RESEARCH PAPER

Effects of Acute and Chronic Air Exposure on Growth and Stress Response of Juvenile Olive Flounder, *Paralichthys olivaceus*

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Abstract

We studied the effects of acute and chronic exposure to air on the growth and stress response of juvenile olive flounder, *Paralichthys olivaceus*. To study the stress response, the water was completely drained from the experimental tank, and the stressed group was exposed to air for 5 minutes, after which the tank was refilled with water. This stress was repeated daily for 30 days (between 1200 and 1300 h). From day 31 to day 69, no stress was applied. On day 70, the fish were again exposed to the air. The non-stressed group was not subjected to air exposure during the 70 days. We measured cortisol, glucose and lactic acid levels, osmolality, growth, survival, and feeding responses during the 70-day test period. Our results showed that olive flounder exhibit "typical" physiological responses (in cortisol, glucose, and lactic acid levels and osmolality) to the acute stress induced by air exposure. The response to chronic stress showed a similar increasing tendency. However, the incremental rate of increase decreased as the stress continued. Adverse stress effects, in terms of growth, survival, and response to food, were higher in the stressed than the non-stressed group. The effects of chronic stress from air exposure remained for 20 days after the stressor was terminated. These results will help to minimize stress from aquaculture activities in olive flounder farm.

Keywords: Olive flounder, air exposure, chronic stress, feed effects, growth.

Introduction

The stress response has been differentiated into acute and chronic patterns. In turn, the effects of stress are divided into three categories: primary, secondary, and tertiary (Barton & Iwama, 1991; Lowe, Ryder, Carragher & Wells, 1993; Wendelaar Bonga, 1997; Barton, 2002; Iwama, Afonso & Vijayan, 2006). The primary response involves the endocrine system, with the main purpose to mobilize energy. It is characterized by stimulation of the adrenergic system (leading to a rise in the catecholamines adrenaline and noradrenaline) and the hypothalamus-pituitary-interrenal (HPI) axis (causing an increase in adrenocorticotropic hormone and cortisol). Catecholamines furnish energy over the short term (through glycogenolysis), whereas cortisol furnishes longer-term energy by stimulating the catabolism of glycogen, lipids, and protein (Gamperl, Vijayan & Boutilier, 1994). Glucose and lactate in plasma are often used to assess stress levels (Acerete, Balasch, Espinosa, Josa & Tort, 2004). An important aspect of the secondary response is metabolic adaptation.

Under intensive fish culture conditions, stress disturbances are usually of a prolonged nature, and the accompanying chronic stress results in a loss of homeostasis. Adaptation, if possible at all, occurs only over a long period (Schreck, 1981). The stress response shifts from adaptive to maladaptive (Barton & Iwama, 1991), eventually resulting in decreased disease resistance, impaired reproduction, and reduced growth. Such whole body responses are regarded as the tertiary response level (Barton, Schreck & Barton, 1987).

The olive flounder, *Paralichthys olivaceus*, has an oval body shape resembling an olive. This fish occurs mainly in the benthos at depths of 10 to 200 m and is one of the most important marine fish species for commercial aquaculture in northeast Asia including Korea and Japan (NFRDI, 2005). In 2014, approximately 42,000 tons of this species were farmed in Korea (K FA, 2015). Olive flounder culture and marketing procedures require grouping the fish by size by selecting individuals with similar growth patterns. The selection procedure includes numerous acute and chronic stressors to the fish, such as water-level reduction in the rearing tank, size selection,

capture, confinement, air exposure, and transport (Hur, Chang, Lim & Lee, 2001; Eslamloo, Akhaven, Fallah & Henry, 2014). Olive flounder producers carry out these selection procedures without understanding how these acute and chronic stressors may affect the fish physiologically.

The aim of the present study was to evaluate the effect of acute and chronic stressor (air exposure) on plasma cortisol, glucose lactic acid and osmolality in cultured olive flounder.

