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Review

Biological effects of low concentrations of tributyltin on the caprellid amphipod *Caprella danilevskii*

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Abstract — In order to examine the biological effects of tributyltin (TBT), experiments involving the exposure of 5 levels of TBT concentrations (0, 10, 100, 1000 and 10000 ng l^{-1}) were conducted on the caprellid amphipod *Caprella danilevskii*, both over a generation after hatching (50 days) and embryonic stage (5 days). In TBT exposure after hatching, marked delays in growth and molting during the early developmental stage and mature stage were found in both 100 and 1000 ng TBTCl l^{-1} concentrations in spite of the sex. All specimens died in 10000 ng TBTCl l^{-1} within 4 days after hatching. Inhibition of maturation and reproduction such as delaying in the achievement of maturity and a decrease in the number of juveniles hatched was apparent in 10 and 100 ng TBTCl l^{-1} concentrations. Furthermore, brood loss, and failure in egg formation and hatching were observed as the TBT concentration became higher. No significant changes in sex ratio were seen in response to TBT exposure after hatching. A drastic decrease in survival rate was observed at 10 ng TBTCl l^{-1} which corresponds to the mean level in coastal waters. In embryonic exposure, although the female proportion was 36% of the total in the control, its proportion increased up to 80% at 100 and 1000 ng l^{-1} in the hatched juvenile. All specimens died in 10000 ng TBTCl l^{-1} within 5 days after spawning due to the acute toxic concentration for the species. No significant differences were observed to occur in the sex ratio in response to the exposure after hatching (50 days) in a previous study. Sex disturbance might therefore be induced during the embryonic stage in the caprellid. Reproductive inhibitions such as brood loss and oogenesis inhibition occurred even at 10–100 ng TBTCl l^{-1} exposures in the short-term period in both parental females and their offspring females. The embryo survival rate in the offspring decreased drastically as the TBT concentrations increased, with the decrease being observed at TBT concentrations as low as 10 ng l^{-1} during 5 days. In parental females, the survival rate also decreased at more than 100 ng TBTCl l^{-1}, despite transfer into the no TBT-added seawater after 5 days. Therefore, our data suggest that nanogram concentrations TBT exposure, both short- and long-term, in the coastal environment might critically damage the life history characters of caprellids, and may influence populations of *C. danilevskii* in the coastal ecosystem.

Key words: tributyltin, caprellid, sex ratio, survival rate, growth rate, reproduction, morphological alterations

Introduction

During the past several decades, butyltin compounds (BTs) have been widely used as an antifouling agent in paints for boats, ships, and aquaculture nets (Fent 1996, Champ and Seligman 1996), thus these compounds have been found in a variety of marine organisms, often at concentrations exceeding acute and chronic toxicity levels (Bryan and Gibbs 1991, Alzieu 1996). The serious pollution and hazardous effects of antifouling paints containing butyltins in marine ecosystem have become a significant environmental issue all over the world (Champ and Wade 1996, Bosselmann 1996). To prevent the destruction of marine ecosystem, the use of antifouling paints containing tributyltin on small boats and fish farming equipment has been banned or regulated in developed countries since the late 1980s (Champ and Wade 1996, Bosselmann 1996). Nevertheless, significant accumulation of BTs has been noted at various trophic levels in the marine food chain including plankton, algae, crustaceans, fishes and cetaceans, indicating that BTs impact continues to be felt in marine ecosystem.

BTs, especially tributyltin (TBT), are reported to be the very toxic compounds, and at nanogram-per-litter levels, TBT has adverse effects on many aquatic organisms, i.e. producing retardation of regenerative growth, delay in molt, reduction in burrowing activity and deformities in limbs in the fiddler crab (Weis and Perlmutter 1987, Weis et al. 1987, Weis and Kim

1988), impairment of egg production in the calanoid copepod (Johansen and Møhlenberg 1987), reduction in larval growth in the silverside (Hall et al. 1988) and avoiding reactions in the Baltic amphipod (Laughlin et al. 1984). Recently, a relationship among metabolic capacity, accumulation and toxicity of BTs in marine organisms has been reported in terms of comparisons of BT residue levels in organisms at various trophic levels in the food chain (Fent 1996, Takahashi et al. 1999, Ohji et al. 2002a). The results indicated that though BTs accumulated in most organisms at levels up to 70000 times higher than those in seawater, no considerable biomagnification was observed in the higher levels of the food chain (Takahashi et al. 1999). Especially, high concentrations were found in lower trophic animals such as caprellids because of their lower metabolic capacity to degrade TBT, and therefore these organisms accumulated BTs at elevated concentrations (78–180 ng g^{-1} wet wt) than other organisms in the coastal ecosystem (Takahashi et al. 1999, Ohji et al. 2002a). The BTs seem to be accumulated in a species specific manner. Thus, studying the implications of species-specific accumulation and the biological effects of BTs on the caprellids may provide some clues in understanding the accumulation mechanisms in the coastal ecosystem as well as the mode of action of BTs in organisms. TBT had strong effects on the development of imposex for females in the dog whelk in exposure experiments after hatching (Gibbs et al. 1988). TBT acts as a competitive inhibitor of cytochrome P450-mediated aromatase, resulting in the increase of androgens (Spooner et al. 1991, Bettin et al. 1996) and inhibition of androgen elimination (Bettin et al. 1996) in gastropods. It is reported that intersex individual in the caprellid was observed in the coastal waters (Takeuchi 1990). Therefore, the sex disturbance might also occur in response to TBT exposure after hatching in the caprellid. However, the action mechanism of TBT might differ among organisms and that the effects of TBT exposure might differ according to the developmental stage. Therefore, in the present study, two periods of TBT exposure such as after hatching and during the embryonic stage were set in order to examine the biological effects of TBT on the caprellid.

The caprellid amphipods are small crustaceans (1 to 3 cm in body length), and are distributed worldwide, living especially in algae beds, on buoys, and on aquaculture nets of the subtidal zone in temperate regions (McCain and Steinberg 1970). Caprellids are an important trophic link as one of the dominant secondary producers between unicellular algae and fishes in coastal water ecosystem. Furthermore, these organisms are important prey resources for small fishes in coastal water ecosystem (Fuse 1962, Caine 1989, Holbrook and Schmitt 1992). The generation length and life span of *Caprella* have been well investigated (Takeuchi and Hirano 1991). *Caprella danilevskii* has a short generation duration of 25.6 d which includes the incubation time of embryos and maturation time of hatched juveniles, and has a shortened life-span of 1–3 months (Takeuchi and Hirano 1991). Therefore, caprellids may prove to be a convenient and important model for the study of the biological effects of TBT in the coastal ecosystem. Recently, usage of caprellids to monitor small temporal and spatial changes in baseline concentrations of BTs was proposed, that is *Caprella* watch (Ohji et al. 2002a). However, little information is presently available regarding TBT's biological effects on such characteristics as sex ratio, survival rate, growth rate and reproduction. The determination of such effects is a prerequisite to reliable biomonitoring of the state of coastal ecosystem using *C. danilevskii* as a model.

The objectives of the present study were to examine the biological effects of TBT exposure after hatching and during the embryonic stage of the caprellid amphipod *Caprella danilevskii* Czerniavski. The results form the basis of discussions on the fluctuation of abundance of this species in the coastal ecosystem as well as the biological impact of TBT on it.

