Reduced Variation of Physiological Traits in Ayu Clones, *Plecoglossus altivelis*[†]

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Two types of clones of the ayu *Plecoglossus altivelis* heterozygous and homozygous clones, were produced by crossing second generation gynogenetic diploids with sex reversed clones of a different or the same line, respectively. Samples of these fish, together with normal control diploids, were used in this study to monitor the expected reduction of the genetic variance in the clonal lines. The morphological traits measured (body weight, fork length and gonad weight) showed a significant reduction of the coefficient of variation (C.V.) of 44.8% on average (heterozygous clones 56.17%, homozygous clones 33.4%). Furthermore, from all the physiological traits evaluated, hematocrit, plasma glucose, and mean corpuscular hemoglobin concentration (MCHC), also showed a significant variation reduction of 42.33% (average) for the heterozygous clones and 13.4% (average) for the homozygous clones. An estimation of the heritability of both kind of traits is proposed, based on a substantial elimination of the genetic variation in the clonal lines.

Key words: genetic variation, environmental variation, ayu, clones, physiological traits

It has been previously demonstrated¹⁾ that quantitative characters (*i.e.*, body weight and fork length) of gynogenetic fish show a significant variation increase compared to normal diploid fish. Taniguchi *et al.*²⁾ established that the variation is drastically reduced in clonal lines due to elimination of the part of the variance that is typically attributed to genetic causes, and suggested a method of estimating heritability by comparing the variance between clonal fish and a target group. These statements referred mainly to quantitative traits, but the possibility of applying them to qualitative traits (*i.e.*, physiological performance) has also been suggested,²⁾ making them useful traits for selective breeding.

This study focuses on physiological traits (*i.e.*, hematological parameters) that assess the effect of environmental stressors: hematocrit and hemoglobin which indicate anemia and/or hemodilution when low, and indicate hemoconcentration due to dehydration when high³⁾; leucocrit, which has proved useful in assessing the health of fish by estimating the white blood cells and/or thrombocyte (WBC-T) counts⁴⁾; plasma glucose which increases during asphyxia, probably mobilized from liver glycogen in readiness for use by the muscles⁵⁾ and mean corpuscular hemoglobin concentration (MCHC), which indicates the swelling of the erythrocytes.⁶⁾

This study tests the variance reduction of morphological or growth-related traits in clonal groups and attempts to extend the above considerations to those hematological parameters related to the health of the fish, foreseeing wide applications in the selective breeding of stress resistant strains.

Materials and Methods

Fish Samples

Gynogenetic fish were initially produced following the procedures of

Taniguchi *et al.*^{7–9)}; hand-stripped eggs of normal diploid ayu were inseminated using UV-irradiated sperm from *Spirinchus lanceolata* ("shishamo" in Japanese) as a marker for confirming the gynogenetic procedure.⁹⁾ The inseminated eggs were then subjected to a hydrostatic pressure shock of 650 kg/cm for 6 min, 80 min after fertilization, in order to suppress the first mitotic division and to produce fully homozygous diploid offspring.⁸⁾

Fig. 1. Schematic diagram for the production of homozygous and heterozygous clones.

* Produced at the Wakayama Prefecture Freshwater Fisheries Center.

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These mitotic gynogenetic fish were reared to maturity and used as broodstock for the production of clones following the procedures of Han *et al.*¹⁰⁾ Diploidization was attained using cold shock (0.4–1.0°C, for 30 min, 5 min after insemination) in order to cause the retention of the second polar body, thus obtaining clonal fish.

The fish used in this study belonged to a second generation of clonal ayu produced at the Wakayama Prefecture Freshwater Fisheries Center. The cross of (female) clones with hormonally sex reversed organisms of the same family produced homozygous offspring (autozygous at all loci) denominated homozygous clones (or inbred clones, referred to hereafter as homo-clones), while crossing a sex reversed clonal male with a mitotic gynogenetic fish (from a different family) produced a group of genetically identical fish (allozygous at all loci), named heterozygous clones (referred to hereafter as hetero-clones) (Fig. 1). The sex reversal method reported by Tsujimura *et al.*¹¹⁾ was used. In the present study, one group of homo-clones and two groups of hetero-clones (hetero-clone 1 and hetero-clone 2) were used. The clonal nature of these fish had been previously confirmed by DNA fingerprinting.¹²⁾ Normal diploid ayu (2*n*-normal) produced at Kochi Prefecture Freshwater Fisheries Center were used as control fish. This fish had no genetic relation with the experimental groups.

Acclimation Period

All the experimental work was performed at the Laboratory of Aquatic Ecology and Genetics in the Faculty of Agriculture of Kochi University, between September 19 and October 8, 1992. After transportation, the experimental fish were placed in 500 l tanks in which commercial salt was added (0.5%) in order to counteract the osmoregulatory problems caused by the transportation confinement, as recommended by Hattingh *et al.*¹³⁾ After 48 h the fresh water circulation system was opened and a food ratio of approximately 1% body weight/day was given to the fish for the following 10 days. Water temperature fluctuated between 19 and 21°C and fish density was set between 1 and 2 g/l.

