Selection of rainbow trout resistant to viral haemorrhagic septicaemia virus and transmission of resistance by gynogenesis

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Summary — In 1984 a programme of selection for resistance to viral haemorrhagic septicaemia virus (VHSV) in rainbow trout was initiated. The progenies of 14 males were submitted to a VHSV waterborne challenge. The mortality ranged from 30 to 95% and the heritability of resistance was estimated to be 0.63 ± 0.26. One male consistently provided the most resistant offspring, and the second generation was produced from sires and dams selected among these families. The mean resistance improved and several females giving birth to resistant offspring were identified (0–10% mortality while the mortality in the controls was from 70 to 90%). The meiotic gynogenetic progeny of these females also demonstrated high resistance (mortality less than 10%). The role of superficial tissues in the resistance was confirmed and there was a striking difference in the growth of VHSV in fins excised and infected *in vitro*. The fins from resistant fish replicated the virus poorly as compared with the fins of susceptible fish.

rainbow trout / selection / viral haemorrhagic septicaemia / resistance / gynogenesis

Résumé — Sélection de truites arc-en-ciel résistant à la septicémie hémorragique virale et transmission de la résistance par gynogenèse. *Un programme de sélection de truites arc-en-ciel résistant à la septicémie hémorragique virale (SHV) a été entrepris en 1984. Les descendances de 14 mâles individuels croisés avec les mêmes femelles ont été soumises à l'infection expérimentale et les mortalités se sont réparties de 30 à 95%. L'héritabilité de la résistance a été estimée à 0,63 ± 0,26. Un mâle a constamment fourni les descendants les plus résistants, et la seconde génération de poissons a été produite à partir de croisements frères x sœurs issus de ces familles. Plusieurs femelles ont donné naissance à des familles résistantes (0 à 10% de mortalité* vs *70 à 90% de mortalité chez les témoins). Ces femelles reproduites par gynogenèse méiotique ont aussi donné des descendances hautement résistantes (mortalités inférieures à 10%). Le rôle des tissus superficiels dans la résistance a été confirmé et une différence importante enregistrée au niveau de la croissance du virus sur*

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des nageoires infectées in vitro *: la multiplication du virus a été beaucoup plus faible dans les nageoires des poissons résistants que dans celles des poissons sensibles.*

truite arc-en-ciel / sélection / septicémie hémorragique virale / résistance / gynogenèse

INTRODUCTION

Viral haemorrhagic septicaemia (VHS) type 1 virus (Egtved virus, Jensen, 1965) produces high losses in rainbow trout *(Oncorhynchus mykiss)* farmed in several European countries. More recently discovered serotypes (de Kinkelin and Leberre, 1977) also kill brown trout *(Salmo trutta),* while strains have been isolated in the wild which have no known pathogenic effect on any of the salmonid species tested (Winton *et al,* 1989). Vaccination against VHS is possible by injection of inactivated VHSV or by waterborne administration of attenuated strains of the virus (de Kinkelin, 1988), but both manufacturers and trout farmers are reluctant to launch such a vaccine. Some salmonid species are resistant to the known VHSV serotypes and can transmit their resistance to interspecific hybrids (Dorson and Chevassus, 1985; Dorson *et al,* 1991). This technique can be used by trout farmers. Intraspecific selection for fish resistant to various diseases has been attempted (reviewed by Price, 1985; Chevassus and Dorson, 1990; Fjalestad *et al,* 1993). Hopeful results concerning viral diseases of salmonids have been published for infectious haematopoietic necrosis (Amend and Nelson, 1977; McIntyre and Amend, 1978) and infectious pancreatic necrosis (Okamoto *et al,* 1993). Preliminary results concerning the selection of rainbow trout with increased resistance or susceptibility to VHS have also been released (Kaastrup *et al,* 1991; Slierendrecht *et al,* 1994). A French selection programme started in 1984 with the goal of obtaining resistant rainbow trout. These could be used for farming and also for revealing information about disease resistance mechanisms and genetic control of this resistance. Another important goal was to determine resistance criteria which would avoid extensive experimentation. This paper describes the first steps of the selection work and the search for resistance criteria.

MATERIALS AND METHODS

Brood stock

Rainbow trout males used for the first step of selection for resistance to VHSV were randomly sampled from an INRA (Institut National de la Recherche Agronomique, France) 'synthetic strain' (Sy) which had been produced by mating fish from different French and American origins in the early seventies and which is supposed to include a large amount of the species' genetic variation. This strain is maintained at the INRA trout farm of Gournay-sur-Aronde (Oise, France). This experimental farm is free of known viruses and permanently controlled. Sires from a 'golden' (phenotypic dominant trait) strain were used as sperm donors for gynogenesis. Females from the domanial fish farm of Neuville Sainte Gemme (Marne, France) were also used at the beginning. This farm is also under strict sanitary control.

