (e.g., the XY system in most mammals), the major genetic sex determination factor will not contribute to between-family variation in MP (i.e., as all individuals will be offspring of XX females and XY males). If so, no genetic variation in MP should be expected, which is in accordance with results from studies in mammals such as man (Maynard Smith, 1980), cattle and pigs (Toro et al., 2006). However in species such as tilapia, where the sex-determination system is more complex and the phenotypic sex may deviate from the major genetic sex determination factor (XX/XY), genetic variation in phenotypic sex (e.g. male proportion) is to be expected. The occurrence of natural sex reversion in Nile tilapia has been suggested for XY females (Mair et al., 1991a; Bezault et al., 2007), XX males (Bezault et al., 2007), and reproduction of such females will necessarily produce a fraction YY males. When such deviations occur the major sex determining factor will thus to some extent act as major segregating QTL in the population, contributing to the between-family variation and potentially also to the estimated additive genetic variance. If "genetic sex" (i.e. sex determined by XX/XY system) of one or both parents is different from phenotypic sex, this may give substantial deviations from the expected sex-ratios of the offspring in the first generation, but these effects will be rather short-lived (Appendix 1). The XX males will produce normal XX female offspring, △XY females will produce 75% normal (i.e. XX female and XY male) and 25% YY

offspring, while YY males will produce normal XY male offspring, assuming that all these are mated with normal partners. Nevertheless, deviations between genetic and phenotypic sex may appear spontaneously in all subsequent generations and therefore consistently contribute to the between-family variation in sex ratios.

Genetic variation may exist with respect to how likely individual sex phenotypes are to deviate from their major genetic sex determination system (XX/XY). Temperature dependent sex determination may also be controlled by several alleles expressed at the embryo stage as seen in Nile tilapia (Wessels and Hörstgen-Schwark, 2007). Some of the genetic variation in MP may be attributed to female behavior through their different temperature preference, as seen for variation of maternal choice of nest temperature in turtles (Bulmer and Bull, 1982). To the extent that such factors (if present) have a genetic background, they will also contribute to the estimated additive genetic variance in the observed sex ratios. In all cases where other factors (both genetic and environmental) override the effect of the major sex determination factor, the latter factor will necessarily contribute to between-family variation in the following generation and may thus add to the estimated additive genetic variance for sex ratio.

Theoretically, genetic variation in observed sex ratios may also arise for reasons outside the sex determination system, i.e., due to genetic variation in fertilization rate of "male" and "female" sperm or sex-specific variation in survival of offspring.

The main goal of this study was to estimate the magnitude of the additive genetic variance for MP in six consecutive pedigreed generations of Nile tilapia from the GIFT project.

2. Material and methods

2.1 Genetic material

Data from six generations of Nile tilapia from the GIFT project was used. The project started with a performance comparison test of four wild African strains (Egypt, Ghana, Kenya, and Senegal) and four Asian farmed strains (known as Israel, Singapore, Taiwan and Thailand) used in the Philippines. Details of the production and growth performance of the strains can be found in Eknath et al. (1993). This was followed by a complete 8x8 diallel cross experiment involving all the original strains. Estimates of strain additive, strain reciprocal and strain heterosis effects from the diallel cross for growth was documented by Bentsen et al. (1998) and for MP by Lozano et al. (2011). A sample of the best performing individuals for harvest body weight from the diallel cross were used as parents to produce a synthetic base population of full- and half-sib families (G0) which was the basis for selection in subsequent generations (G1-G5). More individuals were selected from the crosses with the highest estimated additive genetic performance for harvest body weight, but ensuring a minimum genetic representation of all the original parent strains (Eknath et al., 2007). Thus, in the production of G0 approximately 27% of the grandparent ancestors were from the Kenya strain, 20% from the Egypt strain, 20% from the Thailand strain, 12% from the Senegal strain, 8% from the Taiwan strain, 6% from the Singapore strain, 4% from the Israel strain and 3% from the Ghana strain (Eknath et al., 2007). This study analyzes data on MP from G0 to G5 of the GIFT project. Genetic parameters for body weight at harvest of the same six generations have recently been reported by Bentsen et al. (Unpublished results).

2.2 Production of the families

To produce full- and half-sib families a nested mating design was used. Each male fish was mated with one or two different females within generation, with the exception of

G0 where 30 of the males were mated with three different females. Dams were mated with only one sire within generation, except in G4 where a single dam was mated with two different sires.

The production of the families was performed as follows: The (previously) selected females were stocked in separate breeding hapas. Breeding hapas were prepared and arranged as described by Eknath et al. (2007). Females in the hapas were scored for sexual maturity as ready to spawn (RS), had spawned (HS) or not ready to spawn (NR) (Longalong et al., 1999). Males were placed in hapas with RS females at a 1:1 ratio. To minimize mortalities during the mating process, the premaxilla of the male breeders was removed and animals of similar size were mated (Palada-de Vera, 1998). After approximately 10-14 days the males were removed from the hapas with swim up fry, or from hapas having females carrying eggs, and were transferred to hapas with single RS females to obtain paternal half-sibs through the nested mating design (Palada-de Vera, 1998; Eknath et al., 2007). HS females with incubated eggs in their mouth remained in the hapas until fry reached the swim up stage (Palada-de Vera, 1998). Swim up fry from each full-sib family were collected and stocked in separate nursery hapas. After approximately 21 days, fingerlings of each family were transferred to larger individual mesh hapas (B-net cages) where they were reared until reaching tagging size (3-6 g) at which they were tagged using external individually labeled Floy® tags (Longalong et al., 1999). The fry and fingerling hapas were placed in fertilized ponds and fed as described by Eknath et al. (2007). The fry collection period lasted on average 64 days, ranging from 40 days in G1 to 98 days in G4 (Table 1). The duration of the separate rearing period for families was 94 days on average across generations, ranging from 78 days in G0 to 114 days in G2 (Table 1). Within generations there was variation among families

in the duration of the separate rearing period since a limited number of the families were produced on the same date.

A total of 1091 full-sib families were produced over the six generations (the offspring of 626 sires and 1053 dams). For this study, observations with only one record within the smallest sub-cell (generation/family/environment) were omitted, thus reducing the data to a total of 1077 full-sib families (the offspring of 616 sires and 1043 dams) (Table 2). Repeated mating of some of the same sire and dam combinations (parents with average breeding values for harvest weight within their generation) in two to three different generations resulted in a total of twenty eight full-sib families that were used as control groups for growth performance. Thus, males were mated in total up to six times and females up to three times (Table 3). Of the 28 control families included in this study, 23 families were replicated in two generations and five in three generations. These replicated families provided useful sex information and also contributed to stronger genetic links across generations (in addition to the parent – offspring genetic links) thus increasing the connectedness of the data.

In this study the window of temperature sensitivity occurred in the family nursery hapa which for each year class were all located in the same earthen pond at the GIFT facilities in Munoz Nueva Ecija, Philippines (150 km north of Metro Manila). Thus, we expect sex determination to take place before the fish were distributed into the different test environments.

2.3 Grow-out testing environments

In total eight different test environments were evaluated, with 2-7 environments per generation, to test the growth performance of the families under different farming

conditions (Table 4). Abbreviations and management used for the different test environments are described in detail by Eknath et al. (1993, 2007) and Bentsen et al. (Unpublished results). These test environments included ponds (S1, S2, P1, P2, W2), cages (C2, C4), and rice paddy (RF, in G5 only) which are the typical grow-out conditions encountered in the Philippines. P1 and P2 environments were replicated in G0 and G1. In this study data from replicate ponds were pooled. The test environments were located at the BFAR satellite stations located in southern Luzon (S1) and north-west Luzon (S2), at the BFAR/NFFTRC and FAC/CLSU facilities at Muñoz in the lowlands of Central Luzon (P1, P2, W2, C2, RF) and a collaborating tilapia farm in Central Luzon (C4). All pond environments had standard commercial fertilization, except for P2 which had supplemental feeding and W2 which was fertilized with ipil–ipil leaves. Water used in the cage environments (C2, C4) was not fertilized and fish were fed daily.

Within each test environment equal numbers of tagged fingerlings from each family were stocked. Stocking weight of the fish was on average 4 g (range 3.2-5.6 g) (Bentsen et al., Unpublished results). After a grow out period ranging from 88-126 days for the different test environments (110 days on average) fish were harvested to perform data recording operations as described by Bentsen et al. (Unpublished results). Sex scoring was done by several skilled persons and errors are expected to be low (Edna Dionisio, Personal communication). Untagged animals were registered at harvest.

2.4 Data analysis

Sex records used in this study come from a selective breeding program which main goal was to increase growth in Nile tilapia. Despite the fact that the design is far from optimal to study MP, the comprehensive dataset provides a good opportunity to evaluate

additive genetic variation under normal rearing temperatures. Nevertheless, the genetic sex of all fish (both parents and offspring) as determined from the major sex chromosome (XX/XY) is unknown and our analyses are thus based solely on the observed phenotypic sex of each fish.

2.4.1 Genetic parameters

Two different models were used to obtain estimates of genetic parameters for MP. Both models were used within generations (from G0 to G5) using all environments, across generations using all environments and also across generations using only cage or pond environments.

Model 1: A univariate linear animal model, which in matrix notation can be written as:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Za} + \mathbf{Wc} + \mathbf{e} \qquad (1)$$

where **y** is a vector of the phenotypic observations for sex (scored as 1 for males and 0 for females, thus expressing sex ratio in the different levels of the fixed (e.g. test environments) and random (full- and half-sib families) effects as MP; **X** is a design matrix that links individual observations to the different levels of the fixed effects; **b** is a vector of fixed effect solutions (effects of test environment for models run within generation; or the generation by test environment effect for the models run across generations); **Z** is an incidence matrix that links the observations to the animal additive genetic effects, $\mathbf{a} \sim N(\mathbf{0}, \mathbf{A}\sigma_a^2)$ is a vector of random animal additive genetic effect, **W** is an incidence matrix that links the observations to the random effects common to full-sibs, $\mathbf{c} \sim N(\mathbf{0}, \mathbf{I}\sigma_c^2)$ is a vector of random effects common to full-sibs other than additive genetics, $\mathbf{e} \sim N(\mathbf{0}, \mathbf{I}\sigma_e^2)$ is a vector of random residuals. Finally, **A** is the

numerator relationship matrix that describes the additive genetic relationship among all individuals included in **a**, and **I** is an identity matrix of appropriate size.

For Model 1, estimates of the fixed effects and variance components for the random effect were obtained by Restricted Maximum Likelihood (REML) using the DMU software (Madsen and Jensen, 2008).

In preliminary analyses age of the fish was fitted (regression up to second order, nested within test environment, or within generation and test environment). However, the regression coefficients for these two covariates were in all cases not significantly different from zero and the covariates were therefore omitted from the final model.

The significance of the additive genetic and the full-sib effect in Model 1 was tested (within and across environments) by excluding each effect separately from the full model. A test of each effect separately was obtained by the following likelihood-ratio test (Lynch and Walsh, 1998):

$$LR = -2\left(LogL_R - LogL_F\right)$$

where $LogL_R$ is the log of the restricted likelihood of the reduced model and $LogL_F$ is the log of the restricted likelihood of the full model. The significance of LR was tested with a χ^2 test with df = 1 (number of omitted variance components) and $\alpha = 0.05$.