Blood Sampling

Prior to each sampling period, the chosen fish were starved for 48 h in order to avoid possible initial differences in glucose levels and, immediately before sampling, a light dose of anesthetic (2-phenoxyethanol; 0.2 ml/l) was poured into the tank without disturbing the fish, in order to reduce "awareness" of the handling procedure.^{14,15)} After the fish had lost equilibrium, the maximum possible volume of blood (about 0.5 ml), was withdrawn from the caudal vein using heparinized 1 ml syringes. Immediately, 20 µl of whole blood were used for hemoglobin determination (Hemoglobin B-Test, Wako) and 2 to 4 heparinized capillary tubes were filled and sealed at one end. After sampling the blood of a group of 10 fish (usually about 15 min), the capillary tubes were centrifuged for 10 min at 4,000 rpm and the hematocrit values were read using a commercial hematocrit reader (Tomy Seiko Co., Ltd.).

Leucocrit (volume of packed leucocytes and thrombocytes as a percentage of whole blood volume¹⁶⁾), was determined by measuring the height of the buffy layer (grayish-white layer of cells separating the erythrocytes from the plasma in the centrifuged capillary tube) using a dissecting microscope (40× magnification) and ocular micrometer. The

leucocrit percentage was calculated as follows: height of buffy layer/height of total blood volume × 100%.¹⁶⁾

After recording these values, the capillary tubes were cracked and the portion containing the plasma was sealed on both sides and stored at –20°C for plasma glucose determination. These were done using a Glucose CII Test (Wako).

Mean corpuscular hemoglobin concentration (MCHC), which is the ratio of blood hemoglobin concentration to hematocrit, was calculated. MCHC is independent of the number of red cells per unit volume and its decrease is a useful indicator of the swelling of the erythrocytes.⁶⁾ Body weight, fork length and gonad weight of the fish were recorded. Finally, the Genetic Variation Index (GVI) proposed by Taniguchi *et al.*,¹⁷⁾ was calculated as follows:

$$GVI = (CV2n - CVCl)/CV2n$$

where: CV = coefficient of variation; 2*n* = 2*n*-normal fish and Cl = clonal fish. This value is inversely proportional to the C.V. and is used to standardize the genetic variation values for comparison with other studies.

Statistical Analysis

Means ± standard deviation are reported throughout. We used Fischer's *F*-test to identify significant variance differences (*p* < 0.05) and Student's two tailed *t*-test (unpaired design) to compare mean values between genetic groups. For all the traits studied, clones were compared against the 2*n*-normal group.

Results

Table 1 shows the mean and standard deviation values of the morphological traits measured, as well as the *t* and *F* tests results. The hetero-clone 1 and the homo-clone groups showed no difference in body weight but had a longer fork length than the 2*n*-normal fish. The hetero-clone 2 group was lighter but showed no difference in fork length. Also, the gonad weight mean value was significantly smaller in the clonal groups. The variances of the clones were significantly smaller than those of the 2*n*-normal fish: 78.5% for B.W., 62.3% for F.L. and 79.0% for G.W. on average. The reduction of variation of the hetero-clone groups was on average 12.7% more than that of the homo-clones.

Table 2 shows the mean and standard deviation values of the physiological traits and the comparisons results. The *t* test (comparison of means) indicates no significant difference between the clonal and the 2*n*-normal fish in almost all cases (86%). The variances of hematocrit, glucose and MCHC values of the hetero-clone 1 and homo-clone groups reduced as expected (70.6 and 11.34%, respectively), although the difference was not statistically significant in

Table 1. Morphological traits of the ayu genetic groups*¹

Genetic groups	BW (g)	<i>F</i>	<i>t</i>	FL (mm)	<i>F</i>	<i>t</i>	G.W. (g)	<i>F</i>
Normal-2N	19.21 ± 6.3 (70)	—* ²	—	126.27 ± 11.5 (70)	—	—	3.20 ± 1.44 (24)	—
Heteroclone 1	20.72 ± 2.7 (54)	**	N.S.	133.92 ± 5.8 (54)	**	*	1.95 ± 0.57 (54)	**
Heteroclone 2	18.40 ± 2.6 (49)	**	*	126.14 ± 7.2 (49)	**	N.S.	—	—
Homoclone	18.96 ± 3.4 (29)	**	N.S.	133.08 ± 8.0 (29)	*	*	2.07 ± 0.74 (10)	*

*¹: Values are means ± standard deviation with number of fish in parenthesis.

*²: Fischer's *F* test and Student's *t* test were used to compare variances and means against the normal-2*n* group, respectively. **p* ≤ 0.05; ***p* ≤ 0.001.

