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Induction of Intersex and Masculinization of the Equilateral Venus, *Gomphina veneriformis* (Bivalvia: Veneridae) by Zinc

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Abstract: This study aims to find out the effect of heavy metals, as is the case of EDCs (endocrine disrupting chemicals), on reproductive endocrine disruption of aquatic animals. In the present experiment zinc, which is a heavy metal well known for its androgenous activity, was used. The experimental period was 24 weeks, starting in November during the inactive stage of the clam's reproductive cycle. The experimental groups were composed of one control condition and three zinc exposure conditions (0.64, 1.07, and 1.79 mg/L). The sex ratio (F:M) was 1:1.06 in the control group and 1:1.70 in all the exposed group, illustrating the tendency for higher proportion of males with increases in zinc concentration. Gonad maturity was higher in 1.07, and 1.79 mg/L groups compared to the control group, with higher maturity observed in males than females. Intersex individuals made up 24.7% of the exposed group, while females exhibited a higher ratio than the males with increasing zinc concentration. The results of this study indicate that zinc functions as an androgenic effector on the reproduction of *Gomphina veneriformis*.

Key words: *Gomphina veneriformis*, sex ratio, gonadal activity, intersex, androgenic effector, zinc

INTRODUCTION

Heavy metals and chemicals in marine environments affect the survival and growth of bivalves (Byrne and O'Halloran, 2001). Furthermore, heavy metals have also been reported to induce histopathological changes, physiological disorders, and abnormal reproduction (Mauri et al., 1990; Rainbow, 1993; Munkittrick and Van Der Kraak, 2000; Gauthier-Clerc et al., 2002; Siah et al., 2003; Quinn et al., 2004, 2006).

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Trace metals are usually divided into two classes. The first is the essential metals such as Fe, Mg, Mn, Co, Zn, and Cu, which play essential roles in biochemical processes. The second group of metals includes Cd, Hg, Cr, and Pb, which have no determined biochemical functions, and are regarded as pollutants in aquatic environments (Viarengo, 1985). However, high concentrations of essential heavy metals can also induce a diverse range of physiological imbalances, which have been linked to structural abnormalities in tissues and impairments in biochemical functions (Olsson, 1998).

Among other non biological uses, zinc can be employed in the prevention of corrosion of iron or steel. Structures plated with zinc are widely used in coastal areas, including aquacultural structures, which may act as sources of zinc pollution into the marine environments.

High concentration of zinc in aquatic environments has been reported to inhibit germ cell development in *Cerastoderma edule* (Timmermans et al., 1996). Furthermore, high zinc exposure impedes respiration in rainbow trout, *Salmo gairdneri* (Skidmore, 1970), and reduces feeding and hatching, and embryo survival rate of zebrafish, *Danio rerio* (Dave et al., 1987). However, data of the zinc effect on the reproduction of aquatic organisms is still scarce.

Equilateral venus, *Gomphina veneriformis* used in this study belongs to Veneridae. Its shell length ranges in size from 2 to 4 cm, inhabits sandy benthic environments at a depth of 1-5 m, and filter-feeds on phytoplankton or low quality organic matter, similar to most other bivalves. This species is dominant in the eastern coastal waters of the Korean peninsula, and is an important commercial clam in the region (Yoo, 1988).

In order to better understand the effects of heavy metals on the reproduction of marine invertebrates, we aim to determine the effects of zinc exposure on sex ratio imbalances, changes in gonadal activity and intersexuality in the equilateral venus.

MATERIALS AND METHODS

Exposure design and test chemical

The equilateral venus specimens used in this research were collected from the coastal region of Jumunjin (N 37°54'34", E 128°49'11"), in the eastern coastal waters of Korea, during in October 2004. Adult animals with shell lengths ranging from 3.5-4.0 cm were used (Fig. 1). The clams were acclimated for a week before being subjected to the zinc exposure experiments in November 2004, which according to Park et al. (2003) is the inactive reproductive stage. The exposure experiment in the laboratory lasted for 24 weeks. Thirty liter glass aquariums were used for the experiments, designed with circulatory systems, and 10 cm deep benthic sediment collected from the same region in the wild. During the experimental period, the average water temperature was 15.3°C (13.3-17.9°C), salinity was 33.7‰, and pH 8.18. A 10^6 cell/mL/tank amount of *Isochrysis galbana* was fed daily.

