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Heritability Estimation of Haematological Traits in Clonal Lines of Ayu, *Plecoglossus altivelis*, under Stressed and Non-Stressed Conditions

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스트레스와 비스트레스 조건에서 Clone 은어의 혈액성상에 대한 유전율 추정

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ABSTRACT: Four clonal lines of ayu, *Plecoglossus altivelis*, were produced through gynogenesis, mixed before hatching and reared communally. After 10 months, a randomly taken sample was subjected to a standardized shallow water stressor. Hematocrit, hemoglobin, red blood cells count (RBC) and mean corpuscular volume (MCV) were obtained from stressed and non-stressed fish. DNA fingerprinting was used to confirm the clonal nature of the organisms and to identified the clonal line to which each fish belonged. I observed significant differences between clonal lines mostly in the hematocrit and MCV measured under no-stress conditions. Such differences are suggested to represent mainly genetic variance, on account of the common environment provided to all the experimental groups. The stress response ratio was lower than expected, mainly due to some unexpectedly high non-stress values. Heritability values (h^2) were medium to high for the no-stress measurements (mean 0.238) and very low or zero for the stressed groups' traits (excepting one high value of 0.484). I conclude that the use of communally reared clonal lines represents a good tool for the characterization of the physiological traits, thus allowing for their utilization as genetic selection criteria.

Key words: Ayu, Clones, Heritability, Haematological trait.

요 약: 본 연구는 은어의 clone을 생산하여 생리적 형질에 대한 유전적 특성을 조사하고 또 clone의 유전율을 추정했다. 성숙한 완전 동형접합형 자성발생 2배체(난할형 2배체)에서 채란한 후 자성발생을 반복하여 4계통의 clone을 생산하였으며 부화 전부터 같은 수조에 혼합하여 사육하였다. 10개월간 사육한 후 사육수조의 수심을 낮추었을 때의 스트레스가 clone의 혈액성상과 유전율에 미치는 영향에 대해서 조사했다. 생산된 각 계통의 clone은 실험에 사용하기 전에 DNA fingerprint법을 이용하여 유전적 균질성을 확인한 후 스트레스어와 비스트레스어의 hematocrit 값, hemoglobin량, 적혈구수 및 평균적혈구용적 (MCV)을 측정하였다. 비스트레스어에서 측정한 hematocrit값과 MCV에서 각 clone간에 유의차가 인정되었다. 실험어는 모두 같은 환경에서 사육하였기 때문에 이러한 유의차는 주로 각 clone의 유전적인 차이를 반영한 것으로 생각한다. 스트레스 반응률 (SRR)은 기대값보다 낮았으며 유전율 (h^2)은 비스트레스 group에서는 평균 0.238로 높았으나 스트레스 group에서는 매우 낮거나 거의 0에 가까웠다. 따라서 혼합 사육한 clone 계통은 생리적 형질의 유전적 특성을 조사하는데 좋은 수단이 된다. 또한 선발 육종의 선발형질로서도 유용하다.

INTRODUCTION

Selective breeding has traditionally been based on the manipulation of characteristics such as growth rate and reproductive performance (Hershberger et al., 1990 ; Gjedrem, 1992), while only recently, attention has become focused on the manipulation of the components of the stress response in a similar fashion (Pickering, 1992; 1993; Fevolden et al., 1993).

Schreck (1981) proposed that the fundamental performance capacity of a fish is limited by its genotype and that the environment further restrains it, creating a realized performance capacity. Therefore, grasping that fundamental or genotypically determined performance capacity may constitute a short cut for the identification of the heritable physiological traits that could eventually be included in genetic stock improvement programmes. Nevertheless, the large amounts of environmental

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variation usually involved seems to be one of the main obstacles to this kind of approach.

Taniguchi et al. (1993) observed that the phenotypic variance within a clonal line consists entirely of environmental variance ($V_p = V_e$) which is composed of different elements depending on the rearing and experimental conditions. In this study, the environmental variation due to tank effects was eliminated, thus minimizing the total V_e that could otherwise exceed and mask the genetic variance that is expected to be found between pure lines :

$$V_p = V_g + V_{ei} + V_{et} \quad (1)$$

where V_p and V_g are the phenotypic and genetic variances for each clonal line, respectively. V_{ei} is the environmental variance inherent to every individuals and V_{et} is the variance due to tanks effect. Heritability values were estimated in order to assess the haematological trait' potential response to selection .

Thus, the present study focuses on the genetic characterization of the physiological traits, and the differentiation of the stress response between several lines of clones of ayu, *Plecoglossus altivelis*.

