Sex Determination in Marine Flatfish

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Introduction

All-female production of *Scophthalmus maximus* (turbot) and *Hippoglossus hippoglossus* (Atlantic halibut) could benefit the industry through avoidance of the growth-depressing effects of male maturation.

Treatment with sex hormones would achieve this, but any direct use of hormones on fish destined for consumption would be unacceptable and so indirect methods, such as those used for salmonids, need to be developed.

This approach requires knowledge of the sex-determining mechanisms, which have yet to be identified in these species.

The primary aim of this study was therefore to develop protocols for:

(a) the induction of meiotic diploid gynogenesis and

(b) sex inversion (masculinisation),

as tools for the investigation of the sex determining systems.

Methods

Gynogenesis

Diploid gynogenetic fish inherit chromosomal material solely from the mother and show increased homozygosity; the sex ratio within groups of gynogens can provide preliminary evidence of the genetic system of sex determination.

Meiotic diploid gynogenesis can be achieved by activating the eggs and then preventing loss of the second polar body by providing a sudden temperature or pressure change (shock). The embryos are then allowed to develop normally. If the polar body is not retained the embryos will be haploid.

A straightforward means of activating eggs is with heterologous spermatozoa of a suitable size to penetrate the micropyle. This approach is only useful if the embryo does not incorporate any of the DNA from the sperm.

The risk of genetic material from the sperm becoming incorporated can be reduced by first irradiating milt with ultraviolet light (UV-C) to denature the DNA. This treatment is usually adequate to allow use of conspecific sperm, but irradiation of heterologous sperm minimises the chances that any pieces paternal DNA remaining intact after the UV treatment would be included in the genome. Both techniques have been used in this study.

Sex Inversion

Changing the phenotypic sex of gynogenetic fish by hormone treatment enables the development of breeding programmes to further clarify the effect of genetic factors on sex determination.

Provision of the correct dose of sex steroid hormone in the diet or rearing water, at the time the gonad is differentiating, can induce sex inversion. Following the practice used to masculinise genetic female rainbow trout to produce functional, phenotypic male fish (neo-males), the effectiveness of various doses of 17α-methyl testosterone in the diet for up to 12 weeks from first feeding was assessed.

Phenotypic sex was checked from the morphology and histology of the gonads after 9 to 15 months.

References

Benfey *et al* 2000, Production of all-female populations of fish for aquaculture. *Bulletin of the Aquaculture Association of Canada* 100, 13-15

Hendry *et al* 2003, Hormonal sex reversal of Atlantic halibut (*Hippoglossus hippoglossus*, L.). *Aquaculture* 219, 769-781

Piferrer *et al* 2004, Induction of gynogenesis in the turbot (*Scophthalmus maximus*): Effects of UV irradiation on sperm motility, the Hertwig effect and viability during the first 6 months of age. *Aquaculture* 238, 403-419.

Acknowledgements

The assistance of Mannin SeaFarms Ltd, Isle of Man, is gratefully acknowledged.

The work was funded by **Defra**.

Results - turbot

Gynogenesis

Meiotic diploid gynogenesis was successfully induced in turbot by:

(a) activating eggs with heterologous (halibut) sperm (not irradiated with UV)

(b) inducing diploidy by cold shock applied three minutes after activation.

The gynogenetic embryos produced (Photo A) were indistinguishable from normal embryos (Photo B). Embryos from eggs activated with halibut sperm, but without application of a cold shock displayed typical haploid features such as small eyes and a short thickened body (Photo C).

The effects of both the duration of the shock and its temperature on the yield of diploid gynogens were tested (Table 1). When shocked at 0.7°C for 60 minutes, three minutes after activation with halibut sperm at 14.5°C, the yield of diploid embryos was considerably higher (23%) than in all other treatments.

Large scale induction of diploid gynogenesis was accomplished on four occasions with batches of 20,000 to 250,000 eggs. The temperature of the cold shock used ranged from 0.4°C to 0.9°C and survival to hatching from 9 to 40%. It was apparent that high quality eggs were needed to be able to induce gynogenesis with good survival.

The outcome of this work led to the proposed protocol for inducing diploid meiotic gynogenesis given in Table 2.

Photo A: Diploid gynogenetic turbot

Photo B: Normal turbot

Photo C: Haploid turbot

Table 1: The effect of temperature and duration of a cold-shock applied to turbot eggs three minutes after activation on the yield of diploid embryos.

Sperm	Cold shock		
	Temperature (°C)	Duration (mins)	Yield of diploid embryos (%)
Halibut	0.0	5	0
Halibut	0.7	5	2
Halibut	0.0	60	4
Halibut	0.7	60	23
Halibut (control)	No cold shock		0
Turbot (control)	No cold shock		82

Table 2: Suggested conditions for inducing meiotic diploid gynogenesis.