Model 2: A univariate threshold (probit) animal model, including the same effects as in the linear Model 1, was used to obtain variance components for MP on the underlying liability scale using Gibbs sampling. Observed binary records (y_{ij}) were assumed to be determined by an underlying liability (λ_{ij}) where the threshold value is set to zero, i.e.,

$\lambda_{ij} \leq 0$ gives $y_{ij} = 0$, and $\lambda_{ij} > 0$ gives $y_{ij} = 1$. In matrix notation Model 2 can be written as:

$$\boldsymbol{\lambda} = \mathbf{Xb} + \mathbf{Za} + \mathbf{Wc} + \mathbf{e} \qquad (2)$$

where $\boldsymbol{\lambda}$ is the vector of all λ_{ij}, while $\mathbf{X}$, $\mathbf{b}$, $\mathbf{Z}$, $\mathbf{W}$, $\mathbf{c}$, and $\mathbf{I}$ are as described in Model 1. Residual variance (σ_e^2) was restricted to 1.0, i.e., $\mathbf{e} \sim N(\mathbf{0}, \mathbf{I})$.

For Model 2, parameters were estimated with a modified version of the Gibbs sampler module of the DMU software (Madsen and Jensen, 2008). Cross-sectional animal threshold models for binary data are frequently biased, with heritability frequently being severely overestimated (Hoeschele and Tier, 1995; Stock et al., 2007). However, Ødegård et al. (2010) recently published an algorithm that allows proper estimation of genetic (co)variance components even for this type of data. Here, additive genetic (co)variance components are estimated based on the estimated parental breeding values only (including information from multiple offspring), rather than based on all breeding values (including non-parents) as in a standard animal threshold model. Due to software limitations, likelihood-ratio tests could not be performed for the random effects in the threshold model.

The effect common to full-sibs (c) included the environmental hapa effects caused by separate rearing of the families until tagging and potential dominance and maternal effects. For each family, these effects are confounded, and thus fitted as a single random effect.

For both models, estimated heritability for MP was calculated as:

$$h^2 = \frac{\hat{\sigma}_a^2}{\hat{\sigma}_a^2 + \hat{\sigma}_c^2 + \hat{\sigma}_e^2} \ ,$$

and the relative proportion of the effects common to full-sibs as:

$$c^2 = \frac{\hat{\sigma}_c^2}{\hat{\sigma}_a^2 + \hat{\sigma}_c^2 + \hat{\sigma}_e^2} \, .$$

2.4.2 Genetic correlations among test environments

Male proportion in different test environment can be defined as a separate trait. Since sex of the fish is assumed to be determined before the fish are stocked in the different test environments, we expect the genetic correlations between MP in the different test environments to be close to unity. This was supported by a non-significant strain by test environment effect for MP in a complete diallel cross among the eight strains used to establish the base population (G0) in this study (Lozano et al., 2011). Still, the genetic correlation between MP in different environments may be lower than unity due to different degree of sex differential mortality among the families reared in these environments. Estimates of the genetic (co)variance components for MP for these traits (test environments) across generations were obtained using a linear bivariate animal model similar to Model 1 with two traits (test environments) at a time, assuming that the same test environment in two or more different generation was the same trait (e.g. P1 in all generation as the same trait, and C2 in G0 and G1 as the same trait). In the cases (7 out of 17) where a bivariate model did not converge the random effect common to full-sibs was removed from the model.

Likewise, estimates of the genetic (co)variance components for MP in ponds and cages was obtained across generations with a linear bivariate model similar to Model 1, fitting

MP in ponds and cages as separate traits. In this case the RF test environment was omitted from the data since it is neither a pond nor a cage test environment.

To test if the estimated genetic correlations (those between the different test environments and that between pond and cages) were significantly different from unity, a bivariate Model 1 was used, constraining the genetic correlation to 0.999, and compared with an unconstrained Model 1 using the likelihood-ratio test previously described.

2.4.3 Polygenic versus major genetic (XX/XY) inheritance of sex

As written in the introduction, the phenotypic sex of a fish may differ from the "genetic sex" (i.e., due to naturally occurring sex reversion). However, we are only able to observe the phenotypic sex, while the major genetic factor (XX/XY system) is unobserved. If sex is mainly controlled by the XX/XY system and if we bear in mind that sex reversion may occur naturally (either as a result of environmental or polygenic factors), it is possible that some full-sib families may appear to be genetically superior or inferior for MP simply because the phenotypic sex of the parents does not match their genetic (XX/XY) sex. Possible examples are shown in Appendix 1, where the "Offspring Generation 1" differs from the expected MP of 50%. Hence, the major genetic factor in the parents may contribute to between-family variation in sex ratios, and as such, contribute to the estimated "additive" genetic variance. To assess to what extent the major genetic (XX/XY) factor actually contribute to the estimated genetic variance, a second analysis was conducted. As described in the introduction, when "genetic sex" of parents is different from phenotypic sex, deviations from the expected MP will occur in the first generation, but MP will normalize after one or maximum two generations. To quantify this, the regression of the observed MP of families on the

corresponding mid-parent breeding values (EBV) was estimated. The EBVs were estimated without using data on the offspring (e.g. EBV for breeders of G0 was obtained using data from all generations except G0, EBV for breeders of G1 was obtained using data from all generations except G1, and so forth). In other words EBVs were largely based on data from previous and later generations thus reducing the bias caused by recurring sex reversal in subsequent generations. The regression was performed excluding the parents in G0, since for these parents MP information is not available for the families which were not used in the production of G1 and thus approximately 40% of the families in G0 had breeding values of zero (thus 921 out of 1044 families were used in analysis).

For this purpose a weighted linear model was fitted with the observed MP as the dependent variable and including the fixed effect of generation and the mid-parent EBV of each family as a covariate, and with number of recorded fish per family as weights (see Table 2). For a polygenic trait the expected regression coefficient from this model is unity (Appendix 2). However, if the major sex determination (XX/XY) system is responsible for most of the estimated "additive" genetic variance for MP (some phenotypes differ from the genetic sex determined by XX/XY system), observed familial differences in MP are expected to be more short-lived; i.e., there should be less association between ancestral and offspring MP, and the estimated regression coefficient should therefore be substantially lower than unity.

To test homogeneity of slopes across generations the interaction between generation and mid-parent EBV was included in the preliminary model, but this effect was not significant (P=0.36) and thus excluded. Analyses were done using the R software package (R Development Core Team, 2008). To remove all offspring data for the actual

sets of breeders, only the first occurring offspring of those sires and dams repeated across generations were used in the analysis (mid-parent EBV of 1044 out of 1077 families).

3. Results

3.1 Descriptive statistics

Descriptive statistics for the analyzed data are given in Table 4. Average MP across all generations and environments was 45% (range 37% to 56%), which is slightly less than the expected 50%, and showed large variation between test environments within generation as well as between generations within test environment. The two highest MP were found in the cage environment C4 (G0 and G5). However, some cage environments also showed low MP (e.g. C2 in generation G1). Across generations (Model 1), the generation by test environment effect was statistically significant ($P<0.01$), while within generations (Model 1), the effect of test environment was statistically significant ($P<0.01$) only for G0, G1 and G4.

Frequency distributions of observed MP (across environments) of full-sib families in each generation are shown in Figure 1 (1077 families in total). All distributions were bell shaped, but more narrow for G0 (except for the three families with MP higher than 92%) and G1.

Harvest weight, tag-loss and mortality showed large variation between test environments within generation and between generations within test environment. Average harvest weights in G2 to G5 were roughly two to three times higher than that in G0 and G1. However G0 and G1 had shorter grow out period (average 91 days) when compared with the other generations (average 124 days). The males were on average

40% heavier than the females (range 19%-64% across test environments and generations). Tag-loss showed large variation between test environments and generations (from 0 to 39%, overall mean 12.1%) and in general terms increased as the harvest weight increased. Also, mortality showed large variation between test environments and generations (from 1 to 60%, overall mean 19.6%), but shows no association to harvest weight (r=0.13, P>0.05 for males; r=0.12, P>0.05 for females) or tag loss (r=0.00, P>0.05).

3.2 Genetic parameters

3.2.1 Additive genetic effects

Estimates of heritabilities for MP both on the observed (Model 1) and liability (Model 2) scales are given in Table 5. Across all generations there was a low but significant additive genetic component for MP with a heritability estimate of 0.12±0.02 on the observed scale and, as expected, higher (0.22±0.04) on the underlying liability scale. The within-generation estimates varied substantially for both Model 1 and 2. However, in all generations, except for G4, the Model 1 estimates of the animal additive genetic effect were significantly different from zero (P<0.05; Log-likelihood-ratio test).

The Model 1 and 2 heritability estimates for MP in pond test environments were roughly three times higher than those obtained in cage test environments (Table 5). Additive genetic variance was statistically significant both in ponds and cages (P<0.05; Log-likelihood-ratio test, Model 1).

3.2.2 Effect common to full-sibs

Estimates of proportion of phenotypic variance (on observed/liability scale) of MP explained by effects common to full-sibs (c^2) are given in Table 5. In both Model 1 and

2 estimates of c^2 were about one third of the heritability estimate, and significantly different from zero (P<0.05; Log-likelihood-ratio test for Model 1) (Table 5). Estimates varied substantially between generations.

For the pond and cage test environments the magnitude of c^2 for MP was similar (Table 5) for Model 1 and 2. For Model 1 the effect common to full-sibs was significantly different from zero (P<0.05) for both the cages (0.03±0.01) and ponds (0.04±0.01) (Table 5).

3.2.3 Genetic correlations

Estimates of genetic correlations between MP in the different test environments are given in Table 6. Most genetic correlations were above 0.80 (11 out of 17), but nearly all correlations obtained (except that between C4 and S2) were not significantly different from unity (P>0.05; Log-likelihood-ratio test). In many cases the models did not converge because genetic correlations were too close to unity.

With a linear bivariate model fitting MP in ponds and cages as two separate traits (across generations) the genetic correlation between the traits was 0.98±0.05, and was not significantly different from unity (P>0.05; Log-likelihood-ratio test). Results confirm sex determination occurred prior to stocking.

3.2.4 Regression of observed family means on estimated breeding values (EBV)

The estimate of the regression coefficient of observed MP of full-sib families on their mid-parent EBV was positive (0.64 ± 0.12), but significantly (P<0.01) lower than the expected value of 1.0 (Appendix 2). It is therefore likely that the major genetic sex determination factor (XX/XY) to some extent acts as a major segregating QTL in the

population, causing the MP to deviate from the expected 50% in some families due to occasional discrepancies between genetic and observed sex in the parents.

A YY sire is most likely an offspring of a normal male (XY) mated with a spontaneously occurring sex-reversed female (ΔXY), and would therefore be expected to come from a family with an observed MP of approximately 75% (unless this is modified by naturally occurring sex reversal). To investigate whether very high observed MP in some families (see Figure 1) are likely to be offspring of YY sires, families with MP above 85% (8 families, $n>20$) were plotted against the observed MP of their sire family. However, Figure 3 shows that only two of these eight sire families had observed MP close to the expected 75%. This could indicate that two of these sires had a YY genotype. However, these two sires were also mated with other females resulting in normal MP around 50% in these families. Hence, the hypothesis of these sires really being YY seems unlikely. To investigate further, all sires mated with multiple dams having at least 90% males in one of the families were plotted in Figure 4. One sire (Sire 1 in Figure 4A) had extreme MP in two offspring families (both with 94% males, $n\geq100$), but only 66% in a third offspring family. This exceptional sire was a parent for G0, and no familial background information was therefore available. Animals from G0 families with extreme high MP were not used as parents in subsequent generations. A sire used in the production of G2 showed two offspring families with high MP (Sire 3 in Figure 4A).

To investigate the occurrence of sex reversed males (ΔXX), all sires that had offspring with MP below 10% and were mated with at least two different dams were plotted against their offspring MP (Figure 4B). Only two sires (Sires 22 and 23 in Figure 4B) showed MP below 0.1 in both their half-sib families, indicating low frequency of sex

reversed males (ΔXX). None of the male offspring of sires 22 and 23 were used as sires in subsequent generations. However when female offspring of sire 23 were used as dams in G5, one family showed MP of 18% and two families showed MP close to 30%.