MATERIALS AND METHODS

1. Fish Samples

The four clonal lines of ayu used in this study were produced by chromosome manipulation as described by Taniguchi et al. (1994) : mitotic gynogenetic ayu were obtained by hyperpressure-shocking eggs 80 min after fertilization at an incubation temperature of 19℃ (Taniguchi et al., 1988). Eggs from these fish were given a cold shock (0.4~1.0℃) 5 min after insemination, to cause the retention of the second polar body (Taniguchi et al., 1986), thus obtaining fully homozygous clones (Han et al., 1991).

Three of the lines that I used belonged to a first generation of clones that were obtained from several unrelated mitotic gynogenetic female fish, and the other one came from a fifth generation of clones derived from normal crosses between female clones and hormonally sex-reversed fish of the same family (Tsujimura et al., 1991).

Fertilized eggs of each of the four lines were mixed before hatching and placed in a 700 *l* incubation tank (water temperature between 16℃ and 17℃). Thenceforth, the larvae and juveniles were reared in successively bigger, common tanks.

After approximately 10 months, a sample was randomly taken and examined. The mean body weight was 27.82±9.88 g and the mean fork length was 13.86±1.80 cm.

2. Acclimation Period

The fish were randomly placed in four 500 *l* tanks at a density of approximately 2.8 g/ *l* and treated for 24 h with 0.5% of commercial salt. Starting 48 h later, a ration of 3 %/day of body weight was given to the fish for the following 2 weeks. The water temperature fluctuated from 18℃ to 19℃.

3. Stressor Application

Two of the four acclimation tanks containing the fish were randomly chosen for the stressor application and the other two were used as non-stressed controls. Feeding was suspended 24 h prior to the experiments.

The shallow water stressor methodology was adapted from Fevolden et al. (1991) and Thomas & Robertson (1991). In short, the volume of water in the experimental tank was dropped to 10 % (from 450 *l* to 45 *l*) within 5 to 10 min. Starting 15 min later, subsamples of eight fish were netted and placed in a bucket with abundant aeration and 0.2 ml/l of anaesthetic (2-phenoxyethanol), prior to taking the blood sample. It usually took 12 min to bleed eight fish, therefore, the approximate time that a fish was kept in the experimental tank varied between 15 and 90 min.

For the non-stressed controls, the anaesthetic was poured into the experimental tank without distributing the fish, carefully netting them once they had lost equilibrium.

4. Blood Samples Analysis

1) Haematology

The maximum possible volume of blood (usually 0.5ml) was withdrawn from the caudal vein of the fish, using heparinized syringes. Immediately, 0.02ml were used to determine hematocrit, red blood cells count (RBC), hemoglobin and mean corpuscular volume (MCV) using a haematology analyser

(Nihon Koden; MEK-5105). At the same time, 1~3 heparinized capillary tubes were filled, sealed at one end and centrifuged for 10 min at 1,196.8 g. The plasma obtained after cracking the tubes was stored at −20℃. Glucose concentration was determined within approximately 1 month using a glucose C Ⅱ test, Wako. Due to technical limitations, plasma cortisol could not be evaluated here.

2) DNA Extraction and Digestion

DNA was extracted from approximately 0.2 ml of recently withdrawn whole blood (using a DNA extractor WB kit-Wako) and stored at 4℃. Thereafter, the methodology of Mannen & Tsuji (1993), adapted by Takagi & Taniguchi (1994), was followed. In short, DNA was purified, precipitated and washed, before being digested with *Hae* Ⅲ restriction endonuclease ; it was then fractioned by electrophoresis [1.2% agarose gel in TAE (Tris-acetic acid-EDTA) buffer] . The gel was denaturized and neutralized before blotting the DNA onto a nitrocellulose membrane and fixing it.

3) Probe Labelling and Hybridization

I used the YNZ-22 probe (Nakamura et al., 1987), and labelled it with [α-3 2P] -dCTP by random oligonucleotide priming using a BcaBESTTM Labelling Kit (Takara, Japan). After proper pre-hybridization of the membrane, hybridization was performed overnight at 42℃. The membrane was then washed and autoradiographed at −70℃ for 16~24 h with intensifying screen.

5. Clonal Lines Identification

The autoradiographs of 200 fish (8 plates) were compared, identifying four different band patterns that were assigned to each of the four clonal lines. Thus, referring to the fish experimental number, the haematological and morphological data were regrouped into the four clonal lines.