Materials and Methods

Preparation of the Experimental Fish and Conditions

Juvenile olive flounder was donated from Finfish Research Center, National Institute of Fisheries Science, Republic of Korea. Prior to the feeding trial, the fish were acclimated to experimental conditions for 2 weeks. 30 fish (the average body length 9.8±0.8 cm; weight 8.9±1.9 g) were randomly stocked in each of 270-L flow-through tanks (water volume: 180-L) with a 1.5 kg/m^3 density.

During the acclimation and the following experimental period, the fish were fed commercial extruded pellet (Ewha Oil and Fat Industry Co. Ltd., Republic of Korea) containing 56.0% crude protein and 12.0% crude lipid. Fish in each of the groups were hand-fed to apparent satiation, 100% of satiation, twice a day at 09:00 and 18:00. Uneaten feed was removed 30 min after feeding and the amount was deducted from feed consumption calculations. The flow rate of water into each tank was 4.5 L·min^{-1}. The water source was sand-filtered natural seawater and aeration was supplied to each tank. Water temperature ranged from 19.3 to 24.1℃ (22.6±0.5℃) during the feeding trial in summer. Natural photoperiod was used and fish were fed 6 days a week throughout the feeding trial. The salinity and DO of the seawater ranged from 33 to 36 psu and from 5.0 to 6.7 ppm, respectively.

To study stress response, the tank water was completely drained to expose the fish to air for 5 minutes, after which the tank was refilled with water. The stress experiment was repeated daily for 30 days (between 12:00 and 13:00). From day 31 to day 69, no stress was applied. On day 70, the fish were again exposed to air.

Blood Samples and Analysis

The blood of experimental fish was sampled at 0 (before drain water), 1, 3, 12 hours post the drainage for acute stress response and at 0, 2, 7, 15, 30 and 70 days for chronic stress response. Blood samples were obtained from the caudal blood vein of 10 randomly chosen fish from each tank by using a heparinized syringe after a 24 h starvation and without anesthetizing the fish at the end of the feeding trial.

Blood were kept in a 2 mL vacuum container treated with sodium fluoride potassium oxalate (Vacutainer, UK) and in 1.5 mL polypropylene micro centrifuge tubes on ice for less than 5 min before centrifugation at 5,600 g for 5 min. Plasma was then collected and stored in a deep freezer (CLN-500 UW Nihon Freezer; Nihon Co., Japan) at −70℃ until required for analysis. Plasma cortisol levels were determined in 50-l samples using radioimmunoassay kits (Coat-A-Count TKCO Cortisol RIA Kit; DPC, USA). Mixtures of samples in 100 ml of antiserum were incubated for 45 min at 37℃ and then 1,000 mL of separation reagent was added. Mixture was placed in a refrigerator at 4℃ for 15 min and then centrifuged at 1,200 *g* for 15 min. Supernatant was assayed for gamma radiation using an automatic gamma counter (Cobra; Packard Co., USA). Glucose and lactic acid were analyzed by using an automatic chemistry analyzer (Hitachi 7180; Hitachi, Japan). Osmolality was determined using a micro osmometer (Fiske 210; Fiske, USA).

Analysis of Growth, Survival and Feed Effects

For analysis of the growth, fish were measured at 15, 30 and 70 days. A measuring board with 1 mm graduations and an electronic balance with 0.01 g as a minimum unit was used to measure the length and weight of fish. Growth rate for total length [GRL=(final mean total length)-(initial mean total length)×100/(initial mean total length)] and body weight [GRW=(final mean body weight)-(initial mean body weight)×100/(initial mean body weight)], specific growth rate [SGR=(final mean body weight-initial mean body weight)×100/(rearing day/100)], feed intake [FI=(dry feed intake×100)/(rearing day×number of fish)], specific feeding rate [SFR=(dry feed intake×100)/((initial total weight+final total weight+death total weight)/2)×(rearing day)] and feed conversion rate [FCR= (SGR×100)/SFR] of experimental fish, after offering feed, were calculated by these numerical values at day 70. During the period of these experiments, survival rate was calculated based on the daily number of dead fish.