4. Discussion

Male proportion in this study was on average 44%, and is thus somewhat skewed towards females. However (large) variation in MP has been reported for Nile tilapia in the literature at normal temperatures. In temperature treatment experiments (at 27°C-28°C) MP ranged from 49.9% to 55.8% in progeny from six wild populations (Baroiller et al., 2009) and from 48.8% to 54.1% in a population selected for increased temperature sensitivity (Wessels and Hörstgen-Schwark, 2011). In the experiments described by Lester et al. (1989) MP ranged from 42% to 44%. In a selective breeding programs MP at harvest ranged from 52% to 57% in Vietnam (Luan, 2010); from 45% to 51% in Egypt (Rezk et al. 2009); and was 47% in Malaysia (Nguyen et al., 2007). Some of the differences in MP across environments and generations in this study may be due to human error in the visual scoring of sex, non-random stocking of different sex ratios in the different environments and sex differential mortality in the different environments. However, sex differential mortality (if at all present) will not affect the genetic variance in MP unless the magnitude of the sex differential mortality varies among families. No sex differential mortality has been reported for any part of the tilapia lifecycle (Hickling, 1960; Tuan et al., 1999; Wessels and Hörstgen-Schwark, 2007, 2011; Wessels et al., 2011). In this study this is supported by the low correlation between the observed MP and mortality of each environment across all generations ($r=0.18$, $P>0.05$).

Since Floy anchor tags were employed, different MP among environments and families could also be caused by higher tag loss in larger fish (i.e. the males) due to a higher probability of tag anchor dislodgment in fish with larger muscle tissue and increased tag loss due to higher activity (also males) caused by mating behavior (i.e. making nests, chasing out other males). However, this is not likely since neither the correlations

between the average male harvest weight and MP of the families nor the correlation between the average female harvest weight and MP of the families were statistically different from zero (P>0.05) (with correlation coefficients ranging from -0.14 to 0.07 within generations for males and from -0.13 to 0.01 for females). This is in agreement with the results from a study of MP of different Nile tilapia strains and their crosses (Lozano et al., 2011) where no evidence of sex differential tag loss due to body size was found.

The results from this study suggest that MP is a heritable trait in the studied population of Nile tilapia. The heritability estimate on the liability scale across generations (h^2=0.22±0.04) was similar to the comparable estimate obtained by Lester et al. (1989) (h^2=0.26, confidence interval= 0.13-0.48). The within generation estimates obtained from Model 2 were more stable than those from Model 1 indicating that a threshold model is more appropriate as it accounts better for the differences in MP across test environments. However, results must be interpreted with caution due to the complexity of sex determination in Nile tilapia and since the major sex determination factor (XX/XY) of the parents was unknown and visual sex determination of fish is subjected to human error.

More reliable estimates of the polygenic genetic variation for MP could be obtained if the genetic sex of the parents was known, for example through use of sex-linked genetic markers, and this information was included in the statistical model. Eshel et al. (2010) suggested that LG23 is a male-associated allele; hence LG1 may be an additional autosomal gene that influences sex determination. Lee et al. (2003) found that LG1 was family specific, and further research is thus needed to establish accurate genetic markers for prediction of sex. Accurate sexing of offspring can be obtained using gonadal tissue

squashes from a random sample of fish (Guerrero and Shelton, 1974) as done in the study of MP in hybrids between Nile tilapia and blue tilapia (Lozano et al., Unpublished results).

Variation in MP could be due to introgression of other tilapia species into the Nile tilapia stocks used, as shown by Macaranas et al. (1986) for some farmed Philippine stocks. In total, 38% of the founders in the base population (G0) in the current study were from the farmed Philippine stocks (Eknath et al., 2007, Figure 2), and the impact of these strains could therefore be substantial in the following generations (G1-G5). Additive genetic differences in MP between the different tilapia strains used to produce the base population in this study were reported by Lozano et al. (2011), however they were low in magnitude.

The regression of MP on estimated mid-parent breeding values (excluding the phenotypic sex of the offspring) was lower than the expected value of unity, a bias that may be attributed to the effect of the major genetic sex determination system (XX/XY) and its possible interactions with environmental factors (spontaneous sex-reversal) and autosomal genes. This is likely to give a fraction of parents whose phenotypic sex does not match the genetic sex determined by the XX/XY-system causing an upward bias in the magnitude of the genetic variation in MP in this study.

Strong evidence of genetic variation in MP in Nile tilapia has also been reported earlier through between-family differences for the Auburn strain (71 families with MP ranging from 31 to 77%) (Shelton et al., 1983), Egypt-Swansea strain (Mair et al., 1991a) (57 families with MP ranging from 35 to 65%), and Thai-Chitralada strain (95 families with MP ranging from 15 to 100%) (Tuan et. al, 1999). When crossing YY males with

normal XX females, differences in MP from the expected value of 1.0 were also found (Mair et al., 1991a; 1997; Tuan et al., 1999). For the Egypt-Swansea strain MP of four progeny tested males (mated to six different females) ranged from 93 to 100% (Mair et al., 1991a), from 90% to 100% in six gynogenetic males tested with 18 different males (Mair et al., 1991a) and from 79.5% to 100% (in 61 males tested) (Mair et. al 1997), whereas for the Thai-Chitralada strain MP of the two males tested (with 12 females and producing 19 families) ranged from 36-100% (Tuan et al., 1999). Thus, this large family variation in MP supports the existence of polygenic sex determination with minor and major genetic effects.

Both masculinization (Baroiller and D'Cotta, 2001; Bezault et al., 2007) and feminization (Bezault et al., 2007) have been suggested to occur spontaneously in wild Nile tilapia. In paired mating experiments with Nile tilapia, Mair et al. (1991a) found full-sib families with very high MP and through progeny testing of the dams from these families concluded they were naturally sex reversed (ΔXY) females. However, very few of the breeders (eight out of 1659) seemed to be naturally occurring sex reversed or YY fish. Furthermore, assumed YY males often produce offspring groups deviating considerably from the expected 100% MP (Mair et al., 1997, Tuan et al., 1999), indicating existence of minor genetic sex determination factors (additive genetic variability) for MP.

The robustness of the heritability estimates were tested by re-running Model 1 within and across generations while excluding families with extreme MP (42 families with MP above 0.90 and below 0.1) since they may be the result of mating YY males and ΔXX males, respectively. As expected, heritability estimates were lower (e.g. h^2=0.07±0.01 across generations, Model 1) since extreme MP were omitted from the data, but

generated the same conclusions of significant additive genetic variation for MP across and within generations (except for G4).

The given nested mating structure in this study, it is generally difficult to separate additive genetic and common environmental effects within generation since each family was reared in one hapa only. This may explain the different estimates of the effects common to full-sibs between generations. Consequently, the estimates of these effects across generations (which also include repeated matings of some sire-dam combinations) are likely more stable and reliable.

Nearly all (16 of the 17) the genetic correlations between MP in the different test environments were not significantly different from unity. This implies no sex differential mortalities (or tag loss) occurred across the families in the different test environments since the observed phenotypic sex was determined before the fish were stocked in the different test environments.

Water temperatures ranged approximately from 26 to 32°C in the nursery and breeding hapas where the full-sib families underwent sex differentiation (Edna Dionisio, Personal. communication). We do not expect this range of temperatures to affect sex determination since sustained temperatures above 32°C are not likely. Nevertheless we lack the exact water temperature information during the period of sexual differentiation for the full-sib families. Furthermore, rearing temperature at sex differentiation may change for families produced at different dates within a generation. However, there is no indication of higher MP when families were produced during the warmer months (April-July) than during colder months (December-February) (see Table 1 and 4). Temperature at sex differentiation may even be different within generation for families

produced on the same date due to thermal differences throughout the pond area where the nursery hapas (full-sibs) were located. However, this is accounted for in Model 1 and 2 by the random effect common to full-sibs.

It should be noted that if selection for increased MP is successfully employed, it may result in selection for increased probability of naturally masculinized XX individuals (as such families likely will have high MP). Using such males as broodstock sires may counteract the response to selection. This can be avoided by test crossing of male breeding candidates, or by using sex chromosome markers if reliable markers become available. Therefore, immediate implementation of selection for sex ratio at normal rearing temperatures in a breeding program would be rather complicated since test crossing requires time and sex markers still need to be tested. If we assume selection successfully increases MP to the desired degree (above 95%), hormone treatment of males may be needed to produce subsequent generations.

Alternative methods to increase MP through selection should therefore be considered, e.g. to select breeders based on MP of their sibs tested at an elevated temperature. Since the reported genetic correlation between MP at normal and high temperatures is not significantly different from zero (Wessels and Hörstgen-Schwark, 2011), we expect availability of females in the breeding nucleus (raised at normal temperatures) without need for hormone treatment. Genetic correlations between MP and other traits of economic importance should be evaluated before implementing selection to avoid any negative effects.

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Tables and figures

Table 1. Fry collection period, days of separate rearing of the families (from collection of fry to stocking in grow-out environment), and average duration of grow-out test across environments are summarized for all generations.

Generation	Fry collection	Separate rearing (days)			Grow-out test (days)		
		Min.	Max.	Average[a]	Min.	Max.	Average
G0	12.Jan.– 6.Mar.1991	40	116	78	83	98	90
G1	11.Sep– 21.Oct.1991	51	110	85	90	97	92
G2	15.Jun.– 20.Aug.1992	71	143	114	124	126	125
G3	30.Jun.–31.Aug.1993	43	112	85	120	125	123
G4	2.May–08.Aug.1994	39	158	93	122	126	124
G5	11.Sep.–17.Nov.1995	48	143	110	124	126	125

[a] Average of the families across all test environments.

Table 2. Number of breeders and half-sib groups produced within each generation.

Generation	No. breeders		No.paternal half-sib groups	No. recorded per full sib family Avg.(min,max) [c]	No. families N<20[d]
	sires	dams			
G0	50	123	43	110 (75,143)	
G1	110	192	82	82 (21,104)	
G2	130	215	85	41 (5,56)	23
G3	124	195	71	36 (2,54)	31
G4	107	171	65	41 (24,53)	
G5	127	180	53	44 (2,67)	2
Total	*616*[a]	*1043*[a]	*400*[b]		

After culling data with 1 record per family within test environment and generation.

[a] Breeders used across generations were only counted once.

[b] Includes one maternal full-sib family produced in G4.

[c] Average number of fish recorded at harvest for sex across environments .

[d] Number of families with less than 20 fish recorded at harvest across environments.

Table 3. Number of times sires and dams were used across generations (G0-G5)

	Number of times used						
	1	2	3	4	5	6	Total
Number of sires	203	378	27	4	3	1	616
Number of dams	1015	22	6				1043

Table 4. Number of fingerlings stocked, tag loss and mortality (not including tag loss) from tagging to harvest, male and female body weight at harvest, and male proportion within each test environment for all the generations analyzed.