The test solution used was prepared by diluting a standard solution of 1,000 mg/L Zn, made with $ZnSO_4 \cdot 7H_2O$ (zinc sulfate heptahydrate, Sigma) and distilled water, for each test concentration. The control group received filtered sea water (φ 0.2 μm) without added zinc, while the zinc exposure groups were subjected to nominal concentrations, namely 0.64, 1.07, and 1.79 mg/L Zn, through two acute toxicity experiments. Forty clams were maintained in each aquarium, and the experiment was replicated.

Analysis

All information presented in the results represents analysis after the 24 weeks study. However, in the case of the 0.64 mg/L group, all clams perished after 8 weeks. Accordingly, the results gathered 8 weeks after the beginning of exposure were analyzed. Wild specimens were collected on the same sampling area when we sampled experimental specimens for study. The total number of individuals used in analysis was 315 (Table 1).

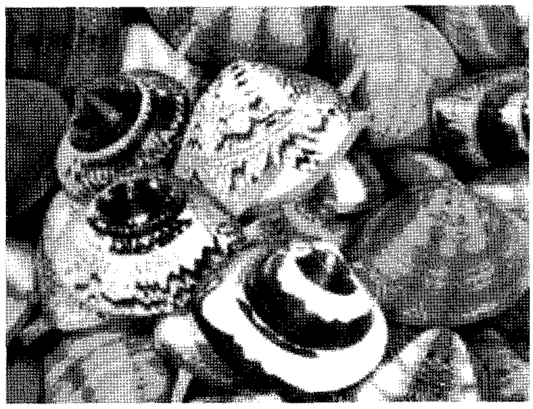

Fig. 1. Equilateral venus, *Gomphina veneriformis*.

Histological analysis: The experimental organisms were dissected, and their visceral mass, which included the gonad, were fixed in aqueous Bouin's solution for 18 hours. Tissues were then embedded in paraffin wax blocks, frozen and then prepared for sectioning. The embedded tissues were serially sectioned at 4-6 μm thickness, mounted on slides and subjected to Mayer's hematoxylin and 0.5% eosin (H-E) double staining.

Gonadal activity: Gonadal maturity was divided into five phases: an inactive stage (In); early active stages I (Ea I), II (Ea II), and III (Ea III); and a late active stage (La), by observing the gonad preparations (Fig. 2). The gonad index (GI) of each individual was formulated by a modified Eversole`s method (1997) (Fig. 3).

Intersexuality: Intersexuality was based on the definitive presence of male germ cells in females, and oocytes in males (Fig. 7).

Statistical analysis: For statistical analysis, significance ($P<0.05$) was tested through examination of the minimum significance of multiple comparisons after having performed Student t-test (for gonad index) and Chi-square (for sex ratio) using the SPSS statistical program (Version 14.0, SPSS Inc., Chicago, IL, USA).

Table 1. Number of analyzed individuals and sex ratio of the equilateral venus, *Gomphina veneriformis* exposed to zinc

Condition		Exposure duration	Number					Sex ratio (F:M)	X^2	*P* value
			Total	Inactive	Intersex	Female	Male			
Wild		-	86	4	-	40	42	1:1.05	0.49	0.825
Control		24W	71	5	2	31	33	1:1.06	0.63	0.803
Zinc exposure group	0.64 mg/L	8W	24	1	3	11	9	1:0.82	0.20	0.655
	1.07 mg/L	24W	67	1	17	19	30	1:1.58	2.47	0.116
	1.79 mg/L	24W	67	2	18	13	34	1:2.62	9.38	0.002