6. Statistical Analyses

Analyses of variance (ANOVAs) were used to test for a possible (acclimation) tank effect and to compare each trait between clonal lines, for both no-stress and stress conditions; unpaired Student's t-test was used to compare no-stress with stress values, for each trait and clonal line.

The heritability h^2 (proportion of the genetic variance in relation to the total variance), was estimated adapting a model for monozygous human twins (Becker, 1964 ; Falconer, 1981), as follows :

$$h^2 = \frac{\sigma_s^2}{\sigma_s^2 + \sigma_w^2} \quad (2)$$

where σ_s^2 and σ_w^2 are the variances between and within groups respectively. σ_s^2 is calculated as follows :

$$\sigma_s^2 = (MS_s - MS_w)/k \quad (3)$$

where MS_s and MS_w are the mean square values between and within groups given by a one-factor ANOVA, and k represents the sample size. σ_w^2 is given by the mean square value within groups (MS_w) of the ANOVA table.

RESULTS

The ANOVA comparison of morphological and physiological traits between tanks showed no significant differences, discarding a potential tank effect during the acclimation period.

Table 1 shows the non-stress mean and standard deviation values for each trait and group. The comparison between clonal lines showed significant differences in all the traits excepting glucose, mainly due to the significantly lower hematocrit, MCV and hemoglobin values of the clone C group ; hemoglobin was significantly lower in the clone A group as well. RBC values were quite similar in all groups, excepting clone D, which showed a significantly higher value.

The heritability estimates for the no-stress condition (Table 1) showed a rather high value for hematocrit (0.421) and MCV (0.408) as compared with those obtained by Del Valle et al. (1995) for morphological traits. Hemoglobin and RBC values were 0.290 and 0.127, respectively, and glucose showed a very low value of 0.025.

Table 2 shows the results for the stressed fish groups. The differences between clonal lines for glucose, hemoglobin and RBC were non-significant. The clone D group had significantly

Table 1. Mean±SD and heritability (h^2) of the haematological traits of non-stressed groups (sample size in parentheses)

Traits	Clone A[1]	Clone B	Clone C	Clone D	h^2
Hematocrit(%)	32.95± 4.0[c] (6)	32.50± 2.4[c] (15)	26.98± 5.4 (18)	33.82± 2.8[c] (19)	0.421±0.23
Glucose(mg dl^{-1})	69.49± 7.1 (7)	78.25±11.6 (18)	72.93±13.8 (8)	78.46±12.9 (12)	0.025±0.06
Mean corpuscular volume(μm^3)	90.33± 2.7[c] (6)	92.0 ± 3.6[c] (14)	84.63± 5.8 (16)	91.37± 3.3[c] (19)	0.408±0.02
Hemoglobin(gr dl^{-1})	15.72± 2.8[c,d] (6)	18.99± 1.9[d] (14)	15.93± 3.1[d] (16)	18.92± 2.3 (19)	0.290±0.21
Red blood cells($\times 10^4 \mu l^{-1}$)	317.3±69.9 (6)	344.8±32.6 (14)	313.4±61.4[d] (16)	358.3±38.2 (19)	0.127±0.04

[1]Superscript indicates difference between the mean value and the one belonging to the numbered clonal line(ANOVA: $P \leq 0.05$)

Table 2. Mean±SD and heritability (h^2) of the haematological traits of stressed groups (sample size in parentheses)

Traits	Clone A[1]	Clone B	Clone C	Clone D	h^2
Hematocrit(%)	35.29± 4.7 (6)	32.54± 6.5[d] (26)	31.94± 5.2[d] (13)	36.75± 5.2 (12)	0.072±0.11
Glucose(mg dl^{-1})	84.55± 8.5 (6)	88.42± 5.9 (13)	81.69±13.9 (11)	84.67±12.5 (18)	0
Mean corpuscular volume(μm^3)	93.80± 4.6[d] (5)	93.9 ± 6.3[c,d] (20)	88.33± 7.9[d] (12)	104.22± 4.5 (9)	0.484±0.024
Hemoglobin(gr dl^{-1})	16.34± 1.8 (5)	17.0 ± 2.6 (25)	16.93± 1.5 (13)	15.78± 1.6 (10)	0
Red blood cells($\times 10^4 \mu l^{-1}$)	323.8±20.2 (5)	331.6 ±79.8 (20)	357.5±84.7 (12)	325.1±68.9 (9)	0

[1]Superscript indicates difference between the mean value and the one belonging to the numbered clonal line(ANOVA: $P \leq 0.05$)

higher hematocrit and MCV values. Heritability was very low for hematocrit (0.072) and zero for glucose, hemoglobin and RBC. MCV was exception, with a high estimate of 0.484.