Species	Activation	Cold shock			
		Initial temp. (°C)	Shock temp. (°C)	Time post-activation (mins)	Duration of shock (mins)
Turbot	Halibut sperm	14.0	0.5	3.0	60
Halibut	UV irradiated halibut or turbot sperm	6.0	-1.0	15.0	120

Species	Activation	Pressure shock			
		Temp. (°C)	Shock pressure ($kg.cm^{-2}$)	Time post-activation (mins)	Duration of shock (mins)
Halibut	UV irradiated halibut or turbot sperm	6.0	600	10.0	5.0

Masculinisation

A comparison of different levels of hormone (1 or 3 $mg.kg^{-1}$ diet) and duration of treatment (400 or 800 day °C) demonstrated 100% masculinisation of normal turbot could be achieved by feeding a 17 α-methyl-testosterone treated (1 $mg.kg^{-1}$) diet for 400 day °C from c. 800 day °C, post-hatch. This treatment was also effective for the gynogens (Table 3).

The groups of untreated and control gynogenetic turbot were found to contain 10-30% males (e.g. control diet treatment in Table 3).

Table 3: Hormone treatment of gynogenetic turbot juveniles.

Treatment with 17α methyl testosterone			No. of individuals		
Age at start (day °C)	Conc. in diet (mg/kg^{-1})	Duration (day °C)	Male	Female	% female
c. 800	1	400	110	0	0
c. 800	0 (Control)	400	34	84	71

Results - halibut

Gynogenesis

The heterologous sperm tested (turbot and cod, *Gadus morhua*) proved to be unsuitable as means of activating halibut eggs because the embryo possibly incorporated a genetic contribution from the sperm. Typical haploid features were not evident in eggs activated by turbot sperm (Photo D), the embryos being similar to normal halibut embryos (Photo E), although they failed to survive hatching. The viability of eggs activated by cod sperm was poor, all embryos aborting by day 6, before they had developed beyond the blastodisc stage (Figure 1). Using UV-irradiated halibut or turbot sperm did result in the production of embryos exhibiting haploid characteristics.

A protocol was defined which enabled activated eggs to be exposed to a cold shock with little loss of viability. Halibut eggs activated with unirradiated turbot sperm and exposed to a cold shock for 2 hours survived as well as unshocked controls, whereas those shocked for 3 hours showed lower survival (Figure 2). The genetic status of these embryos was not confirmed, but it is probable that the conditions used were suitable for retention of the second polar body. Survival of the halibut control groups to hatch was generally low, indicating relatively poor egg quality and this would have made successful induction of gynogenesis more difficult.

Preliminary work using pressure to induce diploidy in activated halibut eggs was also limited by egg quality. However, sufficient information based on these preliminary results was obtained to suggest effective protocols for the induction of diploid meiotic gynogenesis in halibut, by cold or pressure shock (Table 2).

Photo D: Halibut x turbot sperm

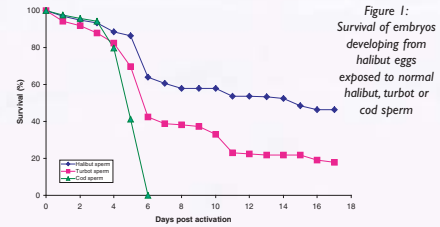

Figure 1: Survival of embryos developing from halibut eggs exposed to normal halibut, turbot or cod sperm

Photo E: Halibut x halibut sperm

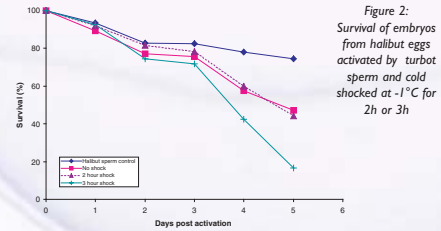

Figure 2: Survival of embryos from halibut eggs activated by turbot sperm and cold shocked at -1°C for 2h or 3h

Masculinisation

Initial work, feeding a 17 α-methyl-testosterone treated (1 $mg.kg^{-1}$) diet for 400 day °C from c. 1000 day °C, post-hatch, gave 96% masculinisation (Table 4). Starting the treatment later in development reduced this percentage. If feeding the treated diet were begun at an earlier stage in development it is likely 100% masculinisation could be achieved.

Table 4: Hormone treatment of normal halibut juveniles.

Treatment with 17α methyl testosterone			No. of individuals			
Age at start (day °C)	Conc. in diet (mg/kg^{-1})	Duration (day °C)	Male	Female	Intersex	% male
c. 1000	1	400	67	3	0	96
c. 1300	1	400	51	18	1	73
c. 1000	0 (Control)	400	35	35	0	50

Conclusions

Protocols for the induction of diploid gynogenesis and sex inversion (masculinisation) in turbot and halibut have been identified. These are in accord with those of Benfey *et al* (2000) and Hendry *et al* (2003) for pressure shock and for sex reversal in halibut respectively, and of Piferrer *et al* (2004) who used UV irradiated sperm and cold shock to induce diploid gynogenesis in turbot.

The sex-determining mechanisms in these species have not yet been determined. For turbot, the presence of up to 30 % males among gynogens, which is a relatively high proportion for groups of fish produced by meiotic gynogenesis, indicates that it may not be the simple XX (female) - XY (male) system. The results may be indicative of a WZ-ZZ system in turbot or one that is influenced by environmental factors (as found in Japanese flounder and seabass), although there is no other evidence of temperature affecting sex ratios in turbot. Further investigations of sex determination will be possible now that these methods of inducing gynogenesis and sex inversion have been developed.

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