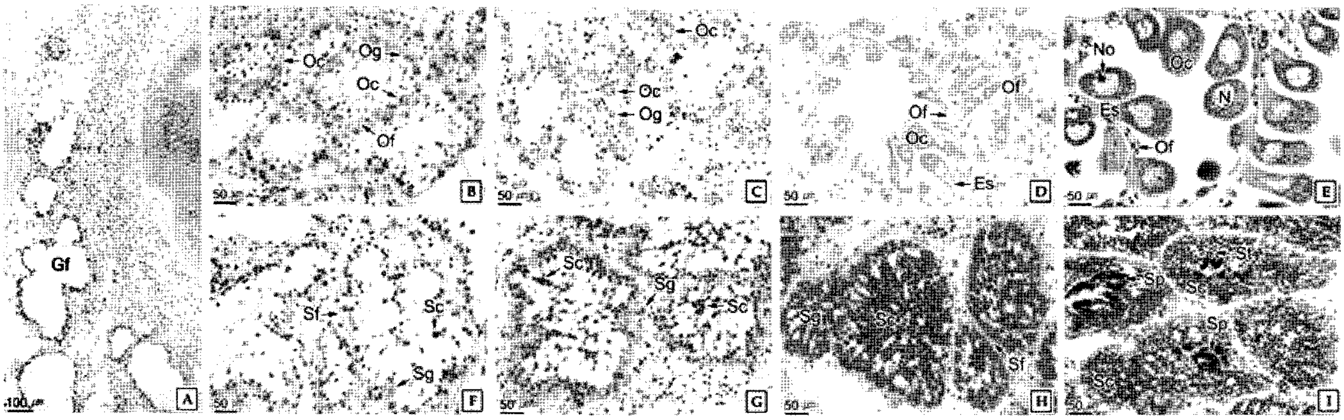

Fig. 2. Photomicrographs on gonadal development of the equilateral venus, *Gomphina veneriformis*. A, inactive stage. B, early active stage I of female; oogonia (Og) in the oogenic follicle (Of) wall. C, early active stage II of female; note proliferation of oogonia in the oogenic follicle. D, early active stage III of female; showing the oocyte (Oc) connected to the oogenic follicle by the egg stalk (Es). E, late active stage of female. F, early active stage I of male; showing the spermatogonia (Sg) near the spermatogenic follicle (Sf). G, early active stage II of male; spermatogonia in the spermatogenic follicle. H, early active stage III of male; numerous spermatocytes (Sc) and spermatids in the lumen. I, late active stage of male, showing the sperm (Sp) in the lumen. Gf, gametogenic follicle; N, nucleus; No, nucleolus.

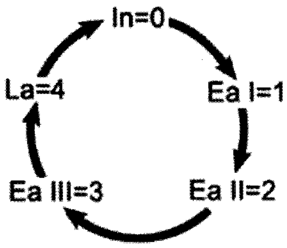

Fig. 3. Scoring system used to rank different stages of gonadal development of the equilateral venus, *Gomphina veneriformis*. In, inactive stage; Ea I, early active stage I; Ea II, early active stage II; Ea III, early active stage III; La, late active stage.

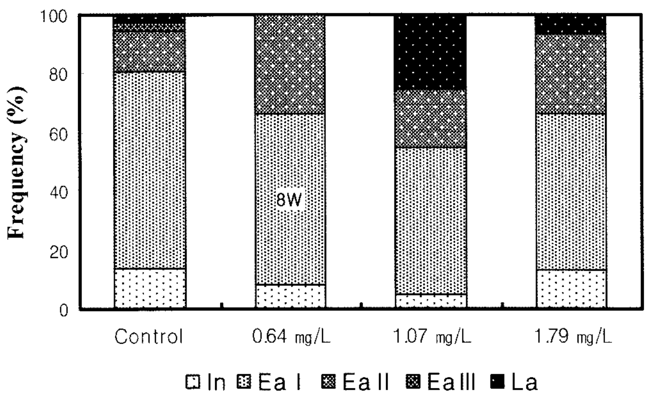

Fig. 4. Frequency of ovarian development stage of the equilateral venus, *Gomphina veneriformis* exposed to zinc for 24 weeks. In, inactive stage; Ea I, early active stage I; Ea II, early active stage II; Ea III, early active stage III; La, late active stage.

Results

Sex ratio

The sex ratio of *Gomphina veneriformis* in the control group was 1:1.06 (female:male). The overall sex ratio for 116 individuals (excluding inactive stage and intersex individuals) in all the exposed groups were 1:1.70 (n=43:73), illustrating a higher proportion of males in comparison to female (Chi-square, 7.759; $P<0.05$). Specific sex ratio in the zinc exposure groups, namely the 0.64, 1.07, and 1.79 mg/L groups, were 1:0.82, 1:1.58, and 1:2.62, respectively. Especially, female in the 1.79 mg/L groups was significantly lower than male ($P<0.05$) (Table 1).

