The influence of rearing density on stress response and disease susceptibility of ayu (Plecoglossus altivelis)

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Abstract

The impact of coldwater disease caused by \textit{Flavobacterium psychrophilum} is a recent important problem in the farming of ayu (\textit{Plecoglossus altivelis}). We hypothesized that stress from high-rearing densities predisposes fish to disease by reducing their immunocompetence, and we sought to reduce fish losses by introducing an alternative culture regime. Fish enclosed at three densities (1250 fish or 8.0 kg, 400 fish or 2.6 kg and 100 fish or 0.6 kg/m\textsuperscript{3}) were examined. Fish in the high-density treatment exhibited more elevated serum cortisol concentrations and more suppressed serum immunoglobulin M (IgM) concentrations accompanied by higher mortality, possibly caused by coldwater disease, than those in the medium- and low-density treatments. These results indicate that high-rearing densities stress fish and evoke subsequent physiological responses which have maladaptive effects. We propose that coldwater disease may be prevented by improving the disease resistance of fish by using moderate stocking densities.

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1. Introduction

Fish under intensive culture conditions are subjected to a variety of stressors due to the economic realities of large-scale production (Burton, 1997). To enhance production, farmers...
often increase rearing densities beyond system capacities. Rearing at high density can cause stress through deterioration in water quality, overcrowding or adverse social interactions (Pickering and Pottinger, 1989; Procarione et al., 1999). Cortisol is the principal corticosteroid in teleost fishes and its concentrations in blood rise dramatically during stress (reviewed in Mommsen et al., 1999). High-rearing density adversely increases fish susceptibility to disease, possibly due to chronically elevated cortisol levels which have immunosuppressive and catabolic actions in fish (Refstie, 1977; Pickering and Stewart, 1984; Pickering and Pottinger, 1989; Mazur and Iwama, 1993, but see Kjartansson et al., 1988; Vijayan and Leatherland, 1988). As for hatchery fish for release, outbreak of disease during rearing can introduce a serious source of infection for wild populations. To minimize disturbance of the native fish community, a tradeoff between economic demand (i.e. increase in rearing density) and maintenance of fish health after release should be considered.

Ayu (Plecoglossus altivelis), a common osmeroid fish in Japan, is commercially important for freshwater fisheries. It is semelparous with an annual life history, with an amphidromous coastal form ranging over the Japanese Archipelago amphidromously (cf. McDowall, 1992) and a lacustrine form landlocked in Biwa Lake, central Japan. A decline in the abundance of amphidromous populations in rivers due to habitat destruction and blockage of migratory routes has promoted transplantation of nonnative fish originating from landlocked and/or hatchery populations (Iguchi, 1997). Recently, bacterial coldwater disease (cf. Holt et al., 1993) has caused mass mortalities of ayu. Flavobacterium psychrophilum (formerly Cytophaga psychrophila) is the causative agent of coldwater disease, infecting fish under rearing conditions, including landlocked fish stocked for short periods for release (Wakabayashi et al., 1994). Secondary outbreaks of bacterial coldwater disease carried by released fish have occurred among wild fish, including native P. altivelis and other feral species (Iida and Mizokami, 1996; Amita et al., 2000). Releasing infected fish could never lead to successful stock enhancement but would damage the wild fish community.

Therapeutic treatments are inappropriate in natural waters and therefore development of vaccines may be needed (Rahman et al., 2000). However, vaccines are costly, and an alternative way to protect rearing fish against coldwater disease was investigated under the hypothesis that stress from high-rearing density predisposes fish to disease by reducing immunocompetence. In the immune system, immunoglobulin produced by lymphocytes is one of the most important anti-disease factors; it neutralizes bacteria and renders them more susceptible to phagocytosis (Ingram, 1980). We manipulated rearing densities of fish in experimental enclosures and measured their stress response and disease susceptibility. We investigated whether or not rearing density affects the mortality of P. altivelis caused by coldwater disease, using serum levels of cortisol and immunoglobulin M (IgM) as one indicator of immunopotency.