Statistical Procedure

The experiment was performed in triplicate and all data are expressed as the mean±standard deviation of the mean (SD); t-test and one-way ANOVA followed by Duncan's multiple range test was used to analyze the data with the SPSS computer package (SPSS Inc., USA). The level of statistical significance was set at $P<0.05$.

Results

Hematochemical Parameters

Figure 1 shows variations of cortisol, glucose,

lactic acid and osmolality in plasma of juvenile olive flounder. In the stressed group, plasma cortisol levels increased significantly from 4.6±0.9 (before the experiment, BE) to 40.1±5.1 at 1 h, to 36.0±6.7 at 3 h, and 16.3±1.2 ng/mL at 12 h. Cortisol levels of the stressed group at 1, 3 and 12 h were significantly higher than levels in the non-stressed group (P<0.05). Glucose levels of the stressed group increased significantly from 25.6±2.1 (BE) to 39.4±4.0, and to 43.8±3.6 mg/dL at 1 and 3 h, respectively (P<0.05). Glucose levels in plasma showed significant difference at 3 h between stressed group and non-stressed group (P<0.05). In the stressed group, plasma levels of lactic acid at 1, 3 and 12 h were significantly higher than that at 0 h (P<0.05). The levels in the stressed group at 1, 3 and 12 h was significantly higher than those in the non-stressed group (P<0.05). Osmolality increased significantly at 1 and 3 h compared to that at 0 h, and showed significant difference between stressed group and non-stressed group (P<0.05) (P<0.05).

Figure 2 shows variations of cortisol and glucose levels in plasma of juvenile olive flounder chronically exposed to air for 70 days. Cortisol levels of non-stressed group during the experimental period did not changed significantly (P>0.05), however cortisol levels of stressed group increased significantly at day 2 and then decreased slowly at day 30 (P<0.05). Plasma glucose levels of the stressed group increased significantly from 25.6±2.1 (0 h) to 41.0±3.1, to

35.0±1.9, and to 38.5±2.6 mg/dL at day 2, 7 and 70, respectively (P<0.05).

Lactic acid levels and osmolality of the non-stressed group did not change significantly during the experimental period (P>0.05), however lactic acid levels of stressed group increased significantly compared to that at day 0 (P<0.05). Osmolality at day 2, 7 and 70 increased significantly from that at day 0 (Figure 3).

Growth, Survival and Feed Effects

Figure 4 shows the growth results of total length and body weight of juvenile olive flounder chronically exposed to air for 70 days. The non-stressed fish grew faster than the stressed fish. Table 1 shows the growth results of SGR, GRL, GRW, FI, SFR and FCR of juvenile olive flounder chronically exposed to air for 70 days. SGR of stressed group (0.86±0.28%) was lower than the rate of the non-stressed group (1.01±0.10%) (P<0.05). GRL, GRW, FI and FCR were significantly higher in non-stressed group than in stressed group (P<0.05).

Survival of non-stressed and stressed group at day 70 were 98.0±1.4% and 88.5±0.7%, respectively (Figure 5).