Fertilization procedure

The ova were separated from the coelomic fluid and their mean weight was calculated from 100. When necessary, the same number of ova from different dams were used to form equivalent groups. Milts were controlled for motility under the microscope by adding 1 drop of buffered saline diluent (Billard, 1977). Sperm was UV-irradiated according to the procedure described by Chourrout (1982), for a minimum duration of 3.5 min. Inhibition of meiosis II was induced by the application of early (20 min after activation of the

ova) heat shock (26.5°C for 20 min) as defined in Cnourrout and Quillet (1982).

Rearing fish

The eggs were incubated in 10 x 10 cm incubators in a recirculated unit, thermoregulated at 10°C. The eyed eggs were disinfected by iodine and transferred to 10 l aquaria supplied with recirculated or with dechlorinated tap water always at 10°C. Survivors were kept in a recirculating unit with 1 x 1 m tanks (up to 6 years). Non-infected fish, which were kept as brood stock, were stocked in the trout farm and marked with PIT tags (Fisheagle, Lechlade, UK).

Virus production

VHSV strain 07-71 (serotype 1) isolated from diseased fish from a French trout farm was propagated in EPC cells as described by Dorson *et al* (1991). After infection, the cells were incubated at 15°C in Stoker's medium buffered at pH 7.4 with 0.16 M Tris-HCl and supplemented with 2% foetal calf serum and antibiotics (penicillin 100 IU/ml, streptomycin 0.1 mg/ml and kanamycin 0.1 mg/ml). The virus was harvested when the cytopathic effect was complete.

Challenge

The VHSV challenge was performed when the fish were 4 to 6 months old according to Dorson *et al* (1991). Briefly, each progeny was duplicated (usually 100 per group) and one group was mock-infected. The water supply of the aquaria was stopped and the infected fish were kept for 3 h in a 5 x 10^4 pfu/ml virus suspension under a vigorous aeration. The level of mortality was monitored for 1 month. When an unexpected mortality (more than 5%) was recorded in control group, the results were disregarded.

Virus growth in excised fins

Pelvic fins were sampled and processed immediately following the procedure described by Dorson and Torhy (1993): each fin was immersed 1 h in 2 ml of Stoker's medium containing 2 x 10^5 VHSV pfu/ml and rinsed 3 times. They were incubated for 3 d at 14°C in 6-well plates. The plates were then frozen at –80°C until virus titration. After thawing, the fins were ground with incubation medium, the debris eliminated by centrifugation and the virus titrated. Serial dilutions of the sample were inoculated onto EPC monolayers freshly prepared in 6-well plates (Nunc). After a 1 h adsorption period the cells were covered with 2 ml of Stokers medium containing 0.4% agarose. After 3 d at 14°C the cells were fixed by 10% formalin and stained with 1% crystal-violet, and the plaques were counted.

Statistical and genetic analyses

A nonparametric method was used to test the effect of the date of the challenge on mortality (median scores test on the 25 pairs of data, performed with the SAS system, Sprent, 1992). It was found to be non-significant, so challenges a and b were considered as true replicates. An arcsin transformation was applied to the mortality data prior to the variance analyses. ANOVA were performed with the SAS system. The model used was:

$$Y_{ijk} = \mu + S_i + T_j + S_i \bullet T_j + R_{ijk}$$

where Y_{ijk} = mortality of the kth progeny

μ = general mortality mean

S_i = fixed effect of ith male

T_j = fixed effect of jth year

$S_i \bullet T_j$ = interaction between male and year

R_{ijk} = residual effect (replicated challenges)

Multiple comparisons of males were performed using the Scheffé's method.

The heritability of the resistance was estimated from the observed mortality after the challenges from year 1984 (see below). The heritability estimates and its standard error were calculated from angular transformed percentages according to Bogyo and Becker (1965), using one-way ANOVA and a genetic model which assumed that the between-sires component of variance estimated the half-sib covariance (1/4 V_A) and the within-sires component, the remainder of the genetic variance plus all the environmental variance.

The virus titres were analysed by the NPAR1WAY procedure of the SAS system and the tests were chosen according to Sprent (1992).

