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NOTE

Long-term and transgenerational effects of nonylphenol exposure at a key stage in the development of *Crassostrea gigas*. Possible endocrine disruption?

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ABSTRACT: The widespread aquatic pollutant nonylphenol has been found to induce long-term and transgenerational effects in the Pacific oyster *Crassostrea gigas* that have not previously been reported. Evidence is provided demonstrating that when larvae are exposed to environmentally relevant concentrations of nonylphenol for a single 48 h exposure at a key stage in their development, long-term sexual developmental effects are induced. Data provided by this study suggest that exposure to 1 and 100 µg l^{-1} nonylphenol at Days 7 to 8 post-fertilization results in a change in the sex ratio towards females and an increase in the incidence of hermaphroditism (10 mo later, up to 30% of the resulting adults were fully functional hermaphrodites). Gamete viability is also affected, resulting in poor embryonic and larval development (up to 100% mortality) of the subsequent generation.

KEY WORDS: *Crassostrea gigas* · Nonylphenol · Endocrine disruption · Transgenerational · Critical exposure period · Larval development · Hermaphrodite · Aquaculture · Oyster

Nonylphenol is a widespread aquatic pollutant used as a plastic additive and surfactant in the production of many household, agricultural and industrial applications (Ahel et al. 1993). It has previously been reported to cause proliferation of human breast-cancer cells (Soto et al. 1991) and intersexuality in fishes (Gray & Metcalfe 1997). Here, we provide evidence that nonylphenol alters sex ratio, induces hermaphroditism and affects gamete viability in the Pacific oyster *Crassostrea gigas*.

In recent years, much research concerning critical exposure periods or critical windows has focussed on aquatic animals, probably because they are continually exposed to a cocktail of environmental pollutants such as pesticides and sewage effluent in the bodies of water in which they live. For example a single exposure of embryos of the Japanese medaka *Oryzias latipes in utero* to the oestrogenic pesticide o,p-DDT immediately after fertilization (a critical period of gonad development), can profoundly bias sexual differentiation towards females (Edmunds et al. 2000); the sex-reversed females were shown to be fully functional by producing viable offspring with normal males. When *O. latipes* was exposed to nonylphenol for a 3 mo period from hatching (Gray & Metcalfe 1997), up to 86% of the male fish developed the intersex condition 'testis-ova', which is characterized by the presence of both testicular and ovarian tissue in the gonad. The exposure period commencing 3 d post-hatch was the most sensitive period for developing the testis-ova condition (Gray et al. 1999a).

The concept of transgenerational effects (effects carried across generations as a consequence of events that happen during the lifetime of the previous generation) has also been demonstrated in *Oryzias latipes*. A 6 mo exposure period to octylphenol from Day 1 post-hatch resulted in a reduction in male courtship activity and a concomitant reduction in overall reproductive success (Gray et al. 1999b). Eggs produced by matings from exposed males and females demonstrated various developmental abnormalities including circulatory defects, incomplete eye development (anisophthalmia), and failure to inflate swim bladders upon hatching (Gray et al.1999b).

In the coho salmon *Oncorhynchus kisutch*, the period during which sex is labile is a narrow window 10 d on either side of hatching. Exposure to hormones during this time can affect sexual differentiation (Piferrer & Donaldson 1989, Sumpter 1995). A bioassay for oestrogenic chemicals using male transgenic zebra-

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fish *Brachydanio rerio* similarly showed the period of gonad differentiation to be the most susceptible for testis abnormalities and intersex (Legler et al. 2000).

Such effects have also been monitored in the field. A study of the River Mimram, UK, showed that approximately 5% of the roach *Rutilis rutilus* living downstream from a sewage effluent outfall were hermaphrodites (Sumpter & Jobling 1995). Hermaphroditism is normally an extremely rare condition in *R. rutilus* (Arme 1965), and it has been suggested that a component of the effluent was responsible for the increased incidence in hermaphroditism during a sexually labile period of the fish's life (Sumpter & Jobling 1995).

Although much research effort has focussed on the effect of environmental contaminants on aquatic organisms, very little research has assessed specific windows of development during which exposure is likely to be critical and to result in transgenerational effects for commercially important organisms such as the Pacific oyster *Crassostrea gigas.* This is unfortunate, because these oysters are of vital importance to the aquaculture industry and are susceptible to contaminant damage. For example, a single exposure to a range of environmentally relevant concentrations (as low as 0.1 µg l^{-1}) of nonylphenol applied immediately after fertilization resulted in delayed development to the D-shape, developmental abnormalities, and significant reductions in larval survival (Nice et al. 2000). With regard to specific critical exposure periods, a previous investigation showed Days 7 to 8 post-fertilization (pf) in *C. gigas* to be a critical period within its development (the period of change between veliger and veliconcha stages), during which exposure to nonylphenol results in developmental effects such as delayed settlement and metamorphosis (Nice et al. 2001).

