The Effect of Packing on Water Quality Parameters, Survival and NNV Load of *Epinephelus coioides* Fry after Simulated Transport

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**Abstract**

Water quality parameters, survival rates, and nervous necrosis virus (NNV) loadings of *Epinephelus coioides* grouper fry packed in 50 L bags after 24 h of simulated transport were examined. All fry packed at 300/bag and with water/oxygen ratios of 10 L/40 L, 12.5 L/37.5 L, and 15 L/35 L at 20°C survived with NNV loads of 4.1×10^4 at 7 d post-release in seawater. All fish packed at 300/bag and a water/oxygen ratio of 12.5 L/37.5 L at 20°C survived with an NNV load of 4.1×10^4, but fish packed at 25°C and 30°C survived 100% and 67.4%, and had NNV loads of 1.6×10^5 and 1.8×10^5, respectively at 7 d post-release. All fish packed at 200, 300, and 400/bag with a water/oxygen ratio of 12.5 L/37.5 L at 20°C survived with DO >5.0 mg/L, CO\(_2\) <95 mg/L, pH >5.6, and ammonia-N <11 mg/L after 24 h. All fish packed at 200 and 300/bag survived with an NNV load of 1.7×10^5, whereas fish packed at 400 and 500/bag survived only 60.8% and 42.6% with NNV loads of 2.4×10^7 and 3.7×10^7, respectively at 7 d post-release. We concluded that grouper fry packed at 300/bag and a water/oxygen ratio of 12.5 L/37.5 L at 20 and 25°C were optimal for maintaining high DO (>5.8 mg/L) and pH (>5.6) and low CO\(_2\) (<89 mg/L), ammonia-N (<10.2 mg/L), and NNV loads (<4.9×10^3) after 24 h of simulated transport. The addition of zeolite increased DO and pH while lowering carbon dioxide and ammonia-N as well as lower NNV loads (2.3×10^3) during simulated transport.

**Keywords:** Grouper; *Epinephelus coioides*; Survival; Simulated transport; Water quality parameter; NNV load; Zeolite

**Introduction**

Nervous Necrosis Virus (NNV) and iridovirus are reported to cause mass mortality in grouper leading to serious economic loss [1,2]. NNV is the causative agent of viral nervous necrosis or viral encephalitis and retinopathy (VER) in fish, which is a non-enveloped, single-strand, positive-sense RNA belonging to the family *Nodaviridae* and genus *Betanodavirus* [3]. Real-time quantitative PCR analyses from 24 grouper farms indicate that NNV is highly infectious horizontally and causes a 100% mortality rate in Taiwan [1,4].

Grouper is a highly-valued and commonly cultured group of marine fishes in Asia, and there is an increasing need to transport larvae and fry. Fish packing density varies with species, size and water quality [5]. Packing conditions for transporting hatchery-reared grouper larvae and wild grouper larvae have been reported [6]. The 35-d larvae are more sensitive to handling stress than 45- and 60-d hatchery-reared grouper larvae, which are transported successfully at a packing density of 50 larvae/L with 100% of survival at a water temperature of 23°C [6]. Real-time quantitative PCR analysis indicates that grouper from 21 of 24 farms are NNV positive [4]. Farmers often observe fry exhibiting erratic swimming behavior followed by sudden death, and suspected that the cause is due to handling stress during shipping. However, little or nothing is known about the relationship between packing conditions and NNV loading that commonly occur after handling and transport.

Prior to transport, fish are commonly deprived of food to decrease their metabolism and metabolic wastes, including ammonia and carbon dioxide, and packed fish are transported at low temperatures [6-8]. It is known that lowering water temperature decreases the growth of bacteria, decreases the stress to and activity of fish, and decreases fish metabolism leading to reductions in ammonia and carbon dioxide production [9].

We assumed that grouper packed in unfavorable conditions negatively affect water quality parameters, decrease survival rates, increase susceptibility to NNV infection, and can enhance viral replication for producing fish damage. Therefore, the objective of this study was to examine the survival of grouper fry, their NNV loads, and water quality parameters in transport bags, when fry were packed at different (1) water/oxygen ratios, (2) water temperatures, and (3) packing densities, and (4) with the addition of zeolite.