Materials and Methods

Exposure after hatching

Specimens

Caprella danilevskii was collected by SCUBA from the rocky shore in Otsuchi Bay, northeastern Japan, and the specimens were brought back to the laboratory and kept in an aquarium provided with running seawater. Premature females and mature males were sorted and provided for the experiments (Fig. 1). Those specimens were kept in deep Petri dishes (6 cm in diameter, 6 cm in height) which contained the filtered seawater with a Teflon mesh piece (2 cm×2 cm) as substrate, and maintained at 20°C under a 12 : 12 hours light : dark photoperiod. One ovigerous mature female was allocated per dish, and a total of 4 females were prepared for an exposure experiment (20 females in 5 concentration-exposure experiments). Diatom colonies *Chaetoceros calcitrans* (Paulsen) Takano were added once a day to each Petri dish this amount was more than sufficient to meet the daily dietary demands of the caprellids. The seawater in each dish was changed every day, and Petri dishes and Teflon mesh pieces were replaced every two days. After the confirmation that premature females had reached the mature stage, mature females were allowed to copulate with males, thus stimulating the release of eggs in the brood pouch. After releasing eggs into the

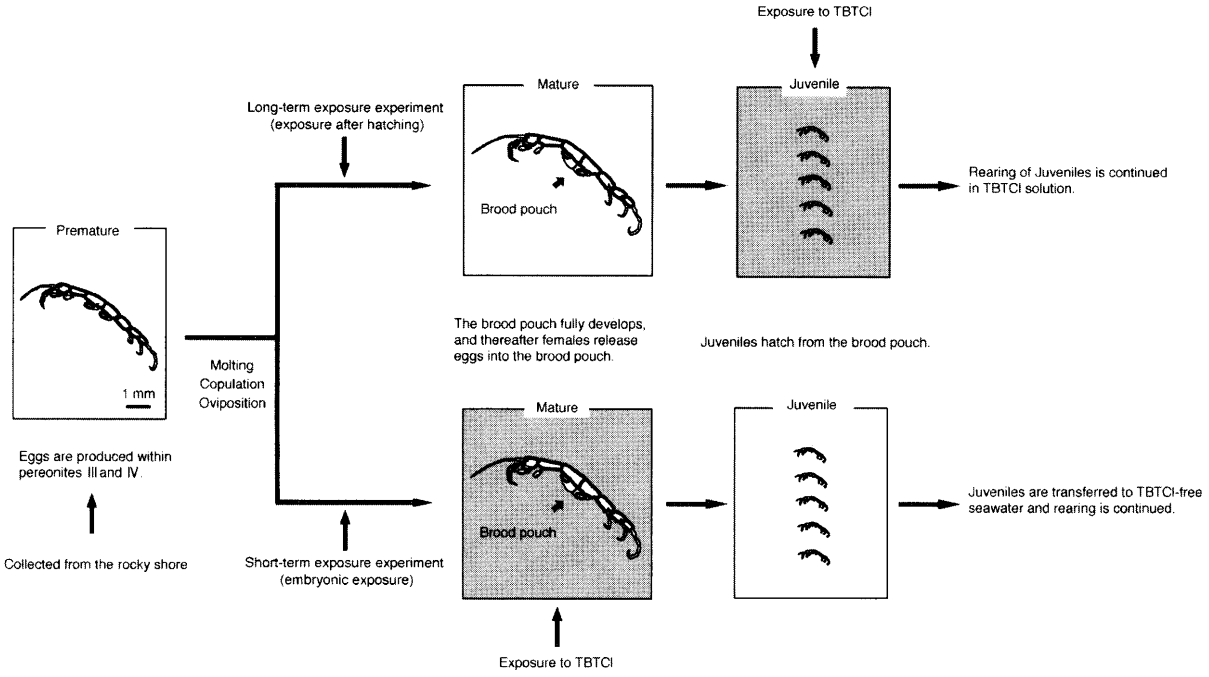

Fig. 1. Schematic view of the experimental methodology used to investigate the biological effects of TBTCl.

brood pouch, mature males were transferred to other Petri dishes, and ovigerous mature females were held here within the filtered seawater. The condition of mature females such as hatching and the emergence of juveniles was observed at 12 hours intervals each day at the same time under a binocular microscope.

Seawater and TBT solution

The seawater used for the present experiments was collected at a 10 m depth outside of Otsuchi Bay, where TBT concentrations at 0.5 and 10 m deep were confirmed to be less than the detection limit (Takahashi et al. 1999, Ohji et al. 2002a), and stored in a 20 l polyethylene tank. The tributyltin-seawater solution and the control seawater that contains only acetone were made in the following procedure. Prior to the TBT exposure experiments, the seawater was filtered through a 0.47 μm Millipore filter. A solution of 10000 ng TBTCl l^{-1} was made by adding 5 μl of 2000 mg TBTCl l^{-1} acetone solution to 1 l of seawater, and thereafter the solution was stirred for 12 hours. Control and dilute solutions were made by adjusting to 5 μl acetone l^{-1} seawater. In the present study, 5 test concentrations of TBTCl (0, 10, 100, 1000 and 10000 ng l^{-1}) were prepared by dilution of the stock solution. Those condensed and dilute solutions were made every week. The 5 test concentrations of TBTCl were measured to confirm the accuracy of TBTCl present in those test solutions during the experiment in the previous report (Ohji et al. 2002a). The concentrations remained the same between pre- and post-experiments.

Chronic toxicity experiments

After hatching from the brood pouch, juveniles were transferred into Petri dishes containing the TBTCl solution at each concentration with the Teflon mesh piece set as a substrate, and continued to rear. Two juveniles were allocated per dish, and a total of 20–27 specimens were used for the exposure experiment (122 juveniles in 5 exposure experiments). The juveniles that emerged from the brood pouch were classified as instar I. Their body lengths were measured from the basal part of the antenna I on the head to the posterior end of pereonite VII. The sex was distinguished from instar II. The maturity of the females was divided into three stages: immature, premature and mature based on the morphology of the oostegites on pereonites III and IV.

Mature females were allowed to copulate with mature males collected from the field and to release eggs in the brood pouch. The number of eggs in the brood pouch was counted at the same time each day. In the present study, oogenesis in the premature stage, and embryo development and new oogenesis in the mature stage were distinguishable under the binocular microscope. Males and females that survived over 50 days were fixed with 10% formalin, as were the animals that died during the experiment period. The sex of the hatched juveniles was determined from the presence of oostegites in fe-

males and the development of gnathopod II and the presence of abdominal appendages in males.

Statistical analysis

Comparisons of the life span between the control (0 ng TBTCl l^{-1}) and each concentration (0, 10, 100, 1000 and 10000 ng l^{-1}) of TBTCl were carried out using the log-rank test. Comparisons between the control and each concentration of TBTCl in regard to sex ratio were carried out using the chi-squared test. Differences in reproduction and growth between the control and each concentration of TBTCl were tested by Mann-Whitney *U*-test. All statistical analyses were carried out using Stat View 5.0 (SAS Institute Inc. 1998).