Gen	Test environment	No. stocked per family	Tag loss (%)[a]	Mortality (%)[a]	Body weight (BW) Total N (harvest)	Males (g)	Females (g)	Male proportion[b]
G0	C2	10	0	16	1016	19	15	0.49
	C4	10	3	31	768	69	47	0.56
	P1	40	11	22	3228	70	44	0.48
	P2	40	15	13	3515	94	61	0.49
	S1	20	22	10	1674	75	54	0.47
	S2	20	23	7	1722	69	54	0.49
	W2	20	8	24	1631	66	43	0.46
	mean	***23***	***13***	***17***	***13554[c]***	***72***	***49***	***0.48***
G1	C2	14	0	4	2565	20	17	0.38
	P1	28	4	23	3875	49	37	0.39
	P2	28	8	8	4485	91	64	0.43
	S1	14	3	9	2357	37	29	0.46
	S2	14	5	35	1616	43	33	0.42
	W2	14	4	60	937	79	59	0.40
	mean	**19**	**5**	**21**	***15835[c]***	***56***	***41***	***0.42***
G2	P1	25	6	10	3990	101	67	0.45
	P2	30	7	8	4997	131	91	0.44
	mean	**28**	**6**	**9**	***8987[c]***	***117***	***80***	***0.44***
G3	P1	27	12	17	3203	140	95	0.40
	P2	33	29	1	3858	208	148	0.40
	mean	**30**	**21**	**8**	***7061[c]***	***177***	***124***	***0.40***
G4	C4	14	6	12	2022	138	103	0.42
	P1	29	8	15	3837	149	105	0.37
	P2	14	17	31	1269	135	110	0.42
	mean	**19**	**10**	**18**	***7128[c]***	***143***	***105***	***0.39***
G5	C4	15	17	28	1413	171	119	0.54
	RF	16	39	39	561	222	135	0.50
	P1	30	34	21	2521	151	113	0.50
	P2	35	9	37	3522	194	146	0.50
	mean	**24**	**23**	**31**	***8017[c]***	***178***	***130***	***0.51***

Pond culture without feed supplement: S1 (lowlands, Southern Luzon), S2 (coastal region, North-West Luzon), P1 (lowlands, Central Luzon).
Pond culture with feed supplement: P2 (lowlands, Central Luzon).
Pond culture without feed supplement and fertilized with ipil-ipil leaves: W2 (lowlands, Central Luzon).
Cage culture with supplement feed: C2 (70% rice bran + 30% fish meal) (lowlands, Central Luzon), C4 (commercial pellet feed) (lowlands, Central Luzon)
Rice-Fish culture: RF (lowlands, Central Luzon)
[a] Based on data from Eknath et al. (2007) and Bentsen et al. (Unpublished results).
[b] N males/(N males+ N females)
[c] Total number of tagged animals harvested per generation (not mean).

Table 5. Estimates of heritability ($h^2 \pm$ se) and of the effect common to full-sibs ($c^2 \pm$ se) for male proportion in Nile tilapia on the observed (Model 1) and the underlying liability (Model 2) scales: within and across all generations, and across all generations in cage and pond environments.

	Model 1						Model 2					
Generation	h^2	±	*Se*	c^2	±	*se*	h^2	±	*se*	c^2	±	*se*
G0	0.05	±	0.03	0.02	±	0.01	0.12	±	0.04	0.03	±	0.02
G1	0.06	±	0.02	0.01	±	0.01	0.11	±	0.02	0.01	±	0.01
G2	0.10	±	0.04	0.05	±	0.02	0.22	±	0.06	0.05	±	0.03
G3	0.10	±	0.05	0.06	±	0.02	0.21	±	0.07	0.06	±	0.03
G4	0.00	±	0.03	0.10	±	0.02	0.26	±	0.09	0.06	±	0.04
G5	0.25	±	0.07	0.01	±	0.02	0.32	±	0.07	0.04	±	0.03
All	0.12	±	0.02	0.04	±	0.00	0.22	±	0.04	0.06	±	0.01
Cages*	0.04	±	0.02	0.03	±	0.01	0.07	±	0.03	0.05	±	0.02
Ponds*	0.12	±	0.02	0.04	±	0.01	0.24	±	0.05	0.06	±	0.01

* Across all generations

Table 6. Estimates of genetic correlations (± standard error) between male proportions in different test environments obtained from a bivariate linear model analysis (Model 1) across generations.

	C2	C4	P1	P2	S1	S2
C4	0.26 ± 0.87					
P1	0.73 ± 0.32	1.00*±0.03*				
P2	0.82 ± 0.25	ne	ne			
S1	0.89*±0.13*	0.98*±0.17*	ne	1.00*±0.03*		
S2	0.14 ± 0.44	***0.57*±0.21****	1.00 ± 0.12	0.97 ± 0.11	0.92*±0.09*	
W2	0.73 ± 0.36	0.50 ± 0.80	ne	0.98 ± 0.16	0.85 ± 0.21	0.87*±0.12*

Pond culture without feed supplement: S1 (lowlands, Southern Luzon), S2 (coastal region, North-West Luzon), P1 (lowlands, Central Luzon).
Pond culture with feed supplement: P2 (lowlands, Central Luzon).
Pond culture without feed supplements but fertilized with ipil-ipil leaves: W2 (lowlands, Central Luzon)
Cage culture with supplement feed: C2 (70% rice bran + 30% fish meal at 20% BW twice daily) (lowlands, Central Luzon), C4 (commercial pellet feed) (lowlands, Central Luzon)
Rice-Fish culture: RF (lowlands, Central Luzon)

ne : Model did not converge or was terminated.
* Estimates obtained after removing the effect common to full-sibs from Model 1.
Correlations significantly different from unity (Log-likelihood ratio test, $P<0.05$) are written in bold and italics.
Environment RF was deleted from the table since no estimates of correlations could be obtained between RF and any other test environment (ne).

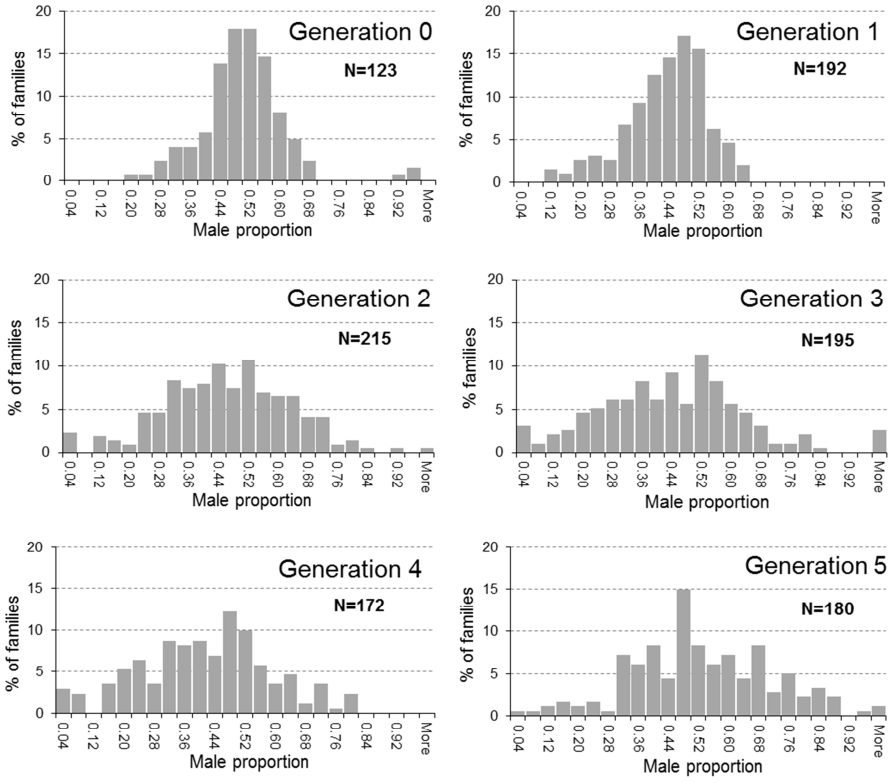


Figure 1. Within generation frequency distribution of the observed male proportion of full-sib families across all test environments.

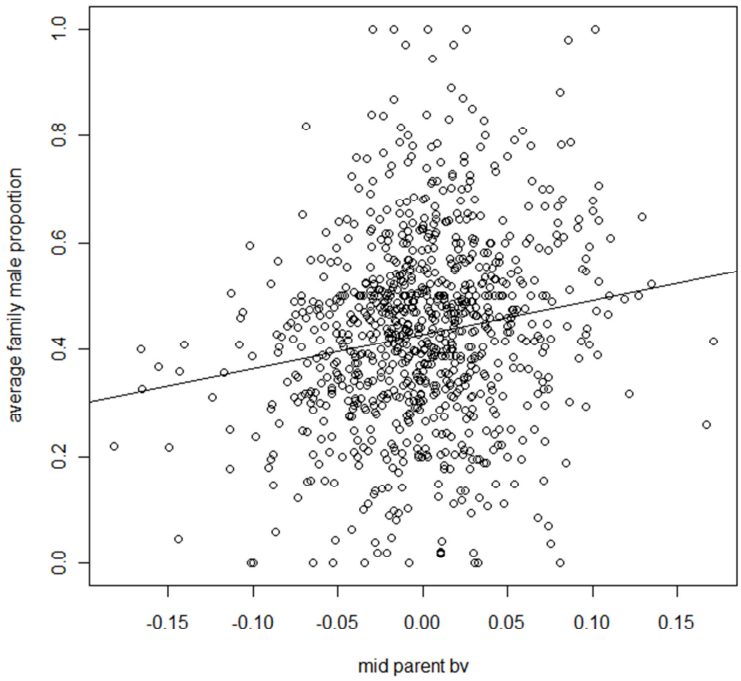


Figure 2. Weighted regression of observed male proportion of full-sib families on the mid-parent EBV corrected for Generation. EBV's were obtained using Model 1 but omitting the recorded sex data of the offspring of each breeder, one generation at a time.

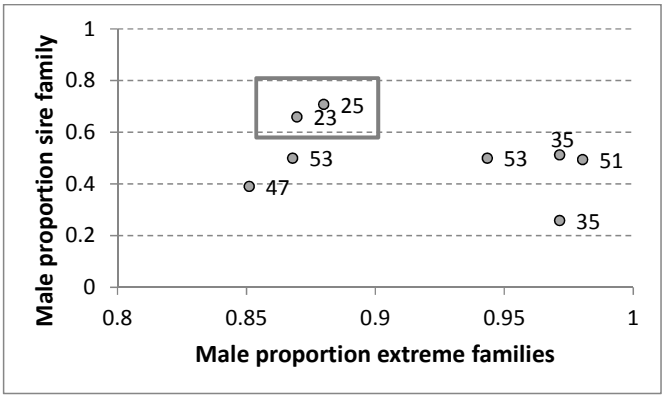
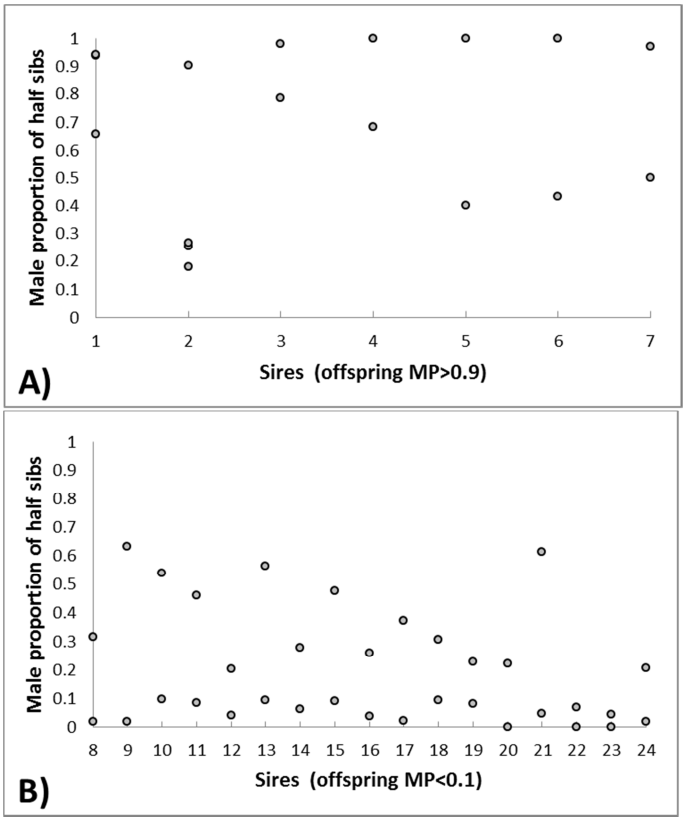


Figure 3. Plot of observed male proportion (MP) of full-sib families with MP above 0.85 and the observed MP of their corresponding sire family (only families with more than 20 records per generation are included). The number of records per extreme family is given above each observation. One of the parents of the full-sib families in the box may have been a YY sire.