**Materials and Methods**

**Experimental design**

Orange-spotted grouper (*Epinephelus coioides*) fry (5.1 ± 0.4 g and 5.1 ± 0.2 cm, 55-d) were obtained from a commercial farm and held in a laboratory tank for one month. Fish were deprived of food for 24 h prior to the experiment. Briefly, grouper fry were placed in double-layered 50 L plastic bags filled with seawater, inflated with oxygen, and tied with rubber-bands. The bags were then placed in styrofoam boxes, and shaken with an orbit shaker. After 24 h of simulated transportation, bags were removed from the shaker, disclosed and their waters sampled for dissolved oxygen (DO), carbon dioxide (CO\(_2\)), pH and ammonia-N (ammonia as nitrogen). Fish survival rates and NNV loads were examined 24 h and 7 d after release into normal seawater. Four experiments were conducted: (1) fish kept at 20°C and packed at 300/bag under different ratios of water/oxygen (10 L/40 L, 12.5 L/37.5 L and 15 L/35 L at 20°C), (2) fish kept at 20°C and packed at 300/bag under different ratios of water/oxygen (10 L/40 L, 12.5 L/37.5 L and 15 L/35 L at 25°C) in seawater. All fish packed at 300/bag and water/oxygen ratios of 10 L/40 L, 12.5 L/37.5 L and 15 L/35 L at 20 and 25°C survived with DO >5.0 mg/L, CO\(_2\) <95 mg/L, pH >5.6, and ammonia-N <11 mg/L, after 24 h. All fish packed at 200 and 300/bag survived with an NNV load of 1.7×10^5, whereas fish packed at 400 and 500/bag survived only 60.8% and 42.6% with NNV loads of 2.4×10^7 and 3.7×10^7, respectively at 7 d post-release. We concluded that grouper fry packed at 300/bag and a water/oxygen ratio of 12.5 L/37.5 L at 20 and 25°C were optimal for maintaining high DO (>5.8 mg/L) and pH (>5.6) and low CO\(_2\) (<89 mg/L), ammonia-N (<10.2 mg/L), and NNV loads (<4.9×10^3) after 24 h of simulated transport. The addition of zeolite increased DO and pH while lowering carbon dioxide and ammonia-N as well as lower NNV loads (2.3×10^3) during simulated transport.

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L, 15 L/35 L), (2) fish packed at 300/bag and a water/oxygen ratio of 12.5 L/37.5 L with different water temperatures (20, 25, 30°C), (3) fish packed at 200, 300, 400, and 500/bag with a water/oxygen ratio of 12.5 L/37.5 L at 20°C, and (4) fish packed at 300/bag and a water/oxygen ratio of 12.5 L/37.5 L with the addition of zeolite particles (5, 20, and 30 g/L). Each experiment was conducted in triplicate.

### Packing and disclosure of bag

Water (10, 12.5, or 15 L) was first added to the bags and grouper fry then placed inside. Bags were purged with pure oxygen and closed with rubber bands and then placed in styrofoam boxes and shaken with an orbit shaker for simulated transport. Bags were disclosed after 24 h and their waters tested for ammonia-N, DO, CO2, and pH. Fish were then released into normal seawater; with the number of fish surviving being counted after 24 h and 7 d. Fry was also sampled for NNV assay. There were three replicates in each treatment and 5 fry were sampled from each replicate.

#### Water quality parameter analyses

Water pH was measured with a Suntex Model SP-7 pH meter (Suntech, Taipei). DO was measured with YSI Model 58 DO meter having an electrode probe (YSI, USA) attached to a battery powered stirrer. Ammonia-N (un-ionized plus ionized ammonia as nitrogen) was measured using a phenol hypochlorite method [10]. CO2 concentration was measured using a phenol hypochlorite method [11].

**RNA isolation and real time RT-PCR:** Grouper head total RNA was extracted using TRIzol reagent (Invitrogen) after 24 h and 7 d post-transportation. First-strand cDNA was synthesized using oligo (dT)$_{20}$ primer. NNV R3 primer and Superscript III reverse transcriptase (Invitrogen) according to manufacturer protocols. Real-time PCR was performed using iQ SYBR Green Supermix (BIO-RAD) and CFX384 Real-Time PCR Detection System (BIO-RAD) to determine NNV expression levels [12,13]. Primers used were NV3, RGNV RPCR-F, RGNV RPCR-R, and β-actin (actin-F and actin-R) (Table 1). Reaction conditions were as follows: 95°C for 3 min, followed by 40 cycles at 95°C for 20 s, 60°C for 20 s, 72°C for 20 s and fluorescence detection at 83°C for 20 s. All samples were analyzed in triplicate. Red-Spotted Grouper Nervous Necrosis Virus (RGNV) expression levels were normalized with an internal control (actin) and the normalized gene expression value of the pre-transportation group was regarded as 1. Fish head NNV loads were quantified before transportation. A qPGE plasmid containing NNV RNA2 ORF gene was used to establish the standard curve of NNV RNA 2 copies in real-time PCR. There were 30 copies of NNV RNA 2 per fish.

