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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 4.1~7.9×10^4 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 4.1×10^3, but fish packed at 25°C and 30°C survived 100% and 67.4%, and had NNV loads of 1.6×10^5 and 1.8×10^6, 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 >5.8, and ammonia-N <11 mg/L, after 24 h. All fish packed at 200 and 300/bag survived with an NNV load of 1.7×10^3, 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 *Betanodavirius* [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].

Groupers are 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]. Famers 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

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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 inflated 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, CO_2 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 Sunte×Model SP-7 pH meter (Suntex, 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]. CO_2 concentration was measured using a trimetric 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 Supermi×(BIO-RAD) and CFX384 Real-Time PCR Detection System (BIO-RAD) to determine NNV expression levels [12,13]. Primers used were NV3, RGNNV RPCR-F, RGNNV 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 Necrosis Virus (RGNNV) 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 pQE plasmid containing the 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 required that $p<0.05$.

Name	Sequence
NV R3	5'-CGAGTCAACACGGGTGAAGA-3'
RGNNV RPCR-F	5'-CAGTCCGACCTCAGTACAC-3'
RGNNV RPCR-R	5'-AACACTCCAGCGACACAG-3'
Actin-F	5'-GGCCGCGACCTCACAGACTACCTC-3'
Actin-R	5'-CCTCTGGGCAACGGAACCTCTCAT-3'

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

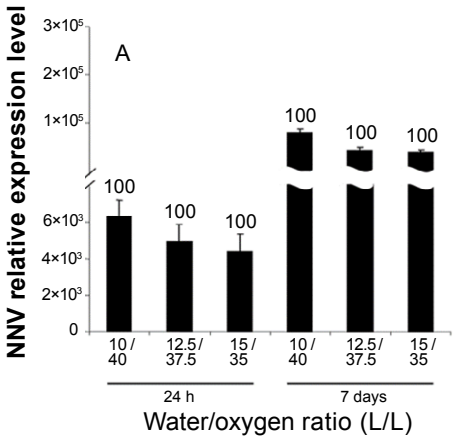

Figure 1a: Relative gene expression level of Nervous Necrosis Virus (NNV) after 24 h transportation and 7 d post-transportation at conditions: different water/oxygen volume ratio.

Water/oxygen ratio	DO (mg/L)	CO_2 (mg/L)	pH	Ammonia-N (mg/L)
10 L/40 L	6.14 ± 0.04^b	85.87 ± 0.38^a	5.63 ± 0.05^a	10.40 ± 0.09^c
12.5 L/37.5 L	6.37 ± 0.03^a	78.03 ± 0.99^b	6.35 ± 0.16^b	9.55 ± 0.10^b
15 L/35 L	6.21 ± 0.03^b	76.91 ± 0.99^b	6.40 ± 0.03^b	8.60 ± 0.14^a

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.

Values are represented as mean ± S.E. Data in the same column with different letters are different ($p<0.05$) among treatments.

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 CO_2 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 4.3×10^3~6.2×10^3 and 4.1×10^4 ~ 7.9×10^4 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 CO_2 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 1.7×10^3~2.8×10^3 and 4.1×10^3~1.6×10^5 at 24 h after disclosure and 7 d post-release, respectively. The NNV load of fry packed at 30°C was 5.6×10^3 and 1.9×10^6 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 CO_2 (92 mg/L) and low DO (5.4 mg/L) and pH (5.57) after 24 h and with high NNV loads (1.9×10^6) 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.

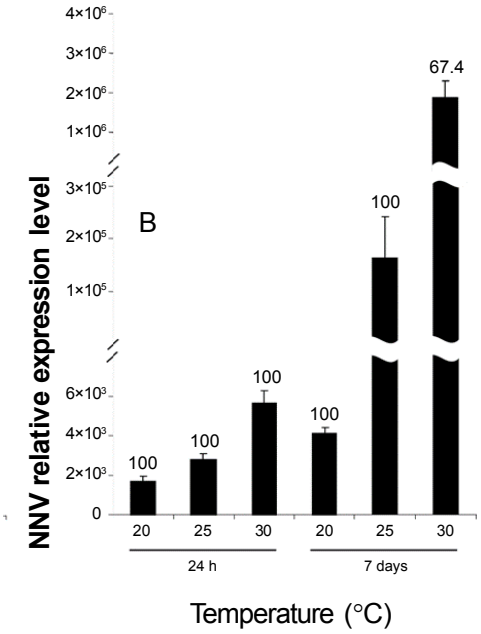

Figure 1b: Relative gene expression level of Nervous Necrosis Virus (NNV) after 24 h transportation and 7 d post-transportation at conditions: different water temperature levels.