Gonadal activity

Gonadal development: The early active stage I (Ea I) of ovarian development accounted for 66.7% of specimens in the control group. In the 0.64 mg/L group, Ea I and Ea II were observed in 58.3 and 33.3% of specimens, respectively. In the 1.07 mg/L group, Ea I and Ea II were observed in 50.0 and 20.0% of specimens, respectively. In the 1.79 mg/L group, Ea I and Ea II were observed in 53.3 and 26.7% of specimens, respectively (Fig. 4).

For males in the control group, 34.2 and 44.7% exhibited Ea I and Ea II were exhibited 34.2 and 44.7%, respectively. The 0.64 mg/L group displayed high proportions of Ea I and Ea II at 40.0 and 50.0%, respectively. In the 1.07 mg/L group, Ea II and Ea III accounted for 51.6 and 19.4% of specimens, respectively, while in the 1.79 mg/L group, Ea I and Ea II accounted for 58.3 and 27.8% of specimens, respectively (Fig. 5).

Gonad index (GI): GI for females was highest in the 1.07 mg/L group at 1.9. The control group and 1.79 mg/L group had indices of 1.1 and 1.3, respectively. In the case of males, GI was highest in the 1.07 mg/L group at 2.2. The control group and 1.79 mg/L group had indices of 1.5 and 1.5, respectively. GI in the 0.64 mg/L group was 1.4 for females and 1.6 for males, illustrating no significant

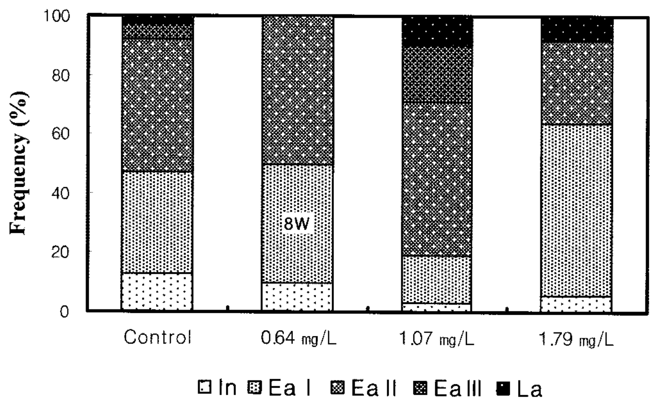

Fig. 5. Frequency of testicular development stage of the equilateral venus, *Gomphina veneriformis* exposed to zinc for 24 weeks. In, inactive stage; Ea I, early active stage I; Ea II, early active stage II; Ea III, early active stage III; La, late active stage.

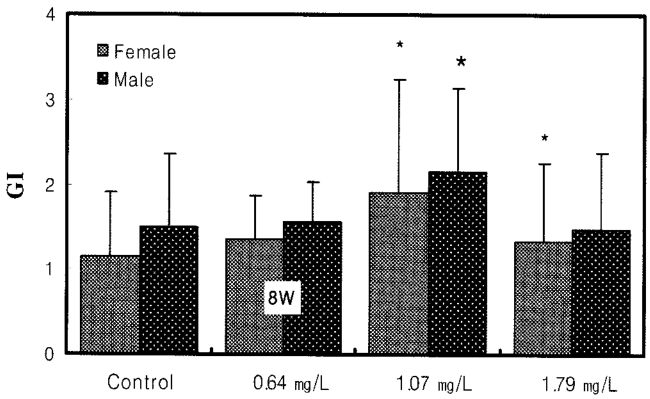

Fig. 6. Gonad index (GI) of the equilateral venus, *Gomphina veneriformis* exposed to zinc for 24 weeks. Vertical bar: SD. *: significantly different from control (P<0.05).

differences (P>0.05) with the control group (Fig. 6).