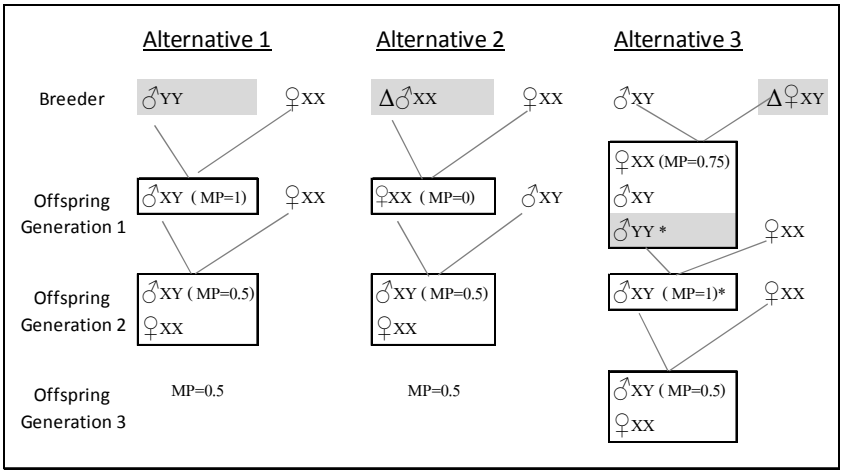


Sires with extreme (high or low) MP offspring but mated with only one dam are excluded from plot.

Figure 4. Plot of observed male proportions (MP) of sires with extreme MP among their offspring (x-axis) vs. the observed MP of their corresponding half-sib families. A) Sires with MP above 0.9 in at least one of their paternal half-sib families (possible YY sires); B) Sires with MP below 0.1 in at least one of their parental half-sib families (possible XX sires).

Appendixes

Appendix 1. Genotypes of sex reversed and YY individuals (grey shaded squares), and possible genotypes of their offspring (outlined squares) if mated with normal fish (genetic sex equal to phenotypic sex) and the expected male proportion (MP) of their offspring assuming solely the XX/XY sex determination system.



*For Alternative 3 only 25% of the offspring is expected be YY, thus only 25% of the offspring in Generation 2 will have MP=1.

Appendix 2.

$P = BV + E$: P is the observed male proportion of a family, BV is the true breeding value for male proportion and E is the error (i.e. random environmental effects, dominance and epistasis effects).

$EBV = \frac{1}{2} EBV_{Sire} + \frac{1}{2} EBV_{Dam}$: EBV is the mid parent estimated family breeding value for male proportion.

$BV = EBV + D$: D is the error that arises when estimating breeding values

If estimated breeding values are unbiased then:

$\text{cov}(BV, E) = 0$ and $\text{cov}(EBV, D) = 0$; $\text{cov}(E, D) = 0$; $\text{cov}(EBV, E) = 0$

Expected regression coefficient of average male proportion of families on mid parent EBV.

$$b_{P,EBV} = \frac{\text{cov}(P, EBV)}{\sigma^2_{EBV}} = \frac{\text{cov}(EBV + D + E, EBV)}{\sigma^2_{EBV}}$$

$$= \frac{\text{cov}(EBV, EBV) + \text{cov}(D, EBV) + \text{cov}(E, EBV)}{\sigma^2_{EBV}} = \frac{\sigma^2_{EBV}}{\sigma^2_{EBV}} = 1$$

Paper III

Paper III

Heritability estimates for male proportion in hybrids between Nile tilapia females (*Oreochromis niloticus*) and blue tilapia males (*Oreochromis aureus*).

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Key words: Hybrids, *O. niloticus*, *O. aureus*, heritability, male proportion, sex ratio

ABSTRACT

Estimates of the genetic variation in male proportion (MP) were obtained from a total of 82 hybrids families, produced by crossing 82 Nile tilapia females (*Oreochromis niloticus*) with 35 blue tilapia males (*O. aureus*), which were stocked in 132 hapas in a common pond. The parents of each species originated from three different countries (Israel, Taiwan, China), but only seven of the nine possible sire-dam origin combinations (crosses) were produced. After on average 53 days in the hapas (April-July) a random sample of, on average, 94 fish per hapa were slaughtered and the sex of each fish was determined by examining its gonad tissue under the microscope. Overall MP was 77%, and thus below the expected value of 100% assuming a major sex determination system (XX/XY in Nile tilapia and WZ/ZZ in blue tilapia).

The effect of cross on MP was highly significant, but not very reliable as some of the crosses were represented with a limited number of families. The variation in MP among the families was substantial (from 0% to 100%) and with heritability estimates for MP on the observed scale that ranged from 0.38±0.07 to 0.42 ± 0.09 (assuming either equal or different additive genetic sire-dam variance for the parental species), and, as expected, higher on the underlying scale (0.79 ±0.11 to 0.82 ± 0.13). The effect common to full-sibs other than additive genetics was significant, but explained a low proportion of the total variance (0.04±0.01 and 0.08±0.02 on the observed and liability scales, respectively). The magnitude of the estimated heritabilities indicates that MP in hybrids is partly under additive (polygenic) genetic control. However, these estimates may be biased upwards, by interaction with the major genetic (XX/XY and WZ/ZZ) sex determination system, as genetic and phenotypic sex does not necessarily match in all parents. Possibilities for selection for increased MP in hybrids, aiming at commercially required levels (>95% males) is discussed.

1. Introduction

"Tilapia" is a common name given to cichlid warm-water fishes from the genera *Oreochromis*, *Saratherodeon* and *Tilapia*. Tilapias are the second most important Aquaculture fish species (FAO Yearbook, 2008). Tilapias originate from Africa (excluding Madagascar) and the Middle East (Philippart and Ruwet, 1982). However, they have been introduced in several countries for the purpose of aquaculture since 1965 (Philippart and Ruwet, 1982). Nile tilapia (*Oreochromis niloticus*) has been widely used due to its excellent growth potential and general sturdiness, and blue tilapia (*Oreochromis aureus*) has been favored due to its higher cold tolerance.

Sex dimorphism favoring males and early reproduction of this species have made all male tilapia farming the industry standard. All male culture controls unwanted reproduction and increases the amount of fish of marketable size obtained per crop. The most common method currently used by the industry to obtain all male fry is oral administration of hormones (Phelps and Popma, 2000), however this method is not accepted in some countries (e.g. Japan, Marengoni et al., 1998) and Best Aquaculture Management Practices (BAP) encourages the use of other methods (GAA, 2008). Mating of hybrids of two different species which yield high male proportion (MP) is an environmentally friendly alternative for the production of all male tilapia fry. There are three main constraints for the use of hybrids: 1) Limited availability of pure tilapia species used to produce the hybrids since introgression of genes from other species may contribute to variation in MP (Macaranas et al., 1986; Marengoni et al, 1998); 2) Maintaining pure genetic stocks of the actual species is challenging since hybrids are fertile and can easily contaminate the pure stocks (Lovshin, 1982; Beardmore et al., 2001); and 3) Large variation in MP has been found between hybrids of different

species (Wohlfarth and Hulata, 1981) and between strains of the same hybrid combination (Pruginin et al., 1975; Garcia Pinto, 1982; Mair et al. 1991b; Marengoni et al., 1998).

Temperature has a strong effect on tilapia growth. The optimal range for most species is between 25°C and 28°C, but reproduction decreases rapidly when temperatures drop below 22°C, feeding stops at 20°C and exposure to temperatures below 10°C for several days is usually lethal (Wohlfarth and Hulata, 1981; Chervinsky, 1982). Hence, when tilapias are farmed in sub-tropical regions grow-out is restricted to summer (Hepher and Pruginin, 1982). Thus, fry stocked early in the summer have been overwintered, and as a consequence, suffer stunted growth (Hepher and Pruginin, 1982) and high mortalities (Tave et al., 1990). Cold tolerance varies between tilapia species and the most tolerant species are *T. sparmani, T. rendalli, T. zilli, S. galilaeus, O. aureus,* and *O. mossambicus* (Chervinsky, 1982; Cnaani et al., 2000). Nile tilapia (*O. niloticus*) is generally preferred for commercial production due to its high growth rate. However, since Nile tilapias are not tolerant to cold temperatures commonly encountered in subtropical areas, *O. niloticus* x *O. aureus* hybrids are often preferred since these crosses, in addition to yielding higher growth than pure *O. aureus* and higher cold tolerance than pure Nile tilapia, also yield high numbers of male fry (Lahav and Ra'anan, 1998).

Hickling (1960) discovered that hybrids between *O. mossambicus* and *O. urolepis hornorum* (formerly called *T. hornorum*) were fertile and with high MP. He observed that when *O. urolepis hornorum* males were mated with *O. mossambicus* females, the

offspring was nearly 100% males, suggesting that *O. mossambicus* had a sex determination system with male heterogamety (XX/XY) and *O. urolepis hornorum* had a sex determination system with female heterogamety (WY/YY) analogous to what Gordon (1957) described for platyfish (*Xiphophorus maculatus* and *Xiphophorus variatus*). He concluded that the Y chromosome could carry genetic factors of maleness and the W chromosome could carry genetic factors for femaleness. However, the reciprocal cross (*O. mossambicus* males mated with *O. urolepis hornorum* females) resulted in offspring with about 75% males, which does not agree with the expected result of 50% MP (males=XY, YY; females=WY, WX), indicating that in tilapia a more complex system determines sex. Hickling's work incited a myriad of hybridization and sex reversal experiments aimed to study sex determination in different species of tilapia (e.g. Chen, 1969; Pruginin et al., 1975) and results of some of these experiments are summarized by several authors (Wohlfarth and Hulata ,1981; Guerrero , 1982; Lovshin, 1982; Beardmore et al., 2001). A four-gonosomal model (XX/XY and WZ/ZZ) as suggested by Bellamy (1936) could explain the results (75 % MP) obtained when crossing female *O. aureus* with male *O. niloticus* and most of the backcrosses performed by Chen (1969). An autosomal theory was proposed by Hammerman and Avtalion (1979), where sex is determined by the sum of the effects of three alleles (W, X and Z, where Y=Z) of a major sex determining locus and two alleles of an autosomal locus (A, a) (Hammerman and Avtalion, 1979). The latter theory explained results obtained by Chen (1969) better that the four-gonosomal model. However, both theories still failed to explain deviations from the expected MP (50%) observed in purebred strains (Shirak et al., 2002). Additionally, hybrids between female *O. niloticus* and male *O. aureus* have been found to have MP that varies from 34% to 100% (Pruginin et al., 1975; Garcia Pinto, 1982; Mair et al. 1991b) and thus cannot be completely explained by either of these models. A multifactorial sex determination system with underlying

primary mechanism of female heterogamety was proposed for *O. aureus* (Mair, 1991b). A comprehensive review done by Baroiller et al. (2009) proposed a complex sex determination for *O. niloticus* where sex is determined by minor genetic factors, major genetic factors (XX/XY system) and a temperature environmental factor.