An overall comparison of the non-stressed and stressed groups (Table 3) showed that hematocrit, glucose and MCV were the traits that could better characterize the stress response.

The stress ratio (SRR) of each clonal line was obtained by dividing the mean value of the stressed group by the mean value of the non-stressed one (for each trait). It was expected to be bigger than 1.0 in all cases, because of the predicted stress induced increment of the traits under study.

Fig. 1 shows that most of the SRR values (80%) were above unity, although statistical differences were only found for glucose in clone A and B, hematocrit in clone C and MCV in clone D.

Table 3. Mean±SD of all the clonal fish groups haematological traits

Traits	Non-stressed groups	Stressed groups	*t*-test
Haematocrit (%)	31.23± 4.9	34.17± 6.0**	
Glucose (mg dl^{-1})	75.66±11.8	84.42±10.9**	
Mean corpuscular volume(μm^3)	88.74± 5.5	94.04± 7.8**	
Haemoglobin (gr dl^{-1})	17.71± 2.8	17.01± 2.5	P = 0.091
Red blood cells($\times 10^4 \mu l^{-1}$)	340.5±59	340.4±75	P = 0.99

** $P \leq 0.01$

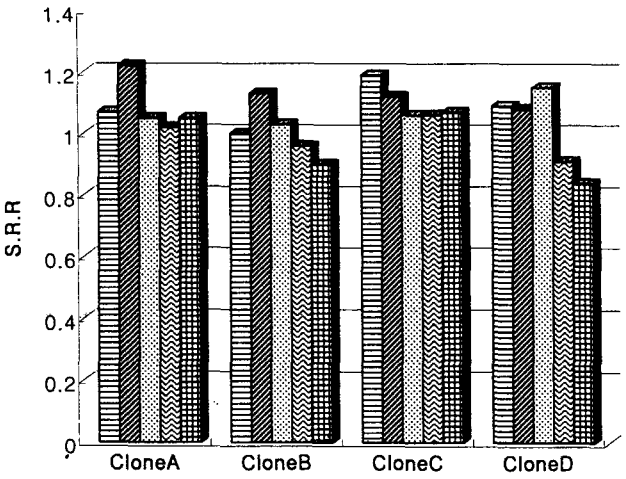

Fig. 1. Stress response ratio (SRR) of the clonal lines under study. From left to right, columns denote haematocrit, glucose, mean corpuscular volume, red blood cells count and hemoglobin.

The DNA fingerprinting identification of the clonal line to which each fish pertained was possible in about 60 % of the cases, probably due to a deficient DNA extraction.

DISCUSSION

In this study, the physiological traits of clonal fish that had been communally reared are studied for the first time. The high genotypic heritability estimates reported for hematocrit, mean corpuscular volume, hemoglobin concentration and red blood cell counts for the no-stress condition, may be said to represent the genetic variation between groups adequately. These results also indicate a markedly reduced variation within groups, which can be assumed to be purely 'environmental', because of a presumably small measurement error and negligible maternal effects (short maturation time and non-pregnancy period).

The results presented here, together with previously reported observations (Del Valle et al., 1994) on the genetic variation index (GVI) of hematocrit and mean corpuscular hemoglobin concentration of separately reared *P. altivelis* heteroclones, indicate that a good selection response can be expected from these physiological traits.

Similarly, Pottinger et al. (1994), who worked with a second generation of rainbow trout, *Oncorhynchus mykiss,* that had been selected for high and low responsiveness to stress, concluded that "manipulation of components of the endocrine system of fish appears to be possible by selectively breeding from fish displaying the desired trait(s)".

Fevolden et al. (1993) reported a mean heritability estimated for rainbow trout cortisol concentration of 0.27 and a very low one for glucose (0.07), which is in the same range as the value reported here (0.025). Nevertheless, plasma glucose increment is still recommended as an easily measurable indicator of the stress response (Table 3 & Fig. 1).

On the other hand, the very low heritability estimates of physiological traits of fish under stress (observed here and reported by Fevolden et al. 1993 for Atlantic salmon, *Salmo salar,* as well), may indicate an increment of the environmental variance caused by the individual degree of response to the stressor, and do not necessarily imply that selection for the trait in question would be ineffective.

Regarding the stress response ratio of the groups studied, we consider that further tests using communally reared clonal lines would permit the appropriate evaluation of the stress response as a quantitative trait. The characterization of the indicators with higher heritabilities, the detection of the best lines through selection, and the fixation of the desired traits, should be priority objectives for further studies.

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