Intersexuality

The developmental phase of male and female sex germ cells, which indicate intersexuality, was different depending on the individual (Fig. 7). Intersex was not observed in the wild group, while in the laboratory experimental groups,

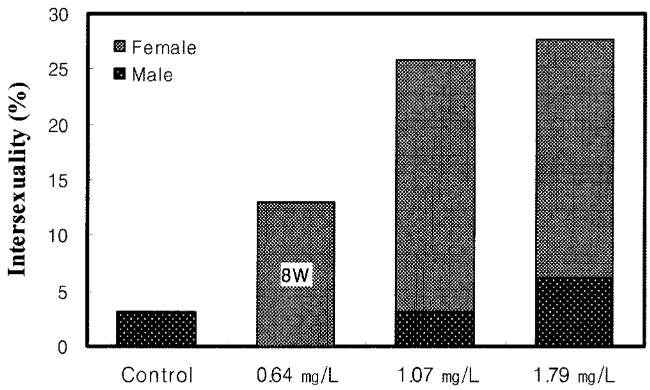

Fig. 8. Intersexuality of the equilateral venus, *Gomphina veneriformis* exposed to zinc for 24 weeks.

including the control group, intersexuality among the 220 individuals (with the exclusion of the individuals in their inactive stage) was 18.2% (n=40/220). Intersexuality in the control group was 3.0% (n=2/66). Overall intersexuality in the zinc exposure groups was 24.7% (n=38/154), while specific ranges in the 0.64, 1.07, and 1.79 mg/L groups were 13.0% (n=3/23), 25.8% (n=17/66), and 27.7% (n=18/65), respectively. Intersexuality of the equilateral venus in the zinc exposure group indicates an increasing tendency with increasing zinc concentration. Furthermore, intersexuality of the female and male was 20.8% (n=32/154) and 3.9% (n=6/154) respectively; it was indicated female higher than males (Fig. 8).

DISCUSSION

Historically, studies on reproductive changes in aquatic animals, including sex ratio, gonadal maturation, and intersexuality, have been focused on EDCs (Siah et al., 2003). Among reports on sex ratio changes in bivalves, the short-necked clam, *Tapes philippinarum,* exhibited different sex ratio between the control group and those exposed to nonylphenol conditions (Matozzo and Marin, 2005). In another example, 63% of soft-shell clams, *Mya*

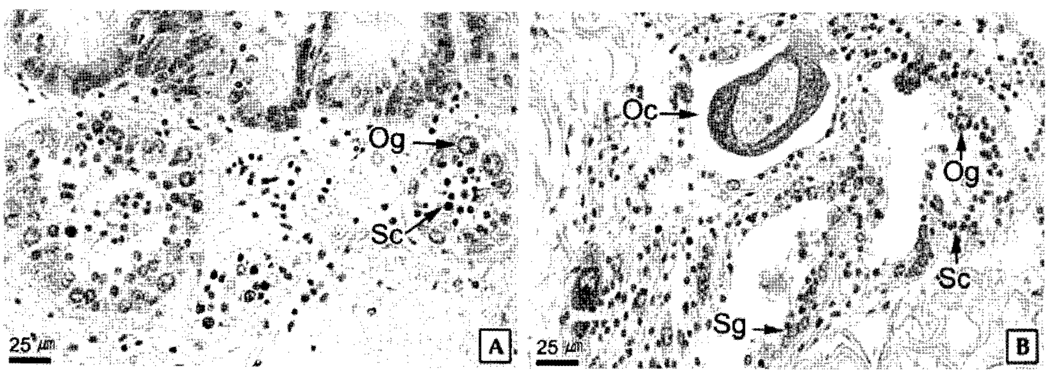

Fig. 7. Photomicrographs of intersexuality of the equilateral venus, *Gomphina veneriformis* exposed to zinc. A, spermatocyte (Sc) in ovary. B, oocyte (Oc) in testis. Og, oogonium; Sg, spermatogoium.

arenaria from Saint Lawrence River (Quebec, Canada) which is characterized by high concentrations of tributyltin were females (Gagné et al., 2003).

In this study, the results indicated similar sex ratios between the wild and laboratory control groups. However, the zinc exposure groups exhibited a trend of increasing males with increasing zinc concentration.

In this study, the need for introduction of new interpretation on reproduction of bivalves with inactive stage during the reproductive cycle, even if it is a dioecism, to the result on changes in sex ratio is presented. If the result on sex ratio of this study is interpreted with the concept that sex that was determined at the initial stage, as in the case of vertebrates including fishes and majority of dioecism, is sustained throughout the life of the animal, then there are aspects that are difficult to understand.