Temperature (ºC)	DO (mg/L)	CO_2 (mg/L)	pH	Ammonia-N (mg/L)
20	6.21 ± 0.02[a]	82.51 ± 0.99[b]	6.19 ± 0.01[a]	9.26 ± 0.10[c]
25	5.79 ± 0.04[b]	88.48 ± 2.33[a]	5.62 ± 0.09[b]	10.22 ± 0.07[b]
30	5.41 ± 0.03[c]	92.21 ± 1.50[a]	5.57 ± 0.03[b]	11.40 ± 0.03[a]

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.

Ammonia-N and CO_2 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 low DO (4.36 mg/L) and pH (5.48) and with high ammonia-N (13.63 mg/L) and CO_2 (112 mg/L) (Table 3) and had an average NNV load of 3.4×10^3 (Figure 1C). 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 of 2.4×10^7 and 3.7×10^7, respectively (Figure 1C). Low survival rates were associated with high ammonia-N (10 and 13 mg/L), CO_2 (95 and 112 mg/L) and NNV loading (2.4×10^7 and 3.7×10^7) and low DO (5 and 4.4 mg/L) and pH (5.8 and 5.5) levels.

Fish packed at 300/bag at 20ºC with a water/oxygen ratio of 12.5 L/37.5 L and the addition of zeolite particles (5.20 and 30 g/L)

All fry survived in all treatments at 24 h of disclosure and at 7 d of post-release into normal seawater. Ammonia-N and CO_2 were inversely related to zeolite and ranged 6.0~9.5 mg/L and 38~82 mg/L, respectively, whereas DO and pH increased directly with zeolite and ranged 6.4~6.6 mg L^{-1} and 6.3~7.6, respectively. Adding zeolite decreased ammonia-N and CO_2 levels and increased DO and pH levels (Tables 4 and 5). Fish packed with zeolite had a 100% survival rate at 7 d post-release into normal seawater, with NNV loads ranging 4.6×10^4 ~5.2×10^4 (Figure 1D).

Discussion

Earlier research on 60-d grouper larvae indicated that packing at 50/L and 23°C 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

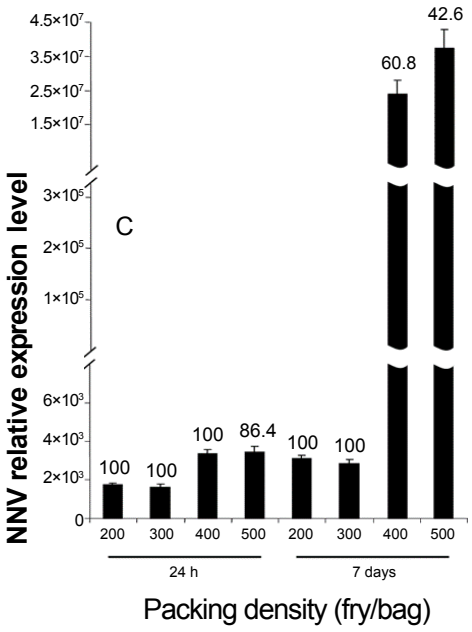

Figure 1c: Relative gene expression level of Nervous Necrosis Virus (NNV) after 24 h transportation and 7 d post-transportation at conditions: different packing density levels.

Packing density (fry/bag)	DO (mg/L)	CO_2 (mg/L)	pH	Ammonia-N (mg/L)
200	8.44 ± 0.12[a]	78.40 ± 2.33[c]	6.59 ± 0.07[a]	8.18 ± 0.08[d]
300	6.35 ± 0.05[b]	81.76 ± 2.33[c]	6.28 ± 0.04[b]	9.47 ± 0.07[c]
400	5.04 ± 0.10[c]	94.83 ± 1.35[b]	5.83 ± 0.06[c]	10.37 ± 0.17[b]
500	4.36 ± 0.08[d]	112.37 ± 2.27[a]	5.48 ± 0.03[d]	13.63 ± 0.10[a]

Table 4: Effect of packing density on water quality in plastic bags packed with grouper at a water/oxygen ratio of 12.5 L/37.5 L and 20˚C after 24 h.

See Table 2 for statistical information.

Zeolite particle (g/L)	DO (mg/L)	CO_2 (mg/L)	pH	Ammonia-N (mg/L)
0	6.35 ± 0.05[b]	81.76 ± 2.33[a]	6.28 ± 0.04[d]	9.47 ± 0.07[a]
5	6.46 ± 0.04[a]	42.19 ± 0.99[b]	6.91 ± 0.03[c]	6.65 ± 0.09[b]
20	6.48 ± 0.03[a]	41.07 ± 0.75[bc]	7.36 ± 0.03[b]	6.21 ± 0.04[c]
30	6.55 ± 0.04[a]	37.71 ± 0.37[d]	7.61 ± 0.04[a]	6.00 ± 0.04[d]

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.