Discussion

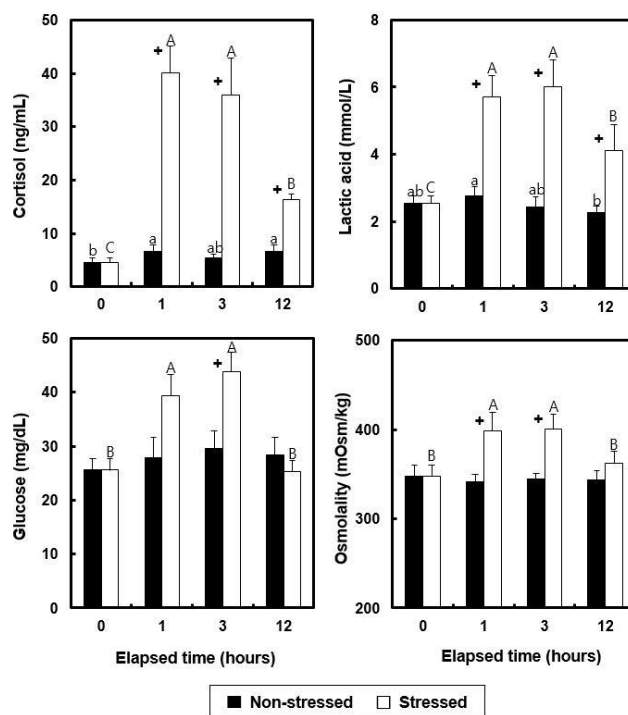If they are severe, acute stressors (e.g., handling,

Figure 1. Plasma cortisol, glucose, lactic acid and osmolality levels of olive flounder (*Paralichthys olivaceus*) to acute air exposure for 5 minutes. The values are mean±SD (*n*=10). The superscripts indicate significant differences between sampling times (P<0.05). Cross marks show significant differences between non-stressed fish and stressed fish at same sampling time (P<0.05).
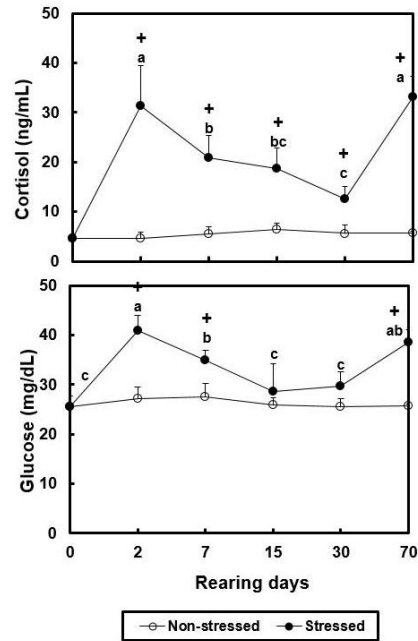
Figure 2. Plasma cortisol and glucose levels of olive flounder (*Paralichthys olivaceus*) to chronic air exposure for 70 days. The values are mean±SD (*n*=10). The superscripts indicate significant differences between rearing days (P<0.05). Cross marks show significant differences between non-stressed fish and stressed fish at same sampling days (P<0.05).
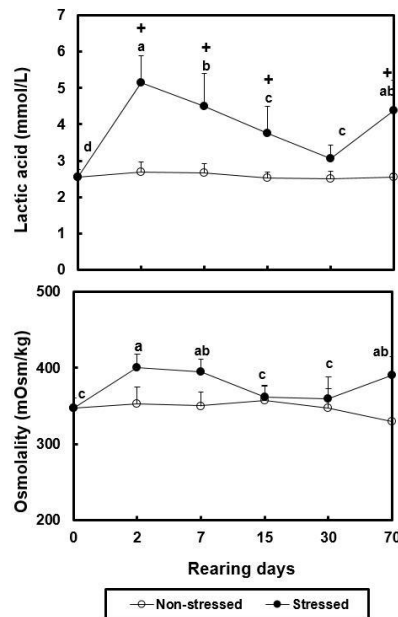

Figure 3. Plasma lactic acid and osmolality levels of olive flounder (*Paralichthys olivaceus*) chronic air exposure for 70 days. The values are mean±SD (*n*=10). The superscripts indicate significant differences between rearing days (P<0.05). Cross marks show significant differences between non-stressed fish and stressed fish at same sampling days (P<0.05).

air exposure, and confinement) can have lethal consequences (Strange, Schreck & Golden, 1977). Chronic stressors (e.g., crowding) are usually associated with reduced growth, changes in behavior, and increased susceptibility to disease (Wedemeyer & Mcleay, 1981). Stressors can reduce the ability of exposed fish to maintain homeostasis (Schreck, 1982) and withstand a second stressor (Specker & Schreck,

1980). The blood parameters of teleost fish may also be affected by acute stress, chronic stress, or both, as reviewed by Barton and Iwama (1991) and Wendelaar Bonga (1997).