If sex determination was to be attributed solely to the effect of a single major sex determination locus (e.g. XX/XY and WZ/ZZ) then there should be no variation in MP between families (except for sampling effects) of the same species and heritabilities should be zero, as seen for some mammals (Maynard Smith, 1980; Toro et al., 2006). Reported heritabilities for MP at normal pond temperatures in *O. niloticus* range from 0.22 to 0.26 in the underlying scale (Lester et al., 1989; Lozano et al., Unpublished results), thus showing evidence of genetic variation in MP. Furthermore, variation in MP between Nile tilapia strains, although of low magnitude, has also been reported (Lozano et al., 2011), and Tuan et al. (1999) found higher variation for MP in the Thai-Chitralada strain than in the Egypt-Swansea strain (Mair et al., 1997). High water temperatures during sexual differentiation have been shown to increase the MP in both *O. niloticus* (Baroiller et al., 1995, 2009; Tessema et al., 2006; Wessels and Hörstgen-Schwark, 2007) and *O. aureus* (Deprez and Mélard, 1998). Temperatures above 32°C have a masculinizing effect in Nile tilapia when treatment is initiated 10 days post fertilization (dpf) and lasts for 10 days (Barroiller et al., 1996**;** Tessema et al., 2006**).** Another window of temperature sensitivity, treatment between 12 and 52 hours post fertilization (hpf) at temperatures ≥34 °C, was found to masculinize Nile tilapias (Rougeot et al., 2008), but during this window Wessels et al. (2011) did not find a masculinizing effect at 34°C. Temperature sensitivity has been proven to be under genetic control and cumulated realized heritabilities reported ranged from 0.63 (high

response line where fish selected had MP ≥ 0.80) to 0.84 (low response line where fish selected had MP ≤ 0.60) in two divergent lines (Wessels and Hörstgen-Schwark, 2011). No significant correlations were found between sex ratios obtained at 28°C and 36°C (Wessels and Hörstgen-Schwark, 2011). However, high water temperatures during sexual differentiation in *O. niloticus* have also been reported to cause a feminizing effect in progenies of YY males (Abucay et al., 1999) and in progenies of normal XY males which have been selected for low temperature sensitivity with respect to MP for three generations (Wessels and Hörstgen-Schwark, 2011).

Evidence of the presence of sex chromosomes by synaptonemal complex (SC) analysis was found for *O. niloticus* (Carrasco et al., 1999) and for *O. aureus* (Campos-Ramos et al., 2001). For *O. niloticus* Carrasco et al. (1999) suggested that the X and Y chromosomes are under initial divergences, whereas for *O. aureus* Campos-Ramos et al. (2001) suggested two pairs of sex chromosomes may be present (ZW, ZZ, Z'W', Z'Z'). Recently sex-linked markers have been identified for six tilapia species, and for *O. niloticus* a sex determining locus was found on linkage group (LG) 1 but for *O. aureus* it was found on both LG1 and LG3 (Cnaani et al., 2008). However Eshel et al. (2010) found that in *O. niloticus* two linkage groups, LG1 and LG23, were associated with sex with the latter group showing the strongest association. Analysis of epistatic interactions among loci in *O. aureus* suggest a dominant male repressor (W on LG3) and a dominant male determiner (Y on LG1) act simultaneously to determine sex, thus a dilocus genotype is considered (Lee et al., 2004). In this case a *O. niloticus* female would be (??XX) and the *O. aureus* male (ZZ??) (where ?? is denoting the unknown allelic state), since we do not know the allelic state for the LG3 locus on *O. niloticus* males nor the allelic state for the LG1 locus on *O. aureus* males (Lee et al., 2004). This may explain

why in some crosses between Nile tilapia females and blue tilapia males not all offspring are males, since hybrids would be (Z?X?) and some females could occur depending of the genotype of the additional (??) sex modifying loci (Lee et al., 2004). Cnaani et al. (2008) suggested that in their ancestral state, tilapias (*Orechromis spp.*) had a female heterogametic system with a major sex determination locus located on LG3 and also a minor male sex determination gene in LG1, but in some lineages the LG1 gene took control of the male sex determination pathway. Lande et al. (2001) suggested yet a different model for cichlid fish, where the ancestral fish had a XX/XY determination system and some X chromosomes carried a dominant sex reversal gene W that changes males to females and an additional polymorphic gene (M) that suppresses W. It is important to bear in mind that environmentally induced sex reversal may also occur in both *O. niloticus* and *O. aureus.* This may contribute to skewed MP among families, and will also produce some animals with non-matching genetic and phenotypic sex. If used as parents, the latter animals will likely contribute to substantial deviations in MP among their offspring in the following generation.

The main objective of this study was to determine the magnitude of the genetic variation in MP among hybrid full-and half-sib families from Nile tilapia females mated with blue tilapia males**.**

2. Material and Methods

2.1 Stocks

Three stocks of Nile tilapia (*O. niloticus*) dams (originating from Israel, China and Taiwan) and three stocks of blue tilapia (*O. aureus*) sires (also from Israel, China and Taiwan) were used as parents for the studied hybrids. Only hybrids between Nile tilapia dams and blue tilapia sires were produced, since this cross is expected to produce high MP and the results of this experiment were intended to be used to select the best stock combinations for commercial fry production. Additionally, only seven of the nine possible stock combinations were produced, referred to herein as "cross" (Table 2).

The Israel and China stocks of each species were introduced to the Research Institute for Aquaculture No. 1 (RIA1), Vietnam in 2005 (as 2-3 cm in size fingerlings of both sexes) from Israel (10,000 fingerlings of Nile tilapia and 10,000 fingerlings of blue tilapia) and China (10,000 fingerlings of Nile tilapia and 10,000 fingerlings of blue tilapia). The Taiwan stock of each species was introduced from Taiwan in 1998. No selection for increased MP was performed in any of the stocks used in this study since their introduction to Vietnam.

2.2 Reproduction

Sixteen breeding hapas (2.5x2.0x1.0 m) were placed in one 2500 m^2 pond at the National broodstock center for fresh water species (NBC), at Thach Khoi commune, Gia Loc district, Hai Duong Province, Vietnam (50 km south of Hanoi). One male blue tilapia and six female Nile tilapias were stocked per breeding hapa; however, not all

females were fertilized or produced viable offspring. Eggs were collected from mated females and placed in separate incubation jars (period from collection until hatching was on average four days). Hatched fry offspring of each sire-dam combination were transferred to separate rearing hapas (2.5x2.0x1.0 m) placed in another pond (2000 m^2) at the NBC station. In some cases fry were collected from the mated females and placed directly into separate rearing hapas. On several occasions females were returned to the breeding hapa, thus the same sire-dam combination (full-sib family) could be collected and placed in different rearing hapas. Over a period of 110 days in 2007 a total of 132 separate rearing hapas were stocked (200 fish per hapa) in the same pond (Table 1), representing 83 families (unique sire-dam combinations); i.e. the offspring of 35 sires and 82 dams (Table 2). Twelve of the sires were mated with one dam each, while the remaining twenty-three sires were mated to from two to five dams. Each dam was mated with one sire only (with the exception of one dam that was mated with two sires).

A total of 51 of the 83 families were stocked only in one rearing hapa. However, 32 of the families were stocked in two or more separate rearing hapas. Since families produced on different dates were stocked in different hapas, offspring of sires were stocked in 1 to 9 breeding hapas and offspring of dams were stocked in 1 to 5 breeding hapas.

In the statistical analyses of the data hapas were grouped into four different batches according to collection date (Table 1). Families with the same sire or sire-dam combination (produced on different dates) which were stocked and reared in separate

hapas facilitated proper separation of additive genetic and environmental effects common to full-sibs.

2.3 Rearing and sexing

The hybrids were kept in the separate rearing hapas for an average of 53 days (range 21-71) until they reached an average of approximately 10 grams. Then, a random sample of fish from each hapa (average 94 fish, range 11-115) were slaughtered and the gonadal tissue of each individual fish was examined in the microscope (magnification from 25 to 100X) using acetocarmine dye solution to determine the sex (Guerrero and Shelton, 1974).

The water temperature in the pond containing the rearing hapas was measured twice a day every second or third day, and was on average 29°C, with the lowest temperatures found in April (average 24°C) and highest in June and July (average 31.5°C) (Figure 1). Fish were fed pellets containing 22% crude protein at a rate of 2-3% of the fish biomass per day. Environmental conditions are expected to have an effect on phenotypic sex which varies according to the genetic makeup of the different strains (Devlin and Nagahama, 2002). Generally, temperatures maintained above 32°C during sex differentiation have a masculinizing effect for Nile tilapia (Baroiller et. al, 2009), however in our study temperatures above 32°C were not maintained for more than 24 hours since temperature fluctuated on average 8°C during the measurements made at 07:00 AM and 14:00 PM . Temperatures recorded at 07:00 AM never exceeded 30°C; but temperatures recorded at 14:00 PM were above 32°C from May 16 onwards (Batch

3 and 4). Baras et al. (2000) found that *O. aureus* exposed to fluctuating temperatures had a masculinizing effect of lesser magnitude (27°C-35°C , 70% males) than at constant high temperatures (35°C, 90% males).

2.4 Data analysis

Since we are dealing with data recorded on hybrids of two different species, estimation of common genetic parameters for male proportion may be debated, and different models were therefore tested. Since phenotypic sex reversion may be influenced by the water temperature during the period the fish were kept in the rearing hapas it may be important to account for both batch (period of production) and rearing hapa in the analyses of male proportions.

Univariate linear (Model 1) and threshold (Model 2) sire-dam models were used to obtain estimates of variance components of the random effects for MP on the observed (Model 1) and the underlying unobserved liability (Model 2) scales using Restricted Maximum Likelihood (REML). Estimates of the fixed effects (batch, cross) and predictions of the random effects (sires, dams, hapa) were obtained using the ASReml software (Gilmour et al., 2009). Cross 2 was eliminated from the dataset used for genetic parameter estimations since both families produced had 100% males. Hence, no variation in MP was observed for this group, and inclusion of such group may lead to extreme-category problems in threshold models for binary traits.

Model 1 (Univariate linear sire-dam model):

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Z_S a_S} + \mathbf{Z_D a_D} + \mathbf{Wc} + \mathbf{e} \qquad (1)$$

where **y** is the vector of the observed phenotypic sex (scored as 1 for males and 0 for females); **X** is a design matrix for the fixed effects that links individual observation to the fixed effect classes; **b** is the vector with the estimates of the fixed effects (including batch as defined in Table 1, and cross as shown in Table 2); $\mathbf{Z_S}$ and $\mathbf{Z_D}$ are incidence matrices linking observations to the sire and dam additive genetic effects; $\mathbf{a_S} \sim N(\mathbf{0}, \mathbf{A}\sigma_S^2)$ and $\mathbf{a_D} \sim N(\mathbf{0}, \mathbf{A}\sigma_D^2)$ are vectors of the random sire and dam additive genetic effects, respectively; **W** is an incidence matrix linking observations to the effects common to full-sibs, $\mathbf{c} \sim N(\mathbf{0}, \mathbf{I}\sigma_C^2)$ is a vector of random effects common to full-sibs other than additive genetics (rearing hapa); **A** is the numerator relationship matrix that describes the additive genetic relationship among all individuals (sires and dams) included in $\mathbf{a_S}$ and $\mathbf{a_D}$, $\mathbf{e} \sim N(\mathbf{0}, \mathbf{I}\sigma_E^2)$ is a vector of random residuals, and **I** is an identity matrix of appropriate size.