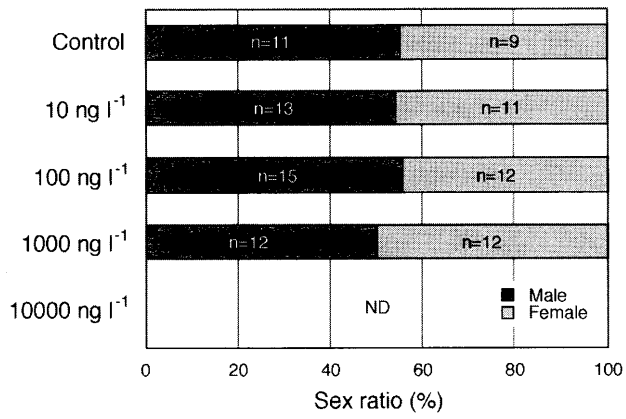

Fig. 2. Sex ratio in juveniles exposed to TBTCl after hatching. ND indicates no data because of the death of all specimens.

Embryonic exposure

Specimens

Caprella danilevskii was collected by SCUBA from the rocky shore in Uchiura Bay, Japan, after which specimens were immediately brought to the laboratory and kept in an aquarium provided with running seawater. Premature females and mature males were sorted and provided for the experiments (Fig. 1).

Seawater and TBT solution

The seawater used for the present experiments was collected from a depth of 10 m outside Otsuchi Bay.

A tributyltin-seawater solution and the control seawater were made according to our previously described method in the section of TBT exposure after hatching. In the present study, 5 test concentrations of TBTCl (0, 10, 100, 1000 and 10000 ng l^{-1}) were prepared using dilute solution. These solutions were made every week. The five test concentrations of TBTCl were measured to confirm the accuracy of TBTCl present in those test solutions during the experiment in the previous report (Ohji et al. 2002a). The concentrations remained the same between pre- and post-experiments.

Embryonic exposure experiments

After confirmation that premature females had reached the mature stage, these parental females were allowed to copulate with males, and spawning was stimulated (first mature stage in parent) (Fig. 2). After spawning in the brood pouch, ovigerous mature females were transferred to Petri dishes (6 cm in diameter, 6 cm in height) containing each concentration of TBTCl, respectively, with a Teflon mesh piece (2 cm×2 cm) as a substrate; specimens were then maintained at 20°C and a 12 : 12 hours light : dark photoperiod. One ovigerous mature female was allocated per dish, and a total of 11 females were used for the exposure experiment (55 females in 5 exposure experiments). Colonies of diatom *Chaetoceros calcitrans* (Paulsen) Takano were added to each Petri dish once a day; this amount was sufficient to supply the daily dietary demands of the caprellids. The seawater in each dish was changed every day, and Petri dishes and Teflon mesh pieces were replaced every two days. The conditions of ovigerous parental females and egg number in the brood pouch were observed each day at the same time under a binocular microscope.

Specimens were exposed to 5 concentrations (0, 10, 100, 1000 and 10000 ng l^{-1}) of TBTCl for 5 days, which corresponded to the period of embryonic development. After being released from the brood pouch, the juveniles were transferred into the filtered seawater containing neither TBTCl nor acetone. Two juveniles were allocated per dish, and a total of 11–25 specimens were used for the exposure experiment (68 juveniles in 5 exposure experiments). The juveniles released from the brood pouch were classified as instar I. At each instar, the body length of every juvenile was measured. The sex was determined from instar II.

Furthermore, parental females were also transferred to the filtered seawater. After molting, these females recopulated with a mature male that was collected from the field. After spawning (second mature stage in parent), the eggs were counted at each concentration of TBTCl to examine the effects of TBTCl on the oogenesis stage.

After reaching maturity, female juveniles exposed to TBTCl during the embryonic period were allowed to copulate with mature males collected from the field, and spawning was stimulated (first generation of offspring). The eggs in the brood pouch were counted at the same time each day. After juveniles were released from the brood pouch, these juveniles were continued to rear until instar II, and the sex was determined under the light microscope (second generation of offspring). Males and females that survived over 50 days were

fixed with 10% formalin. The animals that died during the experiment period were also fixed with 10% formalin.

Statistical analysis

Comparisons of life span between the control condition (0 ng TBTCl l^{-1}) and each concentration (10, 100, 1000 and 10000 ng l^{-1}) of TBTCl were carried out by the log-rank test. A comparison of sex proportion between the control and each concentration of TBTCl was carried out by the chi-squared test. Differences in both reproduction and growth between the control and each concentration of TBTCl were tested by the Mann-Whiney *U*-test. Comparisons between the number of eggs spawned and number of juveniles hatched, and between the number of eggs spawned in the first mature stage and the number of eggs spawned in the second mature stage in the parental female were carried out by Wilcoxon's signed-rank test. All statistical analyses were carried out by Stat View 5.0 (SAS Institute Inc. 1998).

Results

Exposure after hatching

Sex ratio

Sex ratio of male to female was 55.0% and 45.0%, respectively in the control (0 ng TBTCl l^{-1}) (Fig. 2). The ratio was almost constant in spite of increasing of the TBTCl concentrations ranging from 50.0% (1000 ng TBTCl l^{-1}) to 55.6% (100 ng TBTCl l^{-1}) in males and ranging from 44.4% (100 ng TBTCl l^{-1}) to 50.0% (1000 ng TBTCl l^{-1}) in female, although all specimens died in the 10000 ng TBTCl l^{-1} experiment because of acute toxic concentration for this species (Ohji et al. 2002a). No significant differences were found in the sex ratio between the control and other 3 concentrations of TBTCl (chi-squared test, $p>0.5$).

Survival

As the TBTCl concentration were increased, survival rates within 50 days after hatching decreased, 25.0% in 10 ng l^{-1}, 11.1% in 100 ng l^{-1} and 8.3% in 1000 ng l^{-1} (Fig. 3). All specimens died in 10000 ng TBTCl l^{-1} within 4 days after hatching, while all control specimens survived (100%). Significant differences were found in the survival rate between the control and the other 4 concentrations of TBTCl (log-rank test, $p<0.0001$).

Growth

In each concentration of TBTCl except for 10000 ng l^{-1}, body length increased as the organism became older (Fig. 4). However, significant differences were seen in body length between the control and 100 ng TBTCl l^{-1} and between the control and 1000 ng TBTCl l^{-1} in each instar after instar II of either males or females (Mann-Whitney *U*-test, $p<0.05$). No significant difference was found in the body length between the control and 10 ng TBTCl l^{-1} in each instar of either males or females (Mann-Whitney *U*-test, $p<0.05$). Those indicate that a decrease in growth rate results after exposure to 100 and 1000 ng TBTCl l^{-1} in spite of the organism's sex.

The day required from hatching to instar X which corresponds to the experimental period in the control, 10 and

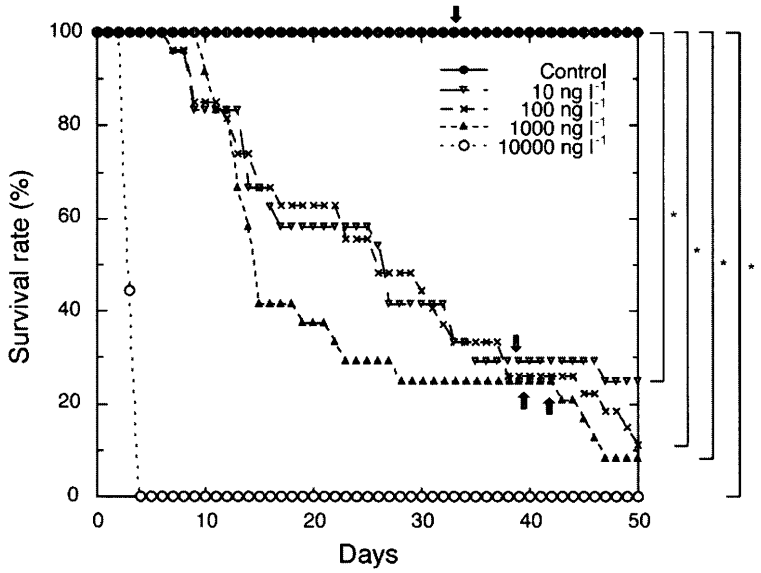

Fig. 3. Survival rate of specimens exposed to TBTCl after hatching. The number of hatched juveniles was calculated as 100%. Arrows indicate the days required from hatching to maturation in females. Log-rank test, *$p<0.0001$.