Table 2. Physiological traits of the ayu genetic group*¹

Genetic groups	Hematocrit %	F	t	Leucocrit %	F	t	Hemoglobin (mg/dl)	F	t	Glucose (mg/dl)	F	t	MCHC (g/dl)	F	t
Normal-2n	24.57 ± 7.2 (24)	—	* ²	0.68 ± 0.2 (22)	—	—	7.21 ± 0.9 (19)	—	—	61.68 ± 12.4 (25)	—	—	33.82 ± 9.0 (19)	—	—
Hetero-clone 1	23.21 ± 3.0 (21)	**	N.S.	0.87 ± 0.2 (15)	N.S.	**	6.82 ± 1.1 (21)	N.S.	N.S.	58.93 ± 8.4 (21)	*	N.S.	29.17 ± 4.5 (20)	**	N.S.
Hetero-clone 2	24.08 ± 7.8 (16)	N.S.	N.S.	0.63 ± 0.2 (11)	N.S.	N.S.	6.95 ± 1.2 (12)	N.S.	N.S.	63.62 ± 12.3 (15)	N.S.	N.S.	27.82 ± 8.3 (12)	N.S.	N.S.
Homo-clone	25.95 ± 6.9 (10)	N.S.	N.S.	0.57 ± 0.2 (7)	N.S.	N.S.	5.60 ± 0.9 (10)	N.S.	**	63.29 ± 11.9 (10)	N.S.	N.S.	22.52 ± 4.6 (10)	*	**

*¹: Values are means ± standard deviation with number of fish in parenthesis.

*²: Fischer's *F* test and Student's *t* test were used to compare variances and means against the normal-2n group, respectively. **p* ≤ 0.05; ***p* ≤ 0.001.

Fig. 2. Coefficient of variation of the morphological (A) and physiological (B) traits of the genetic groups under study.

—○—, gonad weight; —□—, body weight; —△—, fork length; —●—, hematocrit; —■—, MCHC; —▲—, glucose.

Table 3. Genetic Variation Index (G.V.I.)* of the normal-2n group

	B.W.	F.L.	G.W.	Hematocrit	Glucose	MCHC
Hetero-clone 1	0.603	0.525	0.351	0.560	0.291	0.420
Homo-clone	0.453	0.340	0.209	0.009	0.006	0.232

* G.V.I. = (C.V.2n - C.V.Cl)/(C.V.2n); where: C.V. = coefficient of variation, 2n = 2n-normal and Cl = clones.

every case. The hetero-clone 2 group was excluded from further calculations, because it showed no significant variance difference from the 2n-normal group.

Figure 2 illustrates the coefficients of variation of both types of traits. Values of the two clonal groups were smaller than those of the 2n-normal fish in all cases, the heteroclones showing a further reduction when compared to the homoclones.

The genetic variation index values (Table 3) for the heteroclones ranged from 0.603 (body weight) to 0.291 (glucose), while the homoclone values were relatively smaller (0.453 for body weight and 0.006 for glucose).

Discussion

The advantages of the gynogenetic production of inbred lines have been evaluated by several researchers,^{8,18-20} most of whom agree on the usefulness of these organisms to create genetically identical animals. Clonal production of commercially important fish has been reported only for common carp, *Cyprinus carpio*,²¹ flounder, *Paralichthys olivaceus*,²² and ayu, *Plecoglossus altivelis*.¹⁰ If this clonal fish were to be used for selective breeding, an initial approach would focus on superior size or best growth. Nevertheless, selecting for supreme physiological performance increases the possibility of creating specially tolerant strains.

In the present study, the physiological traits of ayu clones are investigated for the first time. When considering the mean values obtained for these traits compared to the morphological ones, we observe consistent values (with even less significant difference than the morphological traits), which suggests that we can exclude potential alterations due to handling stress. In fact, it is suggested that the difference of means (of both types of traits), may rather be due to the random selection of the parental population.

On the other hand, significant variation reduction was found for both types of clones, although the homo-clones reduction was unexpectedly less pronounced than that of the hetero-clones. Similar results were found by Komen *et al.*²⁵ in clones of the common carp, attributing this enlarged variation to reduced homeostasis.²⁴ Also, Leary *et al.*²⁵ showed that developmental instability in rainbow trout is related to homozygosity for certain enzymes involved in major biochemical pathways.

Nevertheless, the significant variation reduction found in this study theoretically implies that (for both types of traits), the genetic variation is substantially eliminated, allowing estimation of the heritability by comparing the variance of the clones and the normal diploids. In the ideal case of providing an identical environment to both groups, the variation due to environmental factors could be eliminated, permitting a theoretical estimation of the 2n-normal fish genetic variation. Furthermore, since the relative *Vg/Vp* can be taken as an estimate of the degree of genetic determination or heritability,²⁶ the estimations for the physiological traits would be in the same range as the

morphological ones, both being relatively high.

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