2. Materials and methods

Young fish were obtained from the prefectural government operated facility in Gunma in mid-May 1999, when they were at a comparative stage to the upstream-migratory stage of wild fish. This hatchery population has practically lost its genetic variation due to inbreeding
through cultivation for multiple generations (Iguchi et al., 1999). Until used in experiments, fish were stocked in outdoor ponds ($1 \times 5$ m, 0.5 m in depth) at a density of 2 kg/m$^3$ and fed assorted commercial food by an autofeeder three times per day (5% of the body weight). Water was supplied from a natural stream through an inlet mouth at a rate of 200 l/min. After a week of acclimation to remove the effect of transport stress, groups of 100 fish at a mean size of 6.4 g in body weight were caged into experimental enclosures situated in other outdoor ponds ($1 \times 5$ m, 0.5 m in depth). These enclosures had different volumes (high-density treatment or HD treatment: $0.4 \times 0.4$ m, medium-density treatment or MD treatment: $0.5 \times 1.0$ m, low-density treatment or LD treatment: $1.0 \times 2.0$ m, each 0.5 m in depth), and accordingly, fish densities/m$^3$ were 1250 (8.0 kg), 400 (2.6 kg) and 100 (0.6 kg) in the high-, medium- and low-density experimental enclosures, respectively. The high fish density of 1250/m$^3$ was within the range of rearing regimes for young fish used by culturists in Japan. The three density treatments consisted of three replicate trials conducted simultaneously over a period of 4 weeks. During the experimental period, fish were fed a quantity of assorted food by hand once a day. Water temperature measured at 10 AM increased gradually from 13.2 to 21.0 $^\circ$C.

Fish were exposed to waterborne infection of coldwater disease through natural stream water contaminated with the pathogen (see Amita et al., 2000). This indicated that the time when exposure to pathogen began was just after transfer of the fish a week before the density treatments. Dead fish were removed every morning and evening and stored in 100% ethanol until infection analysis. After the removal of dead fish, the equivalent number of stocked fish, which could be discriminated from experimental fish by a clipped fin, was added immediately to keep fish density constant. Kidneys from dead fish were examined to identify infection by coldwater disease. Polymerase chain reaction (PCR) was used to detect *F. psychrophilum* with two specific primers, 5'-CGA TCC TAC TTG CGT AG-3' and 5'-GTT GGC ATC AAC ACA CT-3' (Liu et al., 2001).

![Fig. 1. A standard curve for cortisol from the present ELISA system (anti-cortisol dilution, 1:75000; cortisol-HRP dilution, 1:10000) and competition curves for serial two-fold dilution of serum extracts of ayu. Each value represents the mean of duplicate determinations.](image)
Blood samples were collected just before the experiment and at 1 day, 1 week and 3 weeks after the beginning of the experiment. Fish (10) from each enclosure were anesthetized in a solution of 2-phenoxyethanol (ethylene glycol monophenyl ether) and bled from the caudal vasculature. Collection of blood samples was completed within 5 min of capturing the fish to minimize handling stress. Serum was separated by centrifugation for 5 min at 6000 × g and stored at −30 °C until analysis. Cortisol serum concentration was measured by ELISA using anti-cortisol-3-CMO-BSA (FKA-404, Cosmo Bio, Tokyo, Japan) and labeled-steroid cortisol-3-CMO-HRP (FKA-403) according to the method of Asahina et al. (1995). The competition curve for the serum extract and cortisol standard were almost parallel, showing the cortisol ELISA was valid (Fig. 1). IgM serum concentration was measured by single radial immunodiffusion (SRID) on GelBond film (Pharamacia LKB Biotechnology, Uppsala, Sweden) according to the procedure described by Nagae et al. (1994). Decrease in fish density by sampling was compensated for by the addition of fin-clipped stocked fish.

3. Results

Dead fish showed ulceration on the body surface and the specific base arrangement of *F. psychrophilum* was detected from the kidney as the causative agent of coldwater disease. Mortalities in the LD, MD, and HD treatments were 22%, 25% and 46%, respectively (Fig. 2). Variations in mortality differed between treatments both at 1 and 3 weeks after the beginning of the experiment (ANOVA, \( F_{2,6} = 24.14, P = 0.001 \) and \( F_{2,6} = 28.26, P = 0.001 \), respectively). At 1 and 3 weeks, more fish in the HD treatment died than in the other two treatments (Sheffé test, HD vs. MD; \( P = 0.005 \) and \( P = 0.003 \), and HD vs.

![Fig. 2. Cumulative mortality of fish throughout the experiment for the low, medium and high-density treatments. Vertical bars indicate standard deviation of three replicates.](image-url)
LD; \( P = 0.002 \) and \( P = 0.001 \), respectively), while those in the MD and LD treatments showed no significant difference in mortality.