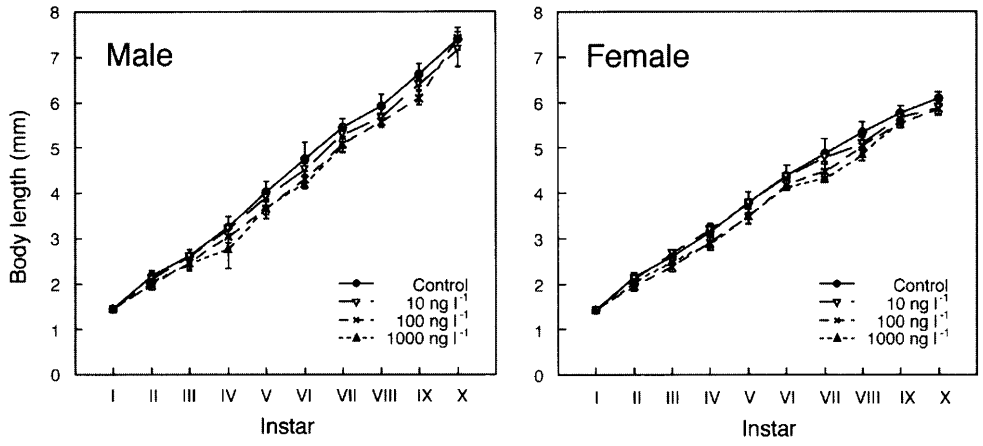

Fig. 4. Body length at each instar of specimens exposed to TBTCl after hatching.

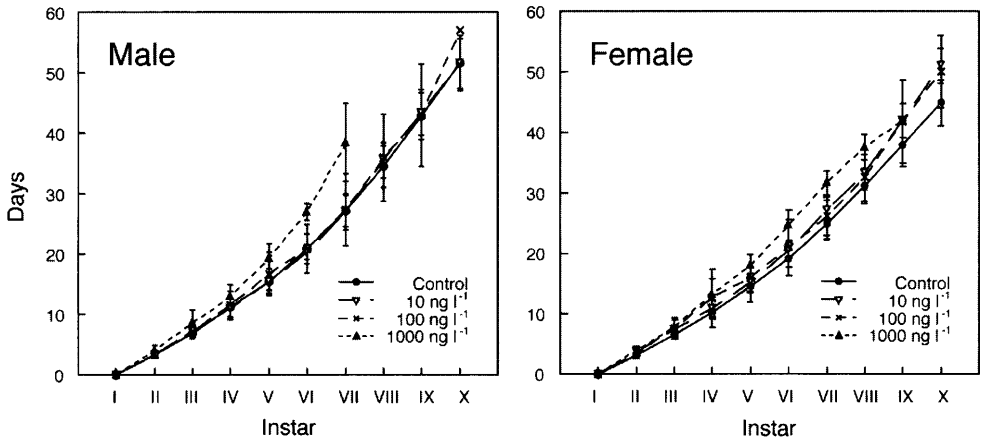

Fig. 5. Days required from hatching to each instar of specimens exposed to TBTCl after hatching.

100 ng TBTCl l^{-1} was approximately 50 days for both males and females. Although all male specimens died after instar VII and females died after instar IX in 1000 ng TBTCl l^{-1} (Fig. 5), a significant difference was found in the day required from hatching to each instar between the control and 1000 ng TBTCl l^{-1} in both males and females (Mann-Whitney *U*-test, $p<0.05$). However, no significant differences were found between other combinations (Mann-Whitney *U*-test, $p>0.05$).

Maturation and reproduction

The instar and day required from hatching to maturity in the female caprellid ranged from VIII to IX and from 33 days to 42 days, respectively (Table 1). Significant differences were seen in the day required from hatching to maturity between the control and 10 ng TBTCl l^{-1} and between the control and 100 ng TBTCl l^{-1} (Mann-Whitney *U*-test, $p<0.05$), while no significant differences were seen in the instar required from hatching to maturity for all other combinations (Mann-Whitney *U*-test, $p>0.05$). Though all specimens were observed to mature completely during instar VIII and instar IX in the control, several specimens died at premature and immature stages during those instars and instar X in other TBTCl concentrations. This suggests that a delay in the day required from hatching to maturity is caused by exposure in TBTCl.

Table 1. Instar and the days required from hatching to maturation of juveniles exposed to TBTCl after hatching.

Concentration (ng TBTC l^{-1})	Instar	Day
Control	VIII±0.5	33±1.6
10	VIII±1.0	39±1.4
100	VIII±0.6	39±2.3
1000	IX	42
10000	ND	ND

Numerical data and ND indicate mean and standard deviation and no data because of death of all specimens, respectively.

Table 2. Reproductive conditions of mature females exposed to TBTCl after hatching.

Concentration (ng TBTCl l^{-1})	Number of embryos spawned	Number of juveniles hatched	Incubation period of embryos	Duration of instar
Control	2.7±1.7	2.7±1.7	5.0±0.0	7.0±0.9
10	2.0±2.8	0.5±0.7	6.0	7.5±2.1
100	2.7±3.1	0.3±0.6	6.0	9.7±2.1
1000	ND	ND	ND	ND
10000	ND	ND	ND	ND

Numerical data and ND indicate mean and standard deviation and no data because of death of all specimens, respectively.

After maturation, the number of eggs in the brood pouch, the number of juveniles hatched and the period from spawning to juvenile hatching ranged from 2.0 to 2.7, from 0.3 to 2.7 and from 5.0 days to 6.0 days in the increasing TBTCl concentration (Table 2). A significant difference was found in the number of juveniles hatched between control and 100 ng TBTCl l^{-1} (Mann-Whitney *U*-test, $p<0.05$).

Molting interval after maturation ranged from 7.0 days to 9.7 days in the each TBTCl concentration. A significant difference was seen between the control and 100 ng TBTCl l^{-1} (Mann-Whitney *U*-test, $p<0.05$). This indicates that a delay in the molting interval is caused by exposure in TBTCl.

The decrease in the number of eggs because of eggs dropping from the brood pouch (brood loss) was found in 1 of 2 females that reached the mature stage, while the other did not succeed in egg formation in 10 ng TBTCl l^{-1}. In 100 ng TBTCl l^{-1}, brood loss was found in 2 of 3 females that reached the mature stage and the other one did not succeed in egg formation. The percentages of hatching success after spawning were decreased as the TBTCl concentration increased except for 1000 and 10000 ng l^{-1}, i.e. 100% in the control, 25.0% in 10 ng l^{-1} and 12.5% in 100 ng l^{-1}. In 1000 and 10000 ng l^{-1}, all specimens died before or after reaching maturation.