In Korea, olive flounder account for more than 70% of all fish aquaculture production. In aquaculture facilities, stress-inducing factors are present at each step of the olive flounder production process. Olive
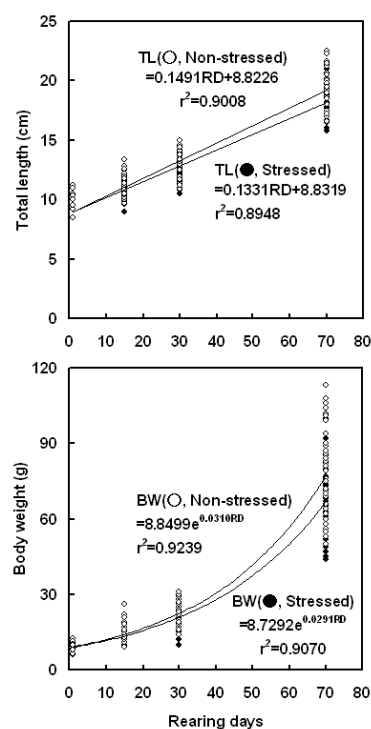
Figure 4. Results of total length and total weight of olive flounder (*Paralichthys olivaceus*) to chronic air exposure for 70 days.-

Table 1. Growth results of olive flounder (*Paralichthys olivaceus*) to repeat air exposure and water level reduction in rearing tank for 70 days

Items	Non-stressed	Stressed
SGR (%)	1.01±0.10	0.86±0.28*
GRL (%)	99.90±0.87	88.45±1.45*
GRW (%)	792.28±27.32	674.92±6.33*
FI (%)	41.13±0.10	40.86±0.28
SFR (%)	4.59±0.16	5.52±0.10*
FCR (%)	22.04±1.52	15.61±0.44*

The values are mean±SD (*n*=30). Asterisks show significant differences between non-stressed fish and stressed fish (P<0.05). SGR: specific growth rate, GRL: growth rate for total length, GRW: growth rate for body weight. FI: feed intake, SFR: specific feeding rate, FCR: feed conversion rate. SGR: (final mean body weight - initial mean body weight)×100/(rearing day/100). GRL: (final mean total length) - (initial mean total length)×100/(initial mean total length). GRW: (final mean body weight) - (initial mean body weight)×100/(initial mean body weight). FI: (dry feed intake×100)/(rearing day×number of fish). SFR: (dry feed intake×100)/((initial total weight + final total weight + death total weight)/2)×(rearing day). FCR: (SGR×100)/SFR.

flounder are euryhaline and very resistant to stress, most likely because in comparison to more active swimmers, olive flounder are less mobile and inhabit a more benthic habitat.

The basal (pre-stress) cortisol level of the experimental fish (4.6 ng/ml) was similar to values previously reported for olive flounder (Hur, Chang, Lim & Lee, 2001; Hur, Choi, Chang & Neill, 2003; Hur, Park & Chang, 2007) and flounder, *Platichthys flesus* (Waring, Stagg & Poxton, 1992). Moreover, the cortisol level of <5 ng/ml that we found in fish sampled immediately after capture from the wild and in aquaculture were similar to values reported for other species sampled under similar conditions (Barton & Iwama, 1991; Bandeen & Leatherland,