Model 2 (Univariate threshold (probit) sire-dam model):

$$\boldsymbol{\lambda} = \mathbf{Xb} + \mathbf{Z_S a_S} + \mathbf{Z_D a_D} + \mathbf{Wc} + \mathbf{e} \qquad (2)$$

where **X**, **b**, $\mathbf{Z_S}$, $\mathbf{Z_D}$, $\mathbf{a_S}$, $\mathbf{a_D}$, **W**, **c** and **I** are as described in Model 1; λ_i is the underlying liability which is assumed to be associated to the binary observation of animal i (y_i) such that $\lambda_i \leq 0$ gives $y_i = 0$, and $\lambda_i > 0$ gives $y_i = 1$. The vector $\boldsymbol{\lambda}$ is a vector of all underlying liabilities. The variance components were estimated on the underlying scale, where residual variance (σ_E^2) was restricted to 1.0, implying that $\mathbf{e} \sim N(\mathbf{0}, \mathbf{I})$.

Not all rearing hapas were sexed on the same date. The longer the period the fish were kept in the separate hapas, the higher the chance of mortality, which theoretically may have some effect on MP, given that mortality is to some extent sex dependent. In a preliminary analysis with the effect of Batch excluded, first and second order polynomial function of age (days from egg collection to sexing) were included as covariates in Models 1 and 2 but were excluded from the final model as their effects on MP were not significantly different from zero.

In tilapia hybrids additive genetic variance of sires (*O. aureus*) and dams (*O. niloticus*) with respect to sex determination in the offspring are not necessarily of identical magnitude. Hence, models allowing for both different and equal additive genetic sire and dam variances were used, both using linear (Model 1) and threshold models (Model 2). This was done by fitting alternative models assuming either $\sigma_S^2 \neq \sigma_D^2$ or $\sigma_S^2 = \sigma_D^2$. For Model 1, differences of sire and dam additive genetic effects were tested using a likelihood ratio test (Lynch and Walsh, 1998):

(4) $LR = -2\left[(L(\hat{\Theta}_r \mid z) - L(\hat{\Theta} \mid z)\right]$

where $L(\hat{\Theta} \mid z)$ is the restricted maximum log-likelihood of the full model (assuming $\sigma_S^2 \neq \sigma_D^2$) and $L(\hat{\Theta}_r \mid z)$ is the corresponding log-likelihood of the reduced model (assuming $\sigma_S^2 = \sigma_D^2$). The LR obtained is compared with the χ^2 value with 1 degree of freedom and $\alpha = 0.05$.

The effect common to full-sibs (other than additive genetics) potentially includes environmental hapa effects (effects of separate rearing of the families until tagging), non-additive genetic effects (dominance and to some extent epistatic effects) and maternal effects on MP. For the data structure used in the current study, all of the above mentioned effects common to full-sibs are confounded and therefore fitted as a single random effect in the model.

To test the significance of the random additive genetic effects, Model 1 (full model) was re-run excluding the additive genetic effect (reduced model) and the restricted maximum likelihood values of the full and reduced models were compared. The LR obtained is compared with a χ^2 value assuming either 1 ($\sigma_S^2 = \sigma_D^2$) or 2 ($\sigma_S^2 \neq \sigma_D^2$) degrees of freedom and $\alpha = 0.05$. Likewise, significance of the effect common to full-sibs was estimated by comparing the full model 1 with a reduced model excluding the effect common to full-sibs, and the LR obtained compared with a χ^2 value with 1 degree of freedom and $\alpha = 0.05$.

For both models heritability was defined as: $h^2 = \frac{2\left(\sigma_S^2 + \sigma_D^2\right)}{\sigma_S^2 + \sigma_D^2 + \sigma_C^2 + \sigma_E^2}$

and the relative proportion of the effects common to full-sibs other than additive genetics as: $c^2 = \frac{\sigma_C^2}{\sigma_S^2 + \sigma_D^2 + \sigma_C^2 + \sigma_E^2}$.

The residual variance in Model 2 was restricted to one.

3. Results

3.1 Data description

On average 93 fish were recorded per hapa (range 11-115). Overall MP was 77%, and the distribution of MP for the 132 hapas is shown in Figure 2A. Contrary to expectations, 3% of the hapas had hybrids with MP close to zero (from 0 to 5% males), while in 40% of the hapas the fish were all-male hybrids.

Average MP of the families within each of the seven different stock combinations (cross) are shown in Table 2. Average MP varied from 31% in cross 4 (female *O. niloticus* from China stock mated with male *O. aureus* from Taiwan stock) to 100% in cross 2 (female *O. niloticus* from China stock mated with male *O. aureus* from Israel stock). Variation of MP among families within the same cross combination was smallest (SD=0, min=max=1) for cross 2 (all-male) and largest (SD=30%, range from 0 to 100%) for cross 5 (female *O. niloticus* from Israel stock mated with male *O. aureus* from China stock) (Table 2).

3.2 Fixed effects

Table 3 shows the least square means (LSM) of MP for the fixed effects of batch and cross obtained from Model 1 (assuming equal sire and dam variances). Batch 1 showed the highest MP (0.76 ± 0.04) and Batch 4 the lowest (0.64 ± 0.04) (Table 3). The batch effect on MP did not follow the trend expected since batches with low average water temperature showed high MP (Batch 1, 24°C, MP=0.76; Batch 2, 27°C, MP=0.70) and batches with high average water temperature low MP (Batch 3, 31°C, MP=0.65; Batch

4, 32°C, MP=0.64). Cross 1 had the highest estimated MP (0.90 ± 0.03) and cross 6 the lowest (0.50 ± 0.10). The crosses 1 and 7 had ~90% males, cross 3 had 75% males, while the crosses 4, 5 and 6 had around 50% or less males, i.e., very large differences in MP were observed among the different crosses. However, some of these differences may be explained by the large between-family variation and a limited number of families per cross.

LSM for MP of the fixed effects of batch and cross obtained for the other models shown in table 4 followed the same trend as described above. For all models the effects of batch and cross were statistically significant ($P < 0.001$ and $P < 0.05$, respectively).

3.2 Genetic Parameters

3.2.1 Additive genetic effects

Assuming $\sigma_S^2 \neq \sigma_D^2$, the sire component for MP was 110% higher than the dam component in Model 1 and 64% higher in Model 2 (Table 4). However, this difference observed in Model 1 was not statistically significant ($P>0.05$; Log-likelihood ratio test). Heritability estimates for MP on the observed (Model 1) and underlying liability scales (Model 2) are given in Table 4. On the observed scale (Model 1) there was a significant additive genetic sire and dam component for MP ($P < 0.05$; Log-likelihood ratio test) with heritability estimates of 0.38±0.07 (assuming $\sigma_S^2 = \sigma_D^2$) and 0.42±0.09 (assuming $\sigma_S^2 \neq \sigma_D^2$), while heritability estimates on the underlying scale (Model 2) were, as expected, higher (0.79±0.11 when $\sigma_S^2 = \sigma_D^2$ and 0.82 ± 0.13 when $\sigma_S^2 \neq \sigma_D^2$).

3.2.2 Effect common to full-sibs

The effect common to full-sibs as a proportion of the total phenotypic variance for MP was about one tenth of the heritability estimates for both Model 1 and 2 (Table 4), but still significantly different from zero ($P<0.05$; Log-likelihood ratio test Model 1). Lozano et al. (Unpublished results) reported a statistically significant effect common to full-sibs for MP of similar magnitude when analyzing six generations of Gift Nile tilapia.

4. Discussion

Given the dual sex determination system proposed by Bellamy (1936), all male offspring (XZ) would be expected from a cross between a homogametic Nile tilapia dam (XX) and a homogametic blue tilapia sire (ZZ). However, in this study large variation in MP among the hybrid families was observed (from 0 to 1), indicating that sex determination in the hybrids is not exclusively under the control of major genetic factors (i.e XX/XY and WZ/ZZ sex determination system). If we instead consider the theory of autosomal influence proposed by Hammerman and Avtalion (1979) and assume that the stocks used were pure, the cross between Nile tilapia dams (AAXX) and blue tilapia sires (aaYY) should also yield all male progeny (AaXY). However, if we assume the Nile tilapia dams and the blue tilapia sires were not necessarily of pure origin, some of the male proportions found in this study (0.00, 0.25, 0.38, 0.44, 0.50, 0.63, 0.75) may be explained if all possible combinations of autosomes (A,a) and major genetic factors (W, X, Y) proposed by Hammerman and Avtalion (1979) occur during the matings (Appendix 1). Likewise, assuming that half of the breeders of each species were pure and the other half possessed all possible combinations of the major gene factors (autosomal and genetic) we obtain the frequency distribution given in Figure 2B which is similar to that obtained in this study (Figure 2A). Thus some of the variation in MP obtained in this study could be due to mixed origin of the broodstock. Macaranas et al. (1986) found introgression of *O. mossambicus* in *O. niloticus* stocks farmed in the Philippines, suggesting introgression is present in tilapias since most of the different tilapia species can breed and produce viable offspring. In this study proper measures were taken to keep the Nile tilapia and blue tilapia breeders of the different stocks separate, but introgression may have occurred prior to their introduction to Vietnam. In

future experiments it is recommended to test the purity of the Nile and blue tilapia stocks using serum protein markers (Avtalion, 1982).

Natural sex reversion may occur in both Nile tilapia (Baroiller and D'Cotta, 2001; Bezault et al. 2007; Wessels and Hörstgen-Schwark, 2011) and blue tilapia (Mair et al., 1991b). When mating Nile tilapia dams with blue tilapia sires, the sex determination model proposed by Bellamy (1936) (one species XX/XY and the other WZ/ZZ) should yield hybrids with all male progeny even if naturally reversed Nile tilapia dams are used. If naturally reversed blue tilapia sires (△WZ) were mated with normal Nile dams (XX) MP of hybrids should be 0.50, and if the same sires (△WZ) were mated with sex reversed Nile dams (△XY) MP of hybrids should be 0.75. If there was a recessive sex-modifying gene (ff) interacting with the major sex determination factor where homozygous recessive fish develop as female (Mair et al.; 1991b), the mating of a homozygote Nile dam (XXff) with a heterozygous blue sire (ZZFf) would yield offspring with MP of 0.50 (XZFf:XZff) as well. Nevertheless none of these proposed theories can fully explain the wide range of MP obtained among hybrid families in this study, nor can they explain deviations from the expected MP of 0.50 in some families of pure Nile tilapia (Mair et al., 1991a; Tuan et al., 1999) or pure blue tilapia (Mair et al., 1991b), supporting the existence of a polygenic sex determination system that affects the phenotypic sex of crossbred individuals. Evidence of additive genetic variation in MP for tilapias reared at normal pond temperatures has been reported by Lester et al. (1989) and Lozano et al. (Unpublished results). No heritability estimate for MP is available for blue tilapia, but a comprehensive study of hormonally sex reversed, hybrid, gyno-genetic and triploid blue tilapias suggested that sex was determined by an autosomal multifactorial mechanism with an underlying mono-factorial mechanism of

female heterogamety (i.e WZ/ZZ sex determination system) (Mair et al., 1991b). The unexpected low proportion of males found in some families in this study (3% of the families with MP close to zero) may be explained by feminizing minor autosomal factors. This is supported by the presence of low proportion of males in a temperature treated (36°C) Nile tilapia line selected for low temperature sensitivity (Wessels and Hörstgen-Schwark, 2011), the presence of spontaneously reversed Nile tilapia dams (XY) (Scott et. al, 1989) identified through gynogenesis, and the presence of females in progeny of hormonally sex reversed blue tilapia dams (ZZ) mated to normal sires (ZZ) (Mair et al., 1991b).