Morphological alterations

During the experiment period, morphological alterations such as loss of gill, contraction of gill, necrosis and loss of pereopod, cramp of pereopod (mis-clinging to the substrate with pereopod), molting disorder, and substances attached on the surface of the body were observed, and their occurrence increased as the TBTCl concentration increased (Fig. 6).

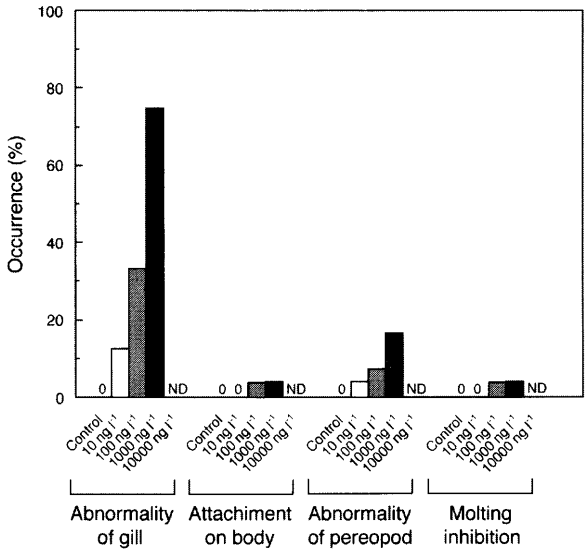

Fig. 6. Occurrence of morphological alterations of specimens exposed to TBTCl. ND indicates no data because of the death of all specimens.

Table 3. Reproductive conditions of mature female exposed to TBTCl during the 5 days which corresponds to the first mature stage.

Concentration (ng TBTCl l^{-1})	Number of embryos spawned	Number of juveniles hatched
First spawning		
Control	2.3±1.7	2.3±1.7
10	2.4±1.3	1.6±1.6
100	3.5±2.2	1.3±1.9
1000	2.9±2.3	1.0±1.3
10000	2.7±1.4	0.0
Second spawning		
Control	3.1±1.8	—
10	1.4±1.4	—
100	1.3±1.9	—
1000	1.0±1.0	—
10000	ND	—

Numerical data, ND and bar indicate mean and standard deviation, no data because of death of all specimens, and no observation, respectively.

Embryonic exposure

Condition of parental females

Eleven ovigerous females were allocated to each concentration compartment of TBTCl (0, 10, 100, 1000, and 10000 ng l^{-1}). The number of eggs per female ranged from 2.3±1.7 (Mean±SD) to 3.5±2.2 in the brood pouch (Table

3). No significant differences were found in the number of eggs spawned between the control and the other 4 concentrations of TBTCl (Mann-Whitney *U*-test, $p>0.1$). A number of deaths of ovigerous females exposed for 5 days were observed at more than 100 ng TBTCl l^{-1} (Fig. 8) and all specimens died at 10000 ng TBTCl l^{-1} due to the acute toxic concentration for the species (Ohji et al. 2002a). Brood loss of the females also occurred at concentrations higher than 10 ng TBTCl l^{-1}, ranging from 3 to 6 specimens, while no brood loss was observed in the control (0 ng TBTCl l^{-1}) (Fig. 7).

The number of eggs per female spawned in the brood pouch in the second mature stage ranged from 1.0±1.0 to 3.1±1.8 (Table 3). Significant differences were found in the number of eggs between the control and 3 concentrations (10, 100 and 1000 ng l^{-1}) of TBTCl (Mann-Whitney *U*-test, $p<0.05$–0.01). Furthermore, significant differences in the number of eggs were found between the first and second mature stages at 100 and 1000 ng TBTCl l^{-1} (Wilcoxon's signed-rank test, $p<0.05$) (Table 3).

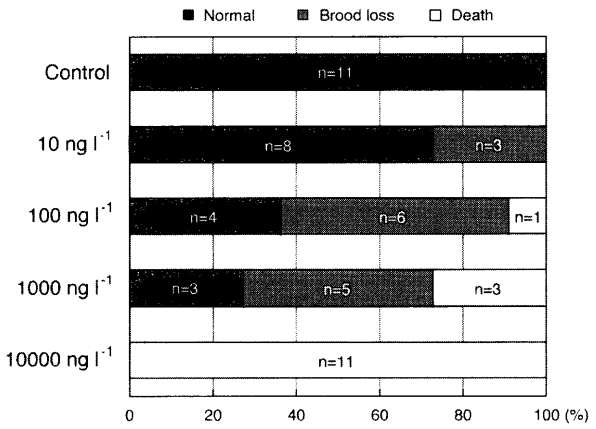

Fig. 7. Condition of the parental female in the first mature stage after 5-day exposure to TBTCl.

Survival rate in the first generation of offspring

The embryo survival rate (estimated from the amount of brood loss, the number of eggs in the brood pouch in dead specimens, and the total number of eggs) during the TBTCl exposure period decreased as the TBTCl concentrations increased, i.e. 69.2% at 10 ng l^{-1}, 36.8% at 100 ng l^{-1}, 34.4% at 1000 ng l^{-1} and 0% at 10000 ng l^{-1} (Fig. 8). Significant differences were found in the embryo survival rates between the control and the other 4 concentrations (log-rank test, $p<0.05$–0.0001).

The number of juveniles hatched per female was 2.3±1.7 in the control. However, it decreased as the TBTCl concentrations increased, ranged from 1.6±1.6 at 10 ng l^{-1} to 0 at 10000 ng l^{-1}. Significant differences were found between control and 1000 ng TBTCl l^{-1} and between the control and 10000 ng TBTCl l^{-1} (Mann-Whitney *U*-test, $p<0.05$–0.0001). Furthermore, significant differences were found between the number of eggs spawned in the brood pouch and the number of juveniles hatched at 100, 1000 and 10000 ng TBTCl l^{-1} (Wilcoxon's signed-rank test, $p<0.05$–0.01) (Table 1).

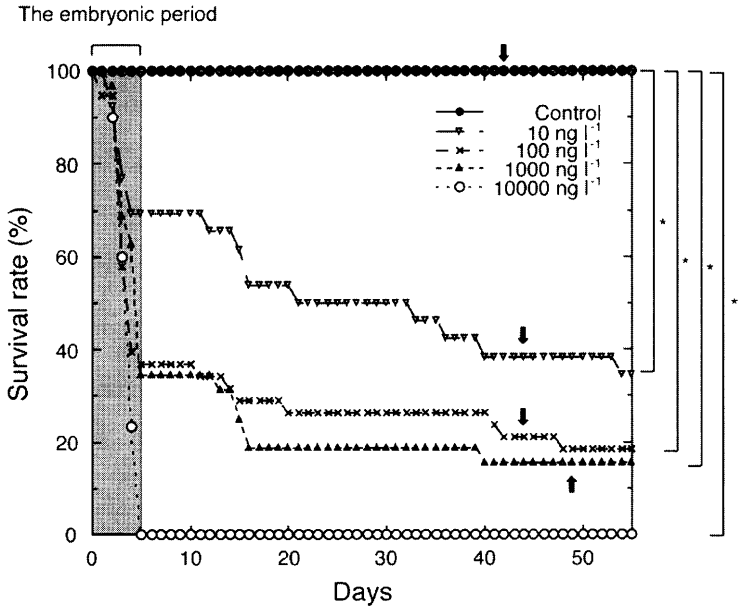

Fig. 8. Changes in the survival rate during spawning and sacrifice in offspring exposed to TBTCl during the embryonic stage and thereafter reared in seawater with no TBTCl added. Arrows indicate the days required from hatching to maturation in females. Log-rank test, *$p<0.0001$.