1997; Carragher & Rees, 1994; Pankhurst & Sharples, 1992). In this study, cortisol levels showed no recovery from 1 to 12 h after acute stress, and the level at 1 h increased similar to other reports (Waring, Stagg & Poxton, 1992). However, in olive flounder, the peak levels and recovery of cortisol after acute stress (e.g., selection, salinity, air exposure, and anesthesia) differed from those previously reported; cortisol levels were increased significantly from 0.5 h to 2 h, then recovered over the period from 3 h to 24 h. This variation may have been due to differences in factors such as the culture system, life stage, physiological status, and level of stress. Barton and Iwama (1991) reported that in a stressed condition, the increased rate and duration of cortisol levels differ
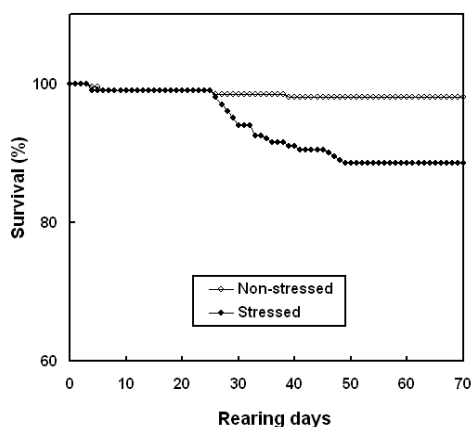
Figure 5. Survival of olive flounder (*Paralichthys olivaceus*) to chronic air exposure for 70 days.

by fish species. The rapid increases in plasma cortisol levels that we observed within 8-240 min after physical disturbances differed from those recorded in other experiments (Robertson, Thomas, Arnold & Trant, 1987; Arends, Mancera, Munoz, Wendelaar & Flik, 1999). The recovery of cortisol levels in salmonid species exposed to acute stress ranges from 4 to 24 h (Barton & Schreck, 1987; Pickering, Pottinger & Christie, 1982), although cortisol in seabream, *Sparus aurata*, returns to basal levels within 2 h of a 3-min air exposure (Arends, Mancera, Munoz, Wendelaar & Flik, 1999). Rainbow trout, *Oncorhynchus mykiss* (Pankhurst & Dedual, 1994), sea raven, *Hemitripterus americanus* (Vijayan & Moon, 1994), and turbot, *Scophthalamus maximus* (Waring, Stagg & Poxton, 1996), recovered within 24 h, as did brown trout, *Salmo trutta* (Pickering & Pottinger, 1989).

The trends in cortisol, glucose, and lactic acid levels observed in this experiment indicated the generalized stress reactions. Glucose formation increased simultaneously with the cortisol level. The increase in lactic acid concentration seemed to stem from the increase in stress induced by air exposure. This finding agrees with previous results showing that the stress level is accompanied by a rise in plasma cortisol and glucose levels (Barton & Schreck, 1987; Robertson, Thomas, Arnold & Trant, 1987; Thomas & Robertson, 1991). Glucose levels may be immediately elevated by catecholamines; however, further increases are usually the result of corticosteriods, which facilitate gluconeogenesis (Barton & Iwama 1991). This response very rapidly provides muscle tissue with large amounts of glucose to assist an organism during an acute stress event. Gluconeogenesis activation toward enzyme increases, and the secretion increases due to cortisol secreted by the fish in response to stress (Barton & Iwama, 1991; Davis, Torrance, Parker & Suttle, 1985). Barton and Iwama (1991) suggested that this increase is the result of a second reaction to the first reaction (hormone response) to stress. In the stressed fish in this study,

the glucose level and osmolality recovered within 12 h; however, cortisol and lactic acid levels had not returned to normal after 12 h, suggesting that stress induced by air exposure is not relieved within that time. Air exposure resulted in tension-induced activity in the fish, thus elevating cortisol and glucose levels, as well as generating lactic acid formation. This suggests that the energy the fish used was supported by anaerobic metabolites. Turner *et al.* (1983) suggested that flatfish store lactic acid in muscle tissue rather than discharging it into the blood, even under low-oxygen conditions. We suggest that the lactic acid increase at 1 h in this study resulted from lactic acid discharging from muscle into blood after air exposure.