The aim of the current study was to explore the long-term transgenerational consequences of exposure to nonylphenol during a known critical stage of development at Days 7 to 8 pf in *Crassostrea gigas*.

Materials and methods. ***Preparation of test solutions:*** Two dilutions (1 and 100 µg l^{-1}) of nonylphenol (Lot No. 74430, Sigma-Aldrich Chemical Company Ltd, Dorset, Kent, UK) were prepared in filtered seawater (35 ‰) obtained from the Seasalter Hatchery, Whitstable, UK. Methanol was used as a solvent at a nominal concentration of 100 µg l^{-1}. This has been shown to have no effect on the development of *Crassostrea gigas* (Nice et al. 2000, 2001) and to have an LC_{50} of 22.0 g l^{-1} for the closely related Sydney rock oyster *Saccostrea commercialis* (A. Mulhall pers. comm.). The experiment was conducted in triplicate.

Preparation of test organisms: The entire investigation was performed at the Seasalter Hatchery using conditioned *Crassostrea gigas* adults (5 of each sex) from a culture maintained at the hatchery. Gametes were obtained for this investigation by the spawning method described by Thompson et al. (1996). Gametes were deposited in separate vessels containing 2 l seawater. Viable eggs were selected (viable eggs are round; non-viable eggs remain teardrop-shaped when discharged into seawater) and pooled, and the egg suspension was filtered. The concentration of eggs was adjusted to 10 000 eggs ml^{-1}. Sperm from each male were assessed microscopically for motility. Motile sperm from different oysters were pooled together and passed through a 60 µm screen to remove any extraneous material. Sufficient sperm suspension was added to the egg suspension to yield 10^5 to 10^7 sperm ml^{-1} in the final mixture. The assessment of fertilization was performed by extracting 1 ml of sample and examining it in a Sedgwick-Rafter chamber under 200× magnification. The entire investigation was performed within the hatchery at a constant temperature of 22 ± 2°C, a dissolved oxygen concentration of 95 to 100%, a salinity of 35‰ and a pH of 7.8 to 8.1.

Experimental procedure: Triplicate test vessels (2 l in volume) were arranged randomly with an airline system connected to aerate and agitate the water. Embryo suspension was added to each of the test vessels using a Gilson pipette to provide a density of 200 ml^{-1}. Temperature, dissolved oxygen, salinity and pH were monitored every other day throughout the duration of the experiment to ensure that conditions were maintained at an optimum. Larvae were fed on a mixture of algae species comprising *Isochrysis galbana*, *Pavlova lutheri*, and *Chaetoceros mureli* cultured at Seasalter Shellfish Hatchery.

On Day 7 pf (the beginning of the exposure period), seawater was replaced with the respective treatments (1 µg l^{-1} nonylphenol in seawater; 100 µg l^{-1} nonylphenol in seawater; 100 µg l^{-1} methanol in seawater for the control). After 48 h, larvae were isolated and all vessels (including controls) were cleaned with a sterilized sponge and rinsed 3 times with filtered seawater before refilling with filtered seawater. The larvae were rinsed by allowing filtered (biological filter consisting of *Crassostrea gigas* spat) seawater to flow over them on a holding sieve (mesh size 40 µm); they were then returned to their respective vessels. Dummy test vessels were also set up on Day 7; they contained test solutions and controls with no larvae and were run for 48 h. Samples were taken from these after 6 and 48 h and immediately put on ice for analysis by gas chromatography mass spectrometry (GC-MS) within 8 h. Analysis was performed at Geochem Analytical Laboratories, Chester, as described by Blackburn & Waldock (1995). The GC-MS analysis was performed to determine actual levels of nonylphenol in the water column during each exposure.

At 1 mo pf, larvae that had developed into spat were transferred to a flow-through system (250 l capacity, pumping 2 l water min^{-1}), where growth and development were monitored to adulthood. This water was pumped directly from a holding pond at Seasalter Hatchery and contained a mixed algae supply (*Isochrysis galbana*, *Pavlova lutheri* and *Chaetoceros mureli*), at 2 mo pf, the spat were transferred to a growing-on tank (500 l capacity) with a water replacement rate of 4 l of water min^{-1} and containing the same algal supply.