At all concentrations, the survival rate in offspring continued to decrease despite the movement of hatched juveniles into seawater that did not contain both TBTCl and acetone (Fig. 8). Significant differences were found in the survival rate between the control and the other 4 concentrations (log-rank test, $p<0.0001$). The survival rate of females at maturity decreased to 38.5% at 10 ng TBTCl l^{-1}, 21.1% at 100 ng TBTCl l^{-1}, 15.6% at 1000 ng TBTCl l^{-1} and 0% at 10000 ng TBTCl l^{-1}, although the survival rate in the control was 100%. The drastic change in survival rate was observed twice, at 10–15 days and during 35–45 days after spawning.

Sex ratio in the first generation of offspring

The female proportions were 36% in the control (Fig. 9), corresponding to previous field observations (Takeuchi and Hirano 1991). However, as the TBTCl concentrations increased, the proportion of females increased, i.e. 55.6% at 10 ng l^{-1}, 85.7% at 100 ng l^{-1} and 81.8% at 1000 ng l^{-1}. Significant differences occurred in the sex proportion between the control and 100 ng TBTCl l^{-1} and between the control and 1000 ng TBTCl l^{-1} (chi-squared test, $p<0.01$).

Growth, maturation and reproduction in the first generation of offspring

In the present study, no significant differences were found in the body length in each instar and in the time taken for each instar from hatching between the control and each concentration of TBTCl in either males or females (Mann-Whitney *U*-test $p>0.05$). These results suggest that no growth or molting inhibition occurs after hatching in response to exposure to TBTCl in the embryonic period.

The instar and day required from hatching to maturity in the female caprellid ranged from VIII to IX and from 37 days to 45 days, respectively (Table 4). Significant differences were seen in the instar required from hatching to maturity between the control and 10 ng TBTCl l^{-1}, between the control and 100 ng TBTCl l^{-1} and between the control and 1000 ng TBTCl l^{-1} (Mann-Whitney *U*-test, $p<0.05$–0.01), while no significant differences were seen in the day required from hatching to maturity for all other combinations (Mann-Whitney *U*-test, $p>0.05$).

In the first mature stage in offspring, oogenesis inhibition and brood loss were observed at 100 and 1000 ng TBTCl l^{-1}. Three of 6 mature females exhibited apparent oogenesis inhibition at 100 ng TBTCl l^{-1} and 3 of 5 at 1000 ng TBTCl l^{-1}. Brood loss was apparent in 1 of 6 mature females at 100 ng TBTCl l^{-1} and in 2 of 5 at 1000 ng TBTCl l^{-1}. These abnormal ratios during the mature stage increased as the TBTCl concentrations increased, i.e. 0% at the control and at 10 ng TBTCl l^{-1}, 66.7% at 100 ng TBTCl l^{-1} and 100% at 1000 ng TBTCl l^{-1}.

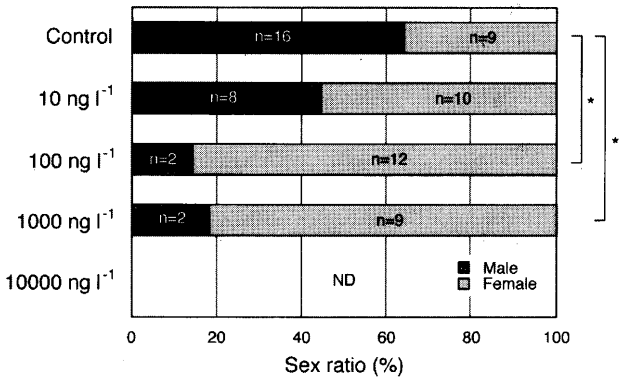

Fig. 9. Sex ratio in offspring of the first generation exposed to TBTCl during the embryonic stage. Chi-squared test, *$p<0.05$. ND indicates no data because of the death of all specimens.

Table 4. First instar and the day required from hatching to maturation of juvenile exposed to TBTCl during the embryonic period.

Concentration (ng TBTCl l^{-1})	Instar	Day
Control	VIII±0.4	37±2.6
10	IX±0.5	39±4.3
100	IX±0.0	39±2.5
1000	IX±0.8	45±12.1
10000	ND	ND

Numerical data and ND indicate mean and standard deviation and no data because of death of all specimens, respectively.

Sex ratio in the second generation of offspring

The proportion of females in the control and at 10, 100 and 1000 ng TBTCl l^{-1} were 28.6%, 28.6%, 22.2% and 33.3%, respectively. No significant differences in the sex proportion between control and other concentrations of TBTCl were observed (chi-squared test, $p>0.5$). These results suggest that TBTCl exposure in the embryonic period does not affect the sex proportion in the second generation.

Discussion

Growth and Morphological alterations

In the TBT exposure experiments after hatching, the marked delay in growth and molting during the early developmental stages and mature stage by exposure to TBT was found in spite of the sex in *Caprella danilevskii* (Ohji et al. 2003) (Fig. 10). However, no significant difference was found in the growth and molting inhibition in the embryonic expo-

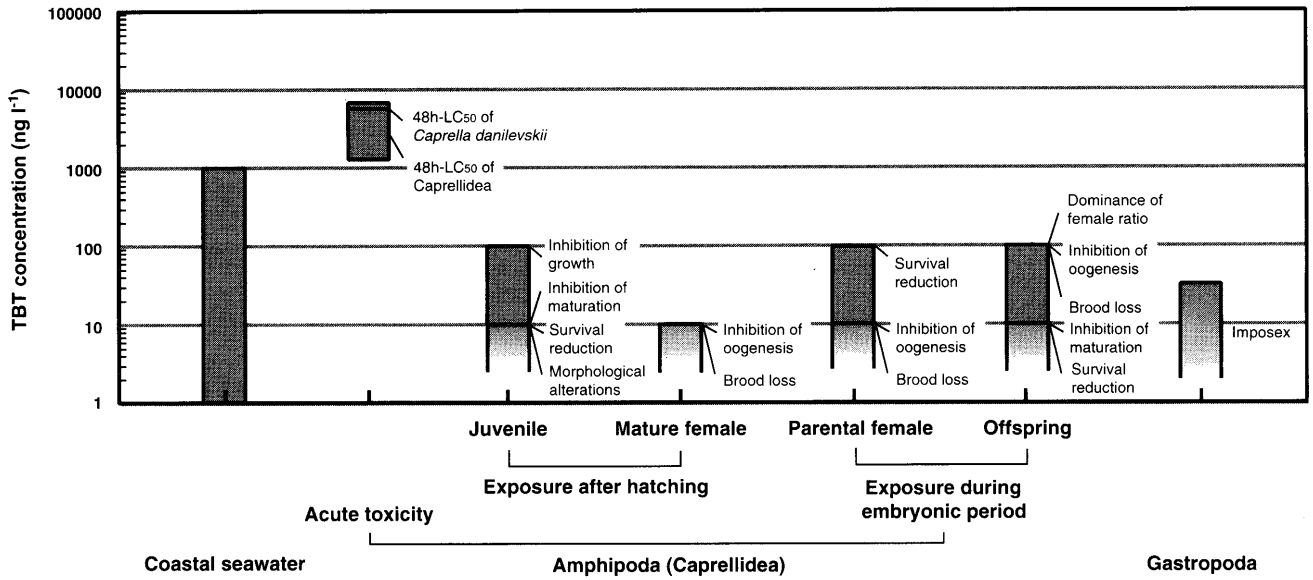

Fig. 10. Summary of biological effects of TBT on caprellids in the present studies, and on gastropods, and TBT concentration in coastal seawater. LC_{50} indicates median lethal concentration. LC_{50} values for caprellids are based on Ohji et al. (2002a). Results of experiments of TBT exposure after hatching and during embryonic period based on Ohji et al. (2003) and Ohji et al. (2002b), respectively. TBT concentrations in coastal seawater are cited from Batley (1996). The occurrence levels of imposex in gastropods are cited from Bryan et al. (1986), Gibbs et al. (1988) and Bettin et al. (1996).