Park et al. (2003) suggested that the sex of *Gomphina veneriformis* begins newly again after it undergoes inactive stage during the annual reproductive cycle. This study began zinc exposure experiment from the inactive stage. It is determined that zinc elevates the ratio of male by inducing the sex of *Gomphina veneriformis* to become male.

Among the various environmental factors that influence gonadal development in invertebrates, water temperature is most important, followed by physical, chemical, and/or biological factors (Mackie, 1984). The results of a comparative analysis between cockle, *Cerastoderma edule* and Baltic clam, *Macoma balthica* in a polluted and non-polluted area, illustrated reductions in the condition index (CI), gonad index (GI), and fertilization rate of the clams within the polluted area (Timmermans et al., 1996). Moreover, the exposure of *Mytilus edulis* to copper and diesel oil resulted in a reduction of its spawning rate in comparison to control group (Stromgren and Nielsen, 1991). Also observed, was the inhibition of gametogenesis in *Rangia cuneata* exposed to high concentrations of cadmium (McConnell et al., 1995).

In addition, *Mya arenaria*, in regions polluted with heavy metals (Hg: 100 ng/g, Pb: 44-66, Zn: 43-145, and Cu: 6-33 μg/g) (Barbeau et al., 1981) and PAHs (500-4500 ng/g) (Martel et al., 1986), exhibited delays in gonadal maturation when compared to clams in non-polluted regions (Blaise et al., 1996). While clams in non-polluted regions were either mature or spawning, those in the polluted regions were in the inactive or active stages. Furthermore, for both males and females, gonadosomatic index (GSI) was substantially lower in the polluted regions (2-4) when compared to the non-polluted regions (6-9) (Gauthier-Clerc et al., 2002; Siah et al., 2003).

In this study, comparative analysis based on GI of equilateral venus illustrated higher gonadal activity in the zinc exposure group as compared to the control groups, for both sexes. In particular, GI was highest in the 1.07 mg/L group. Such results indicate the possibility that even low concentrations of zinc could induce accelerations of gonadal development in equilateral venus.

Intersexuality, due to a broad range of aquatic pollutants, and imposex that was effected by TBT, have been reported in aquatic organisms, including fish and gastropods, which are being used as sentinel of aquatic pollution (Bortone and Davis, 1994; Munkittrick and Van Der Kraak, 2000; Chesman and Langston, 2006). Intersex in the mosquitofish, *Gambusia affinis* exposed to paper mill effluent (Drysdale and Bortone, 1989), and testis-ova in the Japanese medaka, *Oryzias latipes* exposed to nonylphenol (Gray and Metcalfe, 1997), have been reported. Furthermore, imposex in *Nucella lapillus* (Gibbs and Byrne, 1986), *Thais clavigera* and *T. bronniin* (Horiguchi et al., 1994), *T. clavigera*, *T. bronniin*, and *T. luteostoma* (Kahng et al., 1996), and *Haliotis madaka* (Horiguchi et al., 2000) exposed to tributyltin were also reported. In the case of bivalves, Morcillo and Porte (2000) suggested a potential masculinization of clam physiology as a consequence of *Ruditapes decussata* transplanted to an TBT-polluted marina for a period up to 5 weeks and Chesman and Langston (2006) presented evidence that intersex, in the form of ovotestis, is occurring in the common estuarine bivalve *Scrobicularia plana*.

In this study, intersexuality was not observed in the wild group, while only 3.0% of specimens in the laboratory control group were identified as intersex. The zinc exposure groups exhibited a tendency for increasing intersexuality with increasing zinc concentration, with a substantially higher ratio of intersexuality in females than males. Nevertheless, zinc was determined to be a factor for inducing abnormality in the reproductive system of equilateral venus, which was also induced by nonylphenol (Lee and Park, 2007). In existing studies on higher vertebrates, zinc is reported to be a compound with a strong masculinization quality (Bedwal and Bahuguna, 1994).

In this study, it was also found to have masculinization affects in terms of its influence on sex ratio and intersexuality. This study was commenced with the inactive stage during the reproductive cycle of *Gomphina veneriformis*, and it is considered that such result may differ in accordance with the timing of the experiment.

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