### Statistical analysis

All data were subjected to one-way analyses of variance (ANOVA). If significant differences were indicated at the 0.05 level, then a multiple-comparisons (Tukey’s) test was used to examine significant differences among treatments using SAS computer software (SAS Institute, Cary, NC, USA). Statistical significance was determined with p<0.05.

| Name     | Sequence                      |
|----------|-------------------------------|
| NV R3    | 5’-CGAGTCTAACCACGGGTAGAAGA-3’ |
| RGNV RPCR-F | 5’-CATGCGCACTTCAGTACAG-3’     |
| RGNV RPCR-R | 5’-AACACTCCAGGACACAG-3’      |
| Actin-F  | 5’-GCCGCGGACAATCAGACCTAC-3’  |
| Actin-R  | 5’-CCCTGGGCAACGGAACCTCCT-3’  |

**Table 1:** Primers used for the quantitative real-time PCR study of actin and NNV genes of grouper Epinephelus coioides.

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**Figure 1a:** Relative gene expression level of Nervous Necrosis Virus (NNV) after 24 h transportation and 7 d post-transportation at conditions: different water/oxygen volume ratio.

**Table 2:** Effects of water/oxygen volume ratios on the water quality of plastic bags packed with grouper at 300/bag and 20°C after 24 h.

**Results**

Fish packed at 300/bag at 20°C with different ratios of water/oxygen (10 L/40 L, 12.5 L/37.5 L, 15 L/35 L)

All fry survived in all groups at 24 h of disclosure and after 7 d. The pH values increased directly with water/oxygen ratios and ranged 5.6–6.4, whereas ammonia-N and CO2 were inversely related to water/oxygen ratios and ranged 8.6–10.4 mg/L and 76–86 mg/L, respectively (Table 1). Fry NNV loads ranged 4.3×10³–6.2×10⁴ and 4.1×10⁵–7.9×10⁵ at 24 h of disclosure and at 7 d post-release in normal seawater, respectively (Figure 1A).

**Fry packed at 300/bag and a water/oxygen ratio of 12.5 L/37.5 L with different water temperatures (20, 25 and 30°C)**

All fry packed at 20, 25 and 30°C survived at 24 h following disclosure. Ammonia-N and CO2 increased directly with water temperature and ranged 9.3–11.4 mg/L and 83–92 mg/L, respectively, whereas DO and pH were inversely related to water temperature and ranged 5.4–6.2 mg/L and 5.6–6.2, respectively (Table 2). The NNV loads of fry packed at 20 and 25°C ranged 1.7×10³–2.8×10⁴ and 4.1×10⁵–1.6×10⁶ at 24 h after disclosure and 7 d post-release, respectively. The NNV load of fry packed at 30°C was 5.6×10⁵ and 1.9×10⁶ after 24 h of disclosure and at 7 d of post-release in seawater (Figure 1B). The low survival rate (67.4%) of fish packed at 30°C was associated with high ammonia-N (11.4 mg/L) and CO2 (92 mg/L) and low DO (5.4 mg/L) and pH (5.57) after 24 h and with high NNV loads (1.9×10⁶) after 7 d.

**Fry packed at 200, 300, 400 and 500/bag at 20°C with a water/oxygen ratio of 12.5 L/37.5 L**

All fry packed at 200, 300 and 400/bag survived at 24 h of disclosure.
Ammonia-N and CO₂ increased directly with fry density and ranged 8.2–10.4 mg/L and 78–95 mg/L, respectively, whereas DO and pH were inversely related to fry density and ranged 5.0–8.4 mg/L and 5.8–6.6, respectively. Fish packed at 500/bag exhibited 86.4% survival with fish packed at 400 and 500/bag survived 60.8% and 42.6% at 7 day post-release into normal seawater with high NNV loads ranging 4.6×10⁴ and 3.4×10³ (Figure 1C). Fish packed at 300/bag at 20 ºC with a water/oxygen ratio of 12.5 L/37.5 L after 24 h. 