sure experiments (Ohji et al. 2002b). Those considerations suggest that effects on sensitive stages by exposure to TBT may extend over a long period after hatching in the caprellids. Such a growth delay induced by exposure to TBT has also been reported in various other organisms, e.g. the mysid *Acanthomysis sculpta* (Davidson et al. 1986), American oyster *Crassostrea virginica* (Thain 1986), blue mussel *Mytulis edulis* (Strømgren and Bongard 1987) and American lobster *Homarus americanus* (Laughlin and French 1980). Furthermore, several morphological alterations caused by TBT exposure after hatching were observed during the growth of *C. danilevskii*, though no morphological alterations were observed in response to TBT exposure during the embryonic period (Fig. 10). The morphological alterations resulting from exposure to TBT such as imposex in the gastropods *Nucella lapillus* (Bryan et al. 1986, Gibbs and Bryan 1986, 1987), thickened shell in the oyster *Crassostrea gigas* (Thain and Waldock 1986) and deformities in regenerating limbs in the crab *Uca Pugilator* (Weis and Kim 1988) have also been reported. Exposure to TBT after hatching might have also induced the cramp of the pereopod observed in this study, since TBT is also known to be neurotoxic (Watanabe 1980). Those facts suggest that TBT might act as a developmental toxicant or teratogen, affecting the processes of differentiation and morphogenesis during growth.

Maturation and Reproduction

Conspicuous inhibitions of maturation and reproduction occurred in mature females even at nanogram-per-liter levels of TBT exposure (corresponding to present TBT levels in the coastal environment) both after hatching and during the embryonic stage, although such inhibitions were not apparent in the control in *Caprella danilevskii* (Ohji et al. 2002b, 2003) (Fig. 10). In female gastropods, masculinization (imposex) by TBT exposure is the superimposition of male sex organs (development of a penis and vas deferens), with this condition leading to reproductive failure and consequently population decline (Bryan et al. 1986, Gibbs and Bryan 1986, 1987, Bettin et al. 1996, Matthiessen and Gibbs 1998) (Fig. 10). Therefore, TBT exposure might also affect the maturation and reproduction systems of the caprellid, although no external morphological alterations were found in the reproductive organs in the present study. In the caprellids, TBT might not induce morphological alterations in the reproductive organs but cause disruptions in the internal physiological mechanisms concerning maturation and reproduction over whole life stages. Furthermore, a similar phenomenon of impairment of egg production has been reported in the copepod *Acarita tonsa* (Johansen and Møhlenberg 1987) and in the sea urchin *Paracentrouts lividus* (Girard et al. 1997, 2000) in response to TBT exposure. The cytotoxicity of TBT often results in an arrest of cellular dynamics, leading to apoptosis (Stridh et al. 1999) or a blocking of cell division (Girard et al. 1997) primarily oc-

curring through an alteration of macromolecular syntheses (Snoeij et al. 1988, Girard et al. 1997) or membrane-mediated processes controlling cell signaling. These processes consist primarily of a disruption of calcium homeostasis (Chow et al. 1992, Matsuoka and Igisu 1996) or calcium signaling (Corsini et al. 1997, Girard et al. 1997). Girard et al. (1997, 2000) have found that TBT inhibits sea urchin egg cleavage by altering many of the cellular events related to cell division. Furthermore, Girard et al. (2000) have suggested that the inhibition occurs in response to a few hours of TBT exposure and is sufficient to damage the organism during its embryonic life. A similar inhibition related to egg cleavage might occur in the caprellid, resulting in brood loss and oogenesis inhibition in the species. In the present study, impaired reproductive success also occurred in both short- and long-term exposure to TBT. Therefore, our data suggest that nanogram concentrations of TBT similar to those encountered in coastal waters can directly affect reproduction in the caprellid, and that this phenomenon is an environmentally realistic scenario in the coastal ecosystem.

Sex disturbance by TBT exposure in caprellid

It is noteworthy that the no significant differences were seen regarding change in the sex ratio by TBT exposure at all levels after hatching in the present study (Ohji et al. 2003) (Fig. 10). However, an increase in the female ratio in the hatched juveniles was found in an embryonic exposure experiment in *Caprella danilevskii* (Ohji et al. 2002b). As TBT concentrations increase, the proportion of females were found to increase to 55.6% in $10\,ng\,l^{-1}$, 85.7% in $100\,ng\,l^{-1}$ and 81.8% in $1000\,ng\,l^{-1}$. Though the sex proportion in the present study was changed in response to exposure to TBT, the number of females was almost constant (9–12) regardless of increases in TBT concentrations. Accordingly, males seem to have a higher sensitivity to TBT than females. However, the survival rate in response to exposure to TBT has been found to be similar regardless of sex in the juvenile stage (Ohji et al. 2004). Those considerations suggested that sex disturbance might be induced during the embryonic stage in the caprellid. This phenomenon is in contrast to the previous results found in gastropod molluscs in which TBT exposure after hatching induces imposex for females (Matthiessen and Gibbs 1998). TBT had strong effects on the development of imposex in the dog whelk in exposure experiments after hatching (Gibbs et al. 1988). It is reported that TBT acts as a competitive inhibitor of cytochrome P450-mediated aromatase, resulting in the increase of androgens (Spooner et al. 1991, Bettin et al. 1996) and inhibition of androgen elimination (Bettin et al. 1996) in gastropods. Therefore, the increase of androgen in vivo may result in androgenization of organisms. Since this phenomenon differs from our results, it is suggested that the action mechanism of TBT might differ among organisms. Furthermore, as the factor of difference of phenomenon among organisms, it is also considered that the effects of TBT exposure might differ according to the developmental stage. In the gastropods, TBT induce sex disturbance after sex determination, while, in contrast, TBT does sex disturbance before sex determination in caprellids. Sex differentiation in crustaceans, i.e. amphipods, isopods and decapods, is known to control by a hormone secreted from the androgenic gland (Charniaux-Cotton 1954, Katakura 1960, Taketomi et al. 1996). Therefore, it is considered that TBT might affect the production of the androgenic gland or the secretion of androgenic hormone in the caprellid.