See Table 2 for statistical information

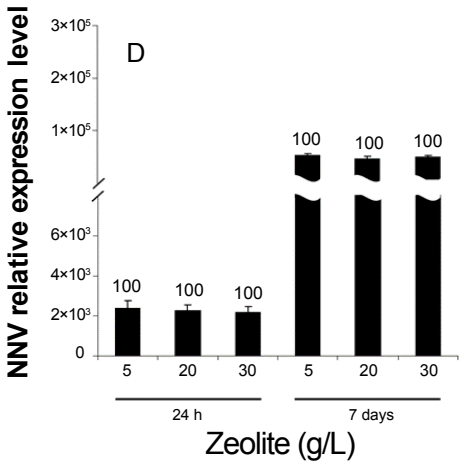

Figure 1d: Relative gene expression level of Nervous Necrosis Virus (NNV) after 24 h transportation and 7 d post-transportation at conditions: different zeolite concentrations. Each bar represents the mean value from 15 determinations with the Standard Error (SE). Data with different letters (a, b, c) (x, y, z) significantly differ (p<0.05) among treatments after 24 h of disclosure and at 7 d post-release in normal seawater. The number above each bar represents survival rate.

packed at 500/bag survived 86.4% and 42.6% after 24 h of stimulated transport and 7 d post-release with NNV loads of 3.3×10^3 and 3.7×10^7, respectively. Grouper fry packed at 400/bag experienced 60.8% survival at 7 d post-release with an NNV load of 2.4×10^7. Therefore, grouper fry should not be packed at 400/bag or higher to avoid proliferation of NNV up to 2.3×10^7. A grouper fry packing density of 400/bag is likely to result in increases in ammonia-N (>10.37 mg/L) and CO_2 (>94.83 mg/L) and decreases in DO (<5.04 mg L^{-1}) and pH (<5.83), leading to increased NNV loads and low grouper survival rates. A (low) survival rate of 42.6% was considered to be associated with a (high) 3.7×10^7 NNV load.

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/bay and 3°C survived 100% after 24 h with high ammonia-N (11.4 mg/L) and CO_2 (92.2 mg/L), low DO (5.41 mg/L) and pH (5.57) and an NNV load of 5.7×10^3, but the survival rate dropped to 67.4% with a significant increase in the NNV load to 1.9×10^6. High NNV replication was considered to be associated with stress caused by high water temperature, ammonia-N and CO_2 and low DO and pH. Previous research indicated that the copy number of WSSV was much higher at 30°C than that of WSSV at 15, 20 and 25°C [15]. The fact that NNV load was higher at 30°C indicated higher temperatures especially 30°C are more susceptible for NNV replication in the present study.

Water quality parameters like temperature, DO, CO_2, pH and ammonia-N are critical factors during live fish transport and are affected by density and other packing conditions. High concentrations of ammonia and CO_2 result in high mortality during transport [16]. Indian major carp after 48 h of transportation in a plastic bag resulted in a deterioration of water quality due to the accumulation of ammonia and depletion of oxygen that subsequently lead to increase mortality [17]. In the present study, grouper fry packed at 500/bag resulted in high CO_2 and ammonia-N and low pH at 112 mg/L, 13.6 mg/L and 5.5, respectively, after 24 h of simulated transport with a survival rate of 86.4%. Therefore, fish packed at high densities suffered deteriorated water quality leading to poor survival rates.

Adding zeolite removes ammonia [18]. Adding zeolite at 7 g/L in bags packed with Indian carp fry reduced the levels of ammonia and increased fry survival rates [17]. In the present study, grouper fry packed in 50 L bag containing zeolite at 5, 20 and 30 g/L significantly reduced ammonia-N to 6.0~6.7 mg/L and CO_2 to 38~42 mg/L, compared to 9.47 mg/L and 82 mg/L, respectively in controls. Adding zeolite improves water quality and reduces NNV load to $2.2\sim2.4\times10^3$ after 24 h and $4.7\sim5.3\times10^4$ after 7 d, respectively with a 100% fish survival rate. It is suggested that adding zeolite reduce ammonia concentration that subsequently leads to less viral replication. Therefore, the addition of zeolite resulted in better water quality, lower NNV loads and higher survival rates of fish during simulated transport.

In conclusion, grouper fry packed at 300/bag and a water/oxygen ratio of 12.5 L/37.5 L at a water temperature of 20°C experienced a 100% survival rate after 24 h of disclosure and 7 d post-release in normal seawater and had NNV loads of 1.7×10^3 and 4.2×10^3, respectively. Fish packed at 500/bag at 20°C increased CO_2 to 112 mg/L and ammonia-N to 13.63 mg/L and decreased DO to 4.36 mg/L and pH to 5.48, leading to high mortality after 24 h. Adding zeolite maintains better water quality (DO >6.56 mg/L, pH>6.9, ammonia-N <6.6 mg/L and CO_2 <42 mg/L) after disclosure and results in lower NNV loads (<5.0×10^4) at 7 d post-release.

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