Cortisol concentrations were highly variable between individuals, but two-way ANOVA based on the mean value within a replicate trial detected an interaction between time and density \( (F_{4,18} = 3.72, P = 0.022) \). Then, variation within treatments differed between treatments at 1 day, 1 week and 3 weeks after the beginning of the experiment \( (F_{2,41} = 4.43, P = 0.018, F_{2,45} = 13.61, P < 0.001, \) and \( F_{2,45} = 15.67, P < 0.001, \) respectively). Cortisol surged to high levels in fish after 1 day, with concentrations differing between the HD and LD treatments (Games–Howell test, \( P = 0.004 \)). Thereafter, cortisol concentrations decreased in the MD and LD treatments but increased in the HD treatment,
with cortisol levels significantly different between treatments after both 1 and 3 weeks (HD vs. MD; \(P=0.013\) and \(P=0.010\), and HD vs. LD; \(P=0.001\) and \(P=0.015\), respectively).

IgM concentrations also varied greatly between individuals, and data in each trial were pooled within treatments because of the small sample sizes. Overall IgM levels increased with time (Fig. 4, \(F_{8,94}=9.25, P<0.001\)). Although mean IgM concentrations were lowest in the HD treatment at all sampling times, variation of IgM levels between treatments differed significantly only after 3 weeks (\(F_{2,26}=3.97, P=0.031\)). Correlation coefficients between cortisol and IgM concentrations were negative at the three sampling times (Fig. 5), although the relationship was suggestive only after 3 weeks (\(r_{26}=-0.357, P=0.062\)).

4. Discussion

During the experimental period, fish in the high-density treatment had elevated cortisol levels and suppressed IgM levels, accompanied by higher mortality of fish infected with coldwater disease, than fish in the low- and medium-density treatments. These results indicate that escalating the rearing density creates stress and evokes subsequent physio-

![Fig. 5. Relationships between cortisol and immunoglobulin M (IgM) serum concentrations sampled 1 day, 1 week and 3 weeks after the beginning of the experiment.](image-url)
logical responses which have maladaptive effects on fish. The hypothesis that stress from high-rearing densities predisposes fish to disease by reducing their immunity appears applicable in the case of *P. altivelis* infected by *F. psychrophilum*.

However, some details of the effects of rearing density remain unclear from our results. This is probably due to other inevitable stressors involved in the experimental procedure. Stress due to transport prior to the experiment, handling at the application of the density treatment and from the progression of the coldwater disease are possible candidates (Burton and Zitzow, 1995; Sharpe et al., 1998; Mesa et al., 2000; Burton, 2000). Generally, stress stimuli and cortisol response do not occur simultaneously, but after a certain time lag, with recovery from stress also taking some time (Nagae et al., 1994; Burton and Zitzow, 1995; Sharpe et al., 1998; Pottinger and Carrick, 2000). At the initiation of the experiment, fish may have been recovering from the transport stress experienced 1 week before. During the early phase of the experiment, handling stress when the density treatments were applied may have influenced the acute physiological reactions of the fish. In the case of handling stress for juvenile walleyes (*Stizostedion vitreum*), cortisol levels reach a maximum just an hour after the treatment (Burton and Zitzow, 1995). Handling stress also causes acute stress responses for ayu, and the time required for recovery from acute stress responses depends upon the magnitude of density stress (Iguchi et al., 2002). This may be the reason why the plasma cortisol levels were low in the HD treatment at day 1 compared to the LD and MD treatments. Stress responses at the initiation of the experiment must have appeared more rapidly in the HD treatment. Increase of cortisol due to infection stress may have accelerated the total cortisol response. Thus, the effects of these potential stressors cannot be separated, but we believe that the basic scenario of density induced stress and cortisol response can be described.

Immune capacity in *Oncorhynchus masou* is known to increase with growth during early development (Fuda et al., 1991), which may explain why overall IgM levels increased with time during the experimental period. The lower IgM levels of the high-density treatment at each sampling time suggest the immunosuppressive effect of cortisol, although the differences were not statistically significant. In *O. masou*, IgM levels in the blood decrease some interval after cortisol administration (Nagae et al., 1994). In the present study, failure to elicit significant relationships between cortisol and IgM concentrations for individuals may be attributed to such a time lag.

Mortality in the medium- and low-density treatments did not differ, suggesting that the threshold rearing density that has an adverse effect on fish health exists at a point higher than 400 individuals (2.6 kg) per m³. Lowering rearing densities may reduce fish production within a limited capacity system; however, loss through disease will be reduced, making it an economically profitable option. We propose this alternative approach to prevent disease by improving the disease resistance of fish through using moderate stocking densities.

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