Furthermore, TBT is known to metabolize by a detoxifying system involving two phases in vivo. The phase-one reactions involve the cytochrome P-450 dependent mixed-function oxygenase system (MFO) which hydroxylates TBT to alpha-, beta-, gamma-, and delta-hydroxydibutyltin derivatives (Fish et al. 1976). The phase-two reactions conjugate sugars or sulfate to hydroxybutyldibutyltin, and these highly polar conjugates are then rapidly eliminated from the organism. The MFO system of vertebrates and invertebrates is associated with the endoplasmic reticulum of the cell and is a multicomponent enzyme system composed of phospholipid, cytochrome p-450, and NADPH cytochrome P-450 reductase (Lu 1976, Lee 1981, Stegeman 1981). Thus, metabolism of a compound generally reduces persistence, increases elimination, and reduces toxicity (Lee 1996). It is reported that the binding of TBT to glutathione S-transferase and cytochrome P-450 results in the inhibition of two detoxifying enzyme systems (Henry and Byington 1976, Rosenberg and Drummond 1983). Cytochrome P-450 systems control the conversion of cholesterol into a variety of hormones. Inhibition or stimulation of cytochrome P-450 systems can result in changes in hormone production or clearance (Levin et al. 1974, Kupfer and Bugler 1976). Therefore, TBT might affect androgenic hormone in the caprellid. Further experiments are needed to clarify TBT action in the endocrine systems in the caprellid.

Survival and biomass of caprellid in the coastal ecosystems

It is reported that the TBT affect the community of the caprellid at present. The biomass of the caprellid inhabiting sea grasses inner of the Otsuchi Bay (49.8–125.0 individuals m^{-2}) were a tenth as many as that of the mouth of the bay (1112.5 individuals m^{-2}) (Takeuchi and Hino 1997). The significant difference of the caprellid biomass between inner and mouth of the Otsuchi Bay might be induced by the difference in TBT concentrations at each site because TBT concentrations were higher ($3.9–19\,ng\,l^{-1}$) at inner of the bay

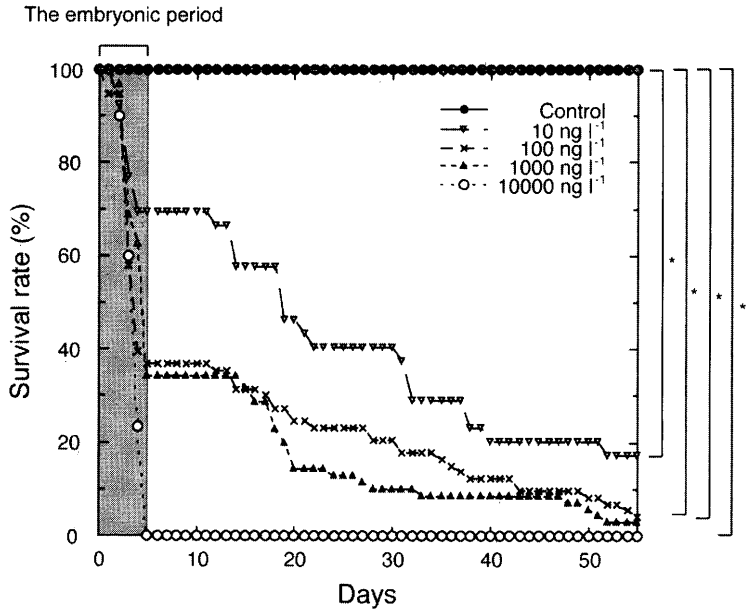

Fig. 11. Survival rate throughout whole life history in specimens based on the results of the TBT exposure after hatching (Ohji et al. 2003) and during the embryonic period (Ohji et al. 2002a). The number of eggs at the beginning of the experiment was calculated as 100%. Log-rank test, *$p<0.0001$.

than that of the mouth (less than the detection limit) (Takahashi et al. 1999, Ohji et al. 2002a). Furthermore, prior to 1960, an extremely high biomass of the caprellid was reported from Japan (Fuse 1962). Seasonal fluctuation of the epifaunal animals living in the *Sargassum* zone in Kasaoka Bay, Japan, from 1956 to 1958 were studied, and it was reported that the biomass of the caprellid was 1.3 kg wet wt m^{-2}. In the past decade, such a high biomass and density in caprellids amphipods has not been reported in the coastal waters of Japan or of those of other developed countries. The biomass of caprellids inhabiting the *Sargassum* zone in Otsuchi Bay, Japan, from1993 to 1995 was estimated at 100 g wet wt m^{-2} (Takeuchi 1998). Though a reduction in TBT contamination was recorded after the ban (Environment Agency Japan 1995), TBT concentrations in Japanese coastal waters still persist, ranging from below the detectable level to 160 ng l^{-1} (180 ng l^{-1} in TBTCl) (Takeuchi et al. 2001) and averaging 10 ng l^{-1} (11 ng l^{-1} in TBTCl). Furthermore, the high TBT contamination in marine organisms and seawater continues in the coastal waters of Spain, France and Canada (Chau et al. 1997, Morcillo et al. 1997, Michel and Averty 1999), as well as in Asian and Oceanian countries (Kannan et al. 1995, Kanantireklap et al. 1997) where no restrictions have been imposed. In small estuaries, marinas and moorings contribute significantly to TBT load, ranging from 24 ng l^{-1} (27 ng l^{-1} in TBTCl) to 2440 ng l^{-1} (2740 ng l^{-1} in TBTCl) (Lau 1991, Batley 1996). In the present study, a drastic decrease in the survival rate was observed in response to TBT exposure in both short- and long-period even at 10 ng TBTCl l^{-1} that corresponds to the mean level in the coastal waters (Ohji et al. 2002b, 2003) (Fig. 10). In the survival rate throughout the whole life history based on the results of the TBT exposure after hatching and during the embryonic period, significant differences were also seen in the survival rate between the control and the other 4 concentrations of TBTCl (Fig. 11). In regard to TBTCl exposure during the embryonic period, the survival rate was 100% in the control, 69.2% in 10 ng l^{-1}, 36.8% in 100 ng l^{-1}, 34.4% in 1000 ng l^{-1} and 0% in 10000 ng l^{-1} (Ohji et al. 2002b). The survival rate during 55 days (5 days in the embryonic period and 50 days from juvenile stage to mature stage after hatching) after spawning decreased in a range from 0% (10000 ng TBTCl l^{-1}) to 17.3% (10 ng TBTCl l^{-1}) (2.9% in 1000 ng TBTCl l^{-1} and 4.1% in 100 ng TBTCl l^{-1}) except for the control (100%). These considerations all lead to the conclusion that TBT exposure threatens the survival of caprellid through their whole life history, and support the decrease in the caprellid biomass in the coastal ecosystem.

Conclusion

Even at ambient water levels, exposure to TBT after hatching (50 days) influences survival, growth, maturation, reproduction and morphological alterations in the caprellids. Adverse effects on sex ratio, reproduction, and survival in the

caprellid have also been observed TBT exposure experiment with exposure during the embryonic stage (5 days) at ambient water levels. Remarkably, the proportion of females increased dramatically in response to exposure to TBT in the embryonic period, though no significant difference was observed in the sex ratio in response to long-term exposure to TBT at these levels after hatching. These findings suggest that sex disturbance might therefore be induced during the embryonic stage in the caprellid. It has been reported that caprellids have a lower metabolic capacity to degrade TBT and therefore accumulate BTs at higher concentrations than other organisms in the coastal ecosystem. Accordingly, TBT exposure, both short- and long-term, in the coastal environment might critically damage the life history characters of caprellids. The impaired reproductive success of a keystone species affects the entire population of species due to drops in the reproductive output below the critical level required for maintaining the population's survival, thus leading to changes in the ecosystem around keystone species. Because caprellids link primary producers to higher consumers in the coastal waters, the high ecological risk to caprellids due to their high sensitivity to TBT over their life history may result in a disturbance in the coastal water ecosystem.

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