RESULTS AND DISCUSSION

In January 1984, 14 two-year-old sires from the 'synthetic strain' (Sy) were individually tagged by fin and operculum clipping. Ova were collected from 6 three-year-old females of the Neuville strain. Approximately 50 ova from each dam were used to make up 14 batches of ova. Each batch was inseminated with the milt of a single male and the eggs were incubated and hatched under identical environmental conditions. At 4 months of age 2 x 35 fish from each group were distributed in duplicate aquaria. One of each pair of aquaria was infected immediately with VHSV and the other one served as a non-infected control. At the end of the course of infection the mortality ranged from 31 to 94% (table I, 1984a). During the same period no fish died in the non-infected aquaria. These fish were then infected and the mortalities after 1 month are given in table I (1984b). Half of the sires survived and could be used again in December 1984 for the fertilization of the ova of 6 other females from the Neuville strain. At the age of 4.5 months, groups of 100 fish were formed and infected. The mortality ranged from 42 to 96% (table I, 1985a). No mortality was observed in the non-infected controls and these fish were infected when they were 5.5 months old (1985b). Finally, the 4 remaining sires were used in December 1985 for the fertilization of 5 Neuville dams according to the same schedule as above.

Table I. Mortality (%) caused by a VHSV experimental challenge in families raised by the insemination of equivalent groups of ova from 6 dams of the Neuville strain with individual milts of sires sampled randomly in the 'synthetic strain' ($n = 35$ in 1984, 100 in 1985–1986).

Sire	*1984*		*1985*		*1986*		*Mean*	*Scheffé grouping*
	a	*b*	*a*	*b*	*a*	*b*		
1	91	97	96	100			96 ± 3.7	A
2	86	94					90 ± 5.7	AB
3	88	86					87 ± 1.4	ABC
13	94	80					87 ± 9.9	ABC
7	94	91	86	89	70	91	86.8 ± 8.6	ABC
5	83	80					81.5 ± 2.1	ABC
9	80	83					81.5 ± 2.1	ABC
12	77	83					80 ± 4.2	ABC
10	68	86	84	80	78	77	78.8 ± 6.3	ABCD
6	68	54	90	63			68.8 ± 15.3	ABCD
8	54	63	76	72			66.3 ± 9.8	BCD
4	51	54	80	61	53	44	57.2 ± 12.4	BCD
14	54	43					48.5 ± 7.8	CD
11	31	28	42	34	51	42	38 ± 8.6	D
M1	72.8	73	79.1	71.3	63	63.5		
M2	72.9		75.9		63.3			

a: First challenge for a given generation. b: Second challenge (1 month later) of the same generation. M1 = mean of the mortalities per challenge. M2 = mean of the 2 challenges within a year.

One hundred fish were challenged at the age of 4 months (table I, 1986a) and again no mortality was recorded for the controls which were then infected when they were 5.5 months old (1986b). This series of experiments permitted the ranking of the males according to the resistance status of their offspring from low (male 1) to high (male 11). A wide range in mortality was observed (28–100%). ANOVA revealed a highly significant male effect, together with a significant year effect. The resistance heritability was estimated to be 0.63 ± 0.26. The use of repeated testing showed that male Sy 11 transmitted particularly high resistance to its progeny (Scheffé's grouping restricted to the males tested over 3 successive years).

In 1984 and 1985 fish from the progeny of male No 11 were kept after VHSV challenge and used as spawners in 1989 and thereafter. Six individual males (designated A to F) were mated with 6 sib females (also designated A to F) or with Sy females and the progeny were tested and compared with Sy x Sy controls. The results are given in table II. Although only a limited number of parents could be tested, the mean mortality observed after the challenges was much lower: 39.6% in crosses involving one Sy parent and only 21% when both parents were selected *versus* 81.7% in the pure Sy controls. In one (sire F x dam F) of the male x female combinations leading to resistant offsprings, all the fish exhibited twisted jaws, narrowed caudal peduncles and poor growth. Dam B, which consistently gave birth to resistant progeny, was chosen for gynogenetic reproduction in 1992. The gynogenetic females obtained also showed good resistance to the challenge.

Table II. Mortality (%) following a VHSV infection in the 'synthetic strain' (pooled Sy sires x pooled Sy dams), in full-sib families from the second generation obtained in 1989 (a), 1991 (b) and 1992 (c) by mating dams and sires issued from sire 11, and in gynogens obtained from dam B (100 fish/group).