**Table 3:** Effect of temperature on water quality in plastic bags packed with grouper at 300/bag with a water/oxygen ratio of 12.5 L/37.5 L after 24 h. See Table 2 for statistical information.

| Temperature (ºC) | DO (mg/L) | CO₂ (mg/L) | pH | Ammonia-N (mg/L) |
|------------------|-----------|------------|----|-----------------|
| 10               | 5.50 ± 0.03  | 92.21 ± 1.50 | 6.28 ± 0.04 | 9.26 ± 0.10 |
| 20               | 5.41 ± 0.03  | 92.21 ± 1.50 | 6.28 ± 0.04 | 9.26 ± 0.10 |
| 25               | 5.79 ± 0.04  | 88.48 ± 2.33 | 5.62 ± 0.09 | 10.22 ± 0.07 |
| 30               | 6.35 ± 0.05  | 82.51 ± 0.99 | 6.19 ± 0.01 | 9.62 ± 0.10 |

Fish packed at 300/bag with water/oxygen ratios of 10 L/40 L, 12.5 L/37.5 L and 15 L/35 L groups were 300/10 L, 300/12.5 L and 300/15 L which are equivalent to 30, 24 and 20 fry/L, respectively. Therefore, 24 fry/L were determined to be the best packing density for transporting of 55-d grouper.

Fish packed at 300/bag at 20 ºC with a water/oxygen ratio of 12.5 L/37.5 L and 20ºC after 24 h.

**Table 4:** Effect of packing density on water quality in plastic bags packed with Epinephelus coioides Fry after Simulated Transport at conditions: different packing density levels.

| Packing density (fry/bag) | DO (mg/L) | CO₂ (mg/L) | pH | Ammonia-N (mg/L) |
|---------------------------|-----------|------------|----|-----------------|
| 200                       | 8.44 ± 0.12  | 78.40 ± 0.37 | 6.59 ± 0.07 | 8.18 ± 0.08 |
| 300                       | 6.35 ± 0.05  | 81.76 ± 0.23 | 6.28 ± 0.04 | 9.47 ± 0.07 |
| 400                       | 5.04 ± 0.10  | 94.83 ± 1.35 | 5.83 ± 0.06 | 10.37 ± 0.17 |
| 500                       | 4.36 ± 0.08  | 112.37 ± 2.27 | 5.48 ± 0.03 | 13.63 ± 1.00 |

**Table 5:** Effect of zeolite on water quality in plastic bags packed with grouper at 300/bag with a water/oxygen ratio of 12.5 L/37.5 L after 24 h.

| Zeolite particle (g/L) | DO (mg/L) | CO₂ (mg/L) | pH | Ammonia-N (mg/L) |
|------------------------|-----------|------------|----|-----------------|
| 0                      | 6.35 ± 0.05  | 81.76 ± 2.33 | 6.28 ± 0.04 | 9.47 ± 0.07 |
| 5                      | 6.46 ± 0.04  | 42.19 ± 0.99 | 6.91 ± 0.03 | 6.65 ± 0.09 |
| 20                     | 6.48 ± 0.03  | 41.07 ± 0.75 | 7.36 ± 0.03 | 6.21 ± 0.04 |
| 30                     | 6.55 ± 0.04  | 37.71 ± 0.37 | 7.61 ± 0.04 | 6.00 ± 0.04 |

**Discussion**

Earlier research on 60-d grouper larvae indicated that packing at 50/L and 23ºC were the best transport conditions in terms of survival when tested at packing densities of 50, 100 and 200 larvae/L after 8 h of simulated transport [6]. In the present study, all fry packed at 300/bag with water/oxygen ratios of 10 L/40 L, 12.5 L/37.5 L and 15 L/35 L, survived after 24 h of disclosure and at 7 d post-release. Fry densities in the 10 L/40 L, 12.5 L/37.5 L and 15 L/35 L groups were 300/10 L, 300/12.5 L and 300/15 L which are equivalent to 30, 24 and 20 fry/L, respectively. Therefore, 24 fry/L were determined to be the best packing density for transporting of 55-d grouper.

Higher packing densities increase stresses on fish and result in viral replication leading to fish death [8,14]. In the present study, grouper fry...
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pH (5.57) and an NNV load of 5.7×10³, but the survival rate dropped.

The survival rate of 60 d grouper larvae packed at 23°C was higher than in fish packed at 28°C after 8 h of transport [6]. In the present study, all grouper fry packed at 300/bag at 20°C survived after 24 h of disclosure and 7 d post-release. It is interesting to note that grouper fry packed at 300/bag and 3°C survived 100% after 24 h of transport [6]. In the present study, all grouper fry packed at 300/bag at 20°C increased CO₂ to 112 mg/L and ammonia-N to 6.0~6.7 mg/L and CO₂ to 38~42 mg/L, compared to 9.47 mg/L and 82 mg/L, respectively in controls. Adding zeolite maintains better water quality (DO >6.56 mg/L, pH>6.9, ammonia-N <6.6 mg/L and CO₂ <42 mg/L) after disclosure and results in lower NNV loads (<5.0×10⁷) at 7 d post-release.

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