Lactic acid levels in flounder rise quickly when they are subjected to acute stress (Hur, Park & Chang, 2007). In this study, the maximum lactic acid level after acute stress was similar to those previously reported in coral trout, *Plectropomus leopardus* (Frisch & Anderson, 2000). However, the post-stress lactic acid level of Atlantic sharpnose shark, *Rhizoprionodon terraenovae* (Hoffmayer & Parsons, 2001), and dusky shark, *Carcharhinus obscurus* (Cliff & Thurman, 1984), was significantly higher. Compared to flounder in other studies (Hur, Park & Chang, 2007), the plasma lactic acid levels in our study may have resulted from swimming and from a lower metabolic rate due to stress.

The correlation between increased production of cortisol and glucose and decreased growth and macrophage functions in salmonids has been well documented (Barton & Iwama, 1991). After each air-exposure, we sampled blood within 3 h to measure the stress level of the stressed group. The incremental rate of hematological factors due to accumulated stress tended to decrease under all conditions. This result seems to be an adaptation to stress. However, the high cortisol, glucose, and lactic acid levels during all experimental periods showed that the flounder do not adapt to chronic stimuli induced by air exposure. In addition, tertiary responses, such as growth, feed

effects, and survival, showed a lack of adjustment to air exposure.

Several studies have evaluated the effects of either acute or chronic stress, but few have assessed the effects of both (Rottlant & Tort, 1997; Barcellos, Nicolaiewsky, Souza & Lulhier, 1999). Adaptations to chronic stress situations have been reported for several teleost species, including Nile tilapia, *Oreochromis niloticus* (Barcellos, Nicolaiewsky, Souza & Lulhier, 1999). One of the indicative tertiary effects to the chronic stress response is impaired growth. Kebus *et al*. (1992) showed that chronic stress greatly reduces growth in rainbow trout. In these trout, the physiological alterations caused by the stress condition mobilize both the energetic input of food and corporal reserves. The effects of stress on growth and feeding was greater in the stressed than in the non-stressed group, as demonstrated by the decreased growth rate due to chronic air exposure stress. Interestingly, the specific feeding rate was higher in the stressed group, whereas the feed conversion rate was greater in the non-stressed group. This is likely the result of chronic stress. Exposed to daily repeated stress, the stressed group showed little difference compared to the non-stressed groups in feeding. However, we assume that the stressed group spent more energy on maintaining homeostasis than on growth. Moreover, when the stressor was terminated after 30 days, the food intake of the stressed group exceeded that of the non-stressed group, possibly to compensate for growth lost to stress. Food intake in fish is generally determined by numerous biotic and abiotic factors, such as water temperature, size, age, social behavior, maturation status, stress, light, salinity, and type of food (Houlihan, Boujard & Jobling, 2001).

Among the stressed group, survival decreased from day 20 to day 50. This result demonstrates that the effects of chronic stress appear after 20 days. Moreover, survival tended to decrease until day 50, even when the stress was not applied after day 30. Thus, the effect of accumulated stress continues at least 20 days after its removal.

In conclusion, our results showed that olive flounder exhibit "typical" physiological responses (in cortisol, glucose, and lactic acid levels and osmolality) to acute stress induced by air exposure. The increase in responses to chronic stress was similar to that of acute stress. However, the rate of increment decreased as the stress continued. The adverse effects of stress on the growth, survival, and feeding responses of the stressed group were greater than on those of the non-stressed group. Thus, the effect of chronic air exposure stress continues at least 20 days after the stressor is removed. These results will be helpful in minimizing the stress from aquaculture activities such as water-level reduction in the rearing tank, size selection, capture, confinement, air exposure, and transport in olive flounder farm.

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