The sire origin by dam origin interaction (labeled cross) effect was significant in this study, indicating that choice of sire and dam origin combination affects MP in the hybrids. Since two of the nine possible stock origin combinations were not produced, estimates of the MP of the three different origins of the Nile tilapia dams and the blue tilapia sires in this study could not be obtained. In addition the number of sires and dams tested for three of the seven strain combinations was low, making MP estimates of these crosses unreliable. Significant strain additive genetic, heterosis and reciprocal effects for MP have been reported for the GIFT Nile tilapia (Lozano et al., 2011). Furthermore differences in MP between hybrids (Nile tilapia dams x blue tilapia sires) of different parental strains have been reported, with the Ghana-74 (Nile) x Mehadrin (blue) strain combination having the highest MP (0.99) (Hulata et al., 1993). The Ghana-74 Nile strain was introduced to Israel in 1974 and has been used commercially for hybridization, and the Mehadrin blue tilapia strain was kept in an isolated irrigated reservoir in Tel Aviv since 1970 (Hulata et al., 1993). In this study the Israel stocks of both Nile and blue yielded the highest MP (e.g. cross 1) and may have possibly

undergone selection for increased MP prior to their introduction, since hybridization of these two species is a common practice in Israel.

Estimated heritability for MP in this study is to be looked upon as an estimate for the samples of Nile dam and blue sire combinations tested, implying that a different sample of Nile dams and blue sires from these stocks may give a heritability estimate of different magnitude. However, the heritability estimates for the hybrids in this study indicate that MP is partly under polygenic control. The estimates are higher than those reported for pure Nile tilapia both on the observed (h^2=0.12) (Lozano et. al, Unpublished results) and underlying liability scales (h^2=0.22-0.26) (Lester et al., 1989; Lozano et. al, Unpublished results). In this and the latter mentioned studies only the phenotypic sex of the parents was known and can thus differ from their genetic sex. In that case the MP among their offspring may differ from the expected MP assuming a major sex determination system only (i.e. in this study differs from expected MP of 100% only when sex reversed blue sires are used). The estimated heritabilities for MP in this study may therefore be biased upwards.

The estimated sire variance was substantial higher than the dam variance (although not statistically significant), suggesting a stronger paternal than maternal effect. An indication of the same was reported by Mair et al., (1991b) who observed less variation in MP between groups of paternal half-sibs (0.47 and 0.59 when one blue tilapia sire ,“211”, was mated with two different Nile tilapia dams), as compared to the variation between groups of maternal half-sibs (0.34 , 0.64 and 1.0 when one Nile tilapia dam, “T17”, was mated with three different blue tilapia sires). However, the larger sire than

the dam variance in MP in the present study could be due to a sampling effect as the total number of Nile sires (35) was lower than the number of blue dams (82).

The estimated heritabilities may also be biased by sex dependent mortality, however only if this sex dependent mortality varies among the families. No evidence of sex differential mortality has been reported for tilapias (Tuan et al., 1999). Hickling (1960) did not find evidence of sex differential mortality in hybrids between *O. mossambicus* and *O. urolepis hornorum*. Baras et al. (2000) did not find different mortalities among groups of temperature treated *O. aureus* which showed different MP, suggesting no sex differential mortality from fry (~10dpf) until 38-55dpf. This is supported by a more recent study in Nile tilapia where survival rates from swim up fry (9 dpf) until sexing of two divergent lines (high and low temperature sensitivity during sex differentiation) under two temperature treatments (28°C and 34-36°C) did not differ, suggesting no sex differential mortality (Wessels and Hörstgen-Schwark, 2007, 2011; Wessels et. al., 2011). Similarly, survival from fertilization until 9 dpf in Nile tilapia was not significantly between the control (28°C) and temperature treated group (36°C) and thus there is no evidence of sex differential mortality during this period either (Wessels et al, 2011).

Wessels and Hörstgen-Schwark (2011) obtained MP of 0.93 in the line selected for increased male proportion after three generations when testing was performed at 36°C. In locations where temperature does not drop below 22°C, it may therefore be more suitable to use Nile tilapia and perform selection for temperature sensitivity to produce all male populations. A MP of 93% is still below the commercially desired MP (>0.95),

but may be increased to desired levels through selection over a few generations. However, in places where cold winters affect production, hybrids between Nile tilapia dams and more cold tolerant blue tilapia sires may be a good way to increase MP if pure lines can be successfully maintained. Selection for specific combining ability among blue tilapia sires mated to Nile tilapia dams using reciprocal recurrent selection (RRS) may be considered as an alternative in this case (Falconer and Mackay, 1996), but implies testing of a high number of sire and dam combinations. Although experiences from practical applications of this method in poultry and plant breeding are conflicting (Falconer and Mackay, 1996), it should be investigated since selection for MP within species reared under normal temperatures would be rather complicated as discussed by Lozano et al. (Unpublished results). However, if selection for increased MP in hybrids also results in increased MP in purebreds, it may be difficult to apply RRS because reproduction of pure populations counteracts selection (natural selection for equal sex contributions). Thus, the relationship between MP in pure lines and crossbreds should also be investigated. Therefore application of RRS for increasing MP in hybrids (Nile tilapia dams mated with blue tilapia sires) requires a thorough study.

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Tables and Figures

Table 1. Egg collection date, number of hapas stocked and classification of batch.

Egg collection date	# hapas stocked	Batch
02.04.2007	10	1
13.04.2007	2	1
16.04.2007	7	1
24.04.2007	5	1
25.04.2007	10	1
05.05.2007	19	2
12.05.2007	14	2
21.05.2007	23	3
25.05.2007	1	3
28.05.2007	9	3
09.06.2007	8	4
20.06.2007	18	4
30.06.2007	3	4
10.07.2007	1	4
21.07.2007	2	4
Total	132	

Table 2. Stock origin and number of sires, dams and hapas per stock combination, and average and standard deviation of the mean male proportion of the full-sib families within each stock combination.

Cross	Stock Origin	Number of			Male proportion of families				
	(♀= O. niloticus , ♂= O.aureus)	dams	sires	hapas	families	average	SD	min	max
1	♀ Israel x ♂ Israel	39	13	62	40	0.90	0.16	0.4	1.0
2	♀ China x ♂ Israel	2	1	2	2	1.00	0.00	1.0	1.0
3	♀ Taiwan x ♂ Israel	16	10	23	16	0.81	0.21	0.5	1.0
4	♀ China x ♂ Taiwan	2	2	2	2	0.31	0.07	0.3	0.4
5	♀ Israel x ♂ China	10	4	20	10	0.42	0.30	0.0	1.0
6	♀ China x ♂ China	7	2	12	7	0.54	0.08	0.5	0.7
7	♀ Taiwan x ♂ China	6	3	11	6	0.90	0.20	0.5	1.0

Table 3. Male proportion (MP) Least Square Means (LSM ± SE) for the fixed effects of batch and cross (stock combination) from Model 1.

Fixed Effect	N	MP ± SE
Batch		
1	3144	0.72 ± 0.03
2	3162	0.67 ± 0.03
3	3156	0.60 ± 0.03
4	2786	0.61 ± 0.03
Cross		
1	5764	0.90 ± 0.03
3	2075	0.75 ± 0.05
4	115	0.36 ± 0.14
5	1960	0.51 ± 0.07
6	1270	0.50 ± 0.10
7	1064	0.88 ± 0.09

Variances of sire and dam are assumed to be equal

Table 4. Estimates of sire and dam genetic variances, variance common to full-sibs, heritability (h^2±se) and effect common to full-sibs (c^2±se) for male proportion in the hybrids between Nile tilapia females and Blue tilapia males (Models 1 and 2).

	Model 1		**Model 2**	
Parameter	$\sigma^2_{sire} = \sigma^2_{dam}$	$\sigma^2_{sire} \neq \sigma^2_{dam}$	$\sigma^2_{sire} = \sigma^2_{dam}$	$\sigma^2_{sire} \neq \sigma^2_{dam}$
σ^2sire	0.014	0.021	0.371	0.496
σ^2dam	0.014	0.010	0.371	0.302
σ^2_c	0.005	0.006	0.149	0.155
σ^2_e	0.114	0.114	1.0	1.0
h^2 ± se	0.38 ± 0.07	0.42 ± 0.09	0.79 ± 0.11	0.82 ± 0.13
c^2 ± se	0.04 ± 0.01	0.04 ± 0.01	0.08 ± 0.02	0.08 ± 0.02

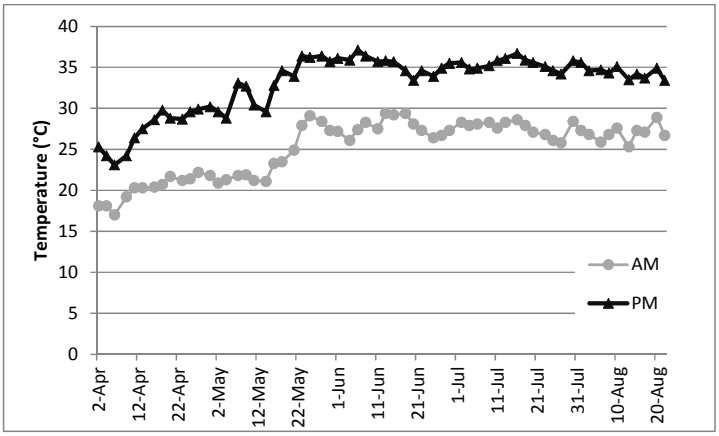

Figure 1. Water temperature (AM recorded at 07:00, PM recorded at 14:00) in the pond where hybrids were reared until sexing.

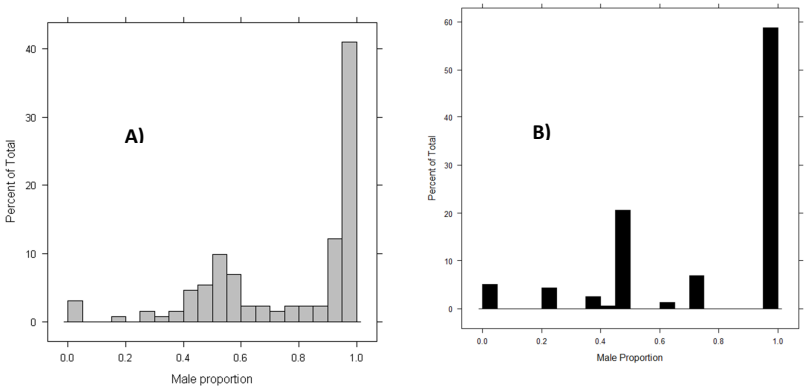

Figure 2. (A) Frequency distribution of male proportion of the hybrids in the 132 hapas, (B) theoretical frequency distribution if we assume half of the sires and damswere pure and half could produce all possible combinations using the autosomal influence theory proposed by Hammerman and Avtalion (1979)(Appendix 1).

Appendixes

Appendix 1. Predicted male proportion of crosses between males and females with different complements of autosomes (A,a) and gonosomes (W, X, Y) (from Avtalion, 1982).

	Males							
Females	AAYY	AaYY	AAWY	AAXY	aaYY	AaWY	AAWW	AaXY
AAWX	1.00	1.00	0.75	0.50	1.00	0.63	0.50	0.50
aaWY	1.00	0.75	0.75	0.75	0.50	0.50	0.50	0.50
AaWW	1.00	0.75	0.75	0.50	0.50	0.50	0.50	0.38
aaXY	1.00	0.75	0.75	0.75	0.50	0.50	0.50	0.50
AAXX	1.00	1.00	0.50	0.50	1.00	0.50	0.00	0.50
AaWX	1.00	0.75	0.63	0.50	0.50	0.44	0.25	0.38
aaWW	1.00	0.50	0.50	0.50	0.00	0.25	0.00	0.25
AaXX	1.00	0.75	0.50	0.50	0.50	0.38	0.00	0.38
aaWX	1.00	0.50	0.50	0.50	0.00	0.25	0.00	0.25
aaXX	1.00	0.50	0.50	0.50	0.00	0.25	0.00	0.25