Dam	*Sire*							
	Sy	*A*	*B*	*C*	*D*	*E*	*F*	*Gynogen*
Sy	89 (a) 65 (b) 91 (c)	67 (a)	60 (a)		78 (a) 55 (b)	32 (a)	12 (a)	
A				13 (a)				
B	10 (a) 10 (b) 9 (c)			2 (a)	0 (a) 3 (b) 3 (c)			11 (c)
C							43 (a)	
D	30 (a)			16 (a)	2 (a)			
E		48 (a)						
F			59 (a)				5 (a)	

After 1992, no infected fish were kept after the challenges and all the fish destined to be used as brood stock were virus free. In 1989 the non-infected fish from the family which was totally resistant (sire D x dam B) were kept and used as brood stock for the third generation in 1992, 1993 and 1994. In 1992, 15 females and 13 males were randomly mated. A large variation in the resistance status was observed in the progeny (range of mortality: 1 to 82% *versus* 91% in Sy control). Unfortunately, these results could not be confirmed the following year due to the loss of almost all the families after a leakage of ethylene glycol from the cooling system to the recirculating unit water. Two females (D1, D2) and 2 males (S1, S2), however, were identified as transmitting resistance. They were used to produce 3 types of progeny (table III). When mated with Sy males, the females transmitted partial resistance (34 and 54% mortality, respectively). The gynogenetic progeny were more resistant (only 10 and 4%) but even better results were obtained in crosses with resistant males.

Table III. Mortality (%) following VHSV infection in groups of 100 trout from the indicated crosses or obtained by meiotic gynogenesis in 1993 (a) and 1994 (b).

Dam	*Sire*	*Mortality*
Sy	Sy	68 (a) 74 (b)
D1	S1	7 (a) 4 (b)
D1	Sy	34 (b)
D1 gynogens		10 (b)
D2	S2	3 (a) 2 (b)
D2	Sy	54 (b)
D2 gynogens		4 (b)

D1, D2, S1, S2 indicate individual dams and sires chosen from the 1993 experiment (a) and Sy indicates pools (from 5–10) of 'synthetic strain' dams and sires.

In 1992, the pelvic fins were sampled from the susceptible Sy control (91% mortality) and from the most resistant family (1% mortality) and infected *in vitro* with VHSV. Fifty fins of each group were processed. A wide range of titres was recorded in the susceptible group (table IV) and the mean of the virus titres was 4 400 pfu/mg. In the case of the resistant family, the highest titre recorded was 60 pfu/mg and the mean was only 9 pfu/mg. The analysis of the results by the ANOVA test concluded that the differences between the 2 groups were non-significant, probably due to the large variability in the Sy group. The Kolmogorov–Smirnov test, however, concluded a significant difference between the groups.

This work demonstrated evidence of a strong genetic background for resistance to VHS. Progeny from single individuals or pairs of parents showed substantial differences in tolerance to the disease. This result is in agreement with previous work in salmonids, which described genetic variation for resistance to VHS (Kaastrup *et al,* 1991), IHN (Amend and Nelson, 1977; McIntyre and Amend, 1978) or IPN (Okamoto *et al,*

Table IV. Mortality in 2 different families (100 fish/group) of rainbow trout infected *in vivo* with VHSV and virus titres (pfu/mg) obtained following *in vitro* infection of excised pelvic fins (50 from each group).

Family	*Mortality*	*Virus titre*	
		Mean	*Range*
Susceptible	91/100	4 400	1–150 000
Resistant	1/100	9.3	0.5–60

1993). More particularly, the preliminary data (table I) demonstrated the paternal influence on susceptibility to VHS in trout. This result is in agreement with the conclusions of Kaastrup *et al* (1991). The heritability estimate calculated here was much higher than those already published for viral diseases in fish and is very promising for future selection programmes. The subsequent data also indicated a female effect on the resistance of the offspring. Females were identified that transmitted resistance to their progeny whatever the mating system involved (crosses with susceptible or selected males, gynogenetic reproduction). It was unlikely that this could result from purely maternal effects including transfer of antiviral molecules *via* the yolk, because of the age of fish when challenged. The improved resistance of gynogenetic progeny is in favour of genetic factors. The level of resistance we obtained by gynogenesis was much higher than the values obtained by Slierendrecht *et al* (1994) but the first step of our selection (choice of male 11) may have been decisive. Nevertheless we have not yet established a resistant line, as has already been achieved for IPN (Okamoto *et al*, 1993).

Little is known about the genetic determinism of disease resistance in fish. An increasing number of studies in higher vertebrates describes the existence of major genes involved in the resistance to several kinds of diseases (Guenet and Montagutelli, 1990). It seems a reasonable hypothesis that similar mechanisms exist in fish (Wiegertjes *et al,* 1993). The general trend of our results seemed consistent with a model relying on the 'dominance' of resistance over susceptibility. Further use of meiotic and mitotic gynogenesis will help clarify this determinism and obtain resistant lines. The correlation obtained here between susceptibility and mean virus growth in excised fins confirmed previous results (Dorson and Torhy, 1993) but was not as complete as was expected. A number of fish from the susceptible group (which comprised several resistant individuals) displayed *in vitro* VHSV growth similar to those in the resistant group. Further work is needed, including individual testing both *in vivo* and *in vitro* of numerous fish, before this test will be capable of predicting the resistance status of a given fish or group of fish.

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