Oysters were sampled monthly, and the length of each individual was recorded according to the method described by Galtsoff (1964), where length is the maximum distance between the anterior and posterior margin, measured parallel with the hinge axis. Temperature, dissolved oxygen, salinity and pH of the water in the growing-on tank were monitored monthly until 10 mo pf, at which time the oysters were sexed (males, females and hermaphrodites). Sex was assessed by taking 5 separate samples along the length of the gonad with the tip of a glass pipette. These were then examined microscopically on a slide. A series of test-crosses were then performed according to methods previously described (Nice et al. 2000), within and between treatments, according to the patterns in Table 1.

The resulting embryos from these crosses were adjusted to a density of 200 ml^{-1} and set up with 10 replicates from each of the crosses in 30 ml glass vessels. After 48 h, the development of embryos and larvae was arrested by the addition of 0.5 ml 20% formalin. Larval densities were assessed by removing a 1 ml aliquot from each test vessel (during agitation of the water), placing in a Sedgwick-Rafter chamber, and counting under 200× magnification.

Data analysis: Growth data were compared separately between treatments using a parametric 1-way ANOVA followed by Tukey's HSD test. Sex ratio data were compared with a chi-squared analysis. Larval densities were compared using a Kruskal-Wallis 1-way ANOVA followed by Tukey's HSD test.

Table 1. *Crassostrea gigas.* Cross-combinations: treatment regime experienced by each parent

Cross	Male	Female
1	Control	Control
2	1 µg l^{-1} nonylphenol	1 µg l^{-1} nonylphenol
3	100 µg l^{-1} nonylphenol	100 µg l^{-1} nonylphenol
4	Control	1 µg l^{-1} nonylphenol
5	1 µg l^{-1} nonylphenol	Control
6	Control	100 µg l^{-1} nonylphenol
7	100 µg l^{-1} nonylphenol	Control

Results. ***Development monitored over time—growth:*** Shell length increased with time; however, there was no significant difference in length between exposed and control individuals at any of the time intervals monitored from juvenile (spat) to adulthood ($p > 0.05$ for all cases; Table 2).

Long-term developmental effects—sex ratio: At 10 mo pf, when the resulting oysters were sexually mature, they showed a sex ratio skewed towards females in addition to a comparatively high percentage of fully functional hermaphrodites (17% in 1 µg l^{-1}; 30% in 100 µg l^{-1} treatments). There was no significant difference between the control and expected sex ratios ($\chi^2 = 2.03$; df = 2; $p > 0.05$; n = 18 for controls). However, there was a highly significant difference in the number of hermaphrodites present between 1 µg l^{-1} nonylphenol and controls ($\chi^2 = 14.80$; df = 2; $p < 0.001$; n = 6 for 1 µg l^{-1} nonylphenol); and also for 100 µg l^{-1} nonylphenol and controls ($\chi^2 = 129.76$; df = 2; $p < 0.001$; n = 17 for 100 µg l^{-1} nonylphenol) (Fig. 1). There was no significant difference in shell length or fresh body weight between males, females and hermaphrodites ($p > 0.05$ for both analyses).

Transgenerational effects—gamete viability: When individuals were crossed and the survival rate of offspring recorded 48 h pf, the offspring from control parents had a significantly higher survival rate than offspring where at least one parent had been exposed to nonylphenol during larval development ($\chi^2 = 39.46$; df = 6; $p < 0.001$; Fig. 2).

Chemical analysis: The actual nonylphenol concentrations in the water column 6 and 48 h after dosing were considerably lower than the initial dose concentrations (Table 3).

Discussion. Whilst growth rate in post-settlement individuals remained unaffected by earlier larval exposure to nonylphenol, sexual differentiation appeared to be affected dramatically and transgenerational effects were clearly evident. Although many studies have recorded female yearling oysters as being

Table 2. *Crassostrea gigas.* Shell length compared between treatments for each month (post-fertilization) separately

Months post-fertilization	Mean shell length (mm)	$F_{(df)}$	p
2	14.4	$0.95_{(3,111)}$	0.46
3	21.1	$1.73_{(3,105)}$	0.12
4	22.2	$2.37_{(3,106)}$	0.06
5	26.9	$0.85_{(3,87)}$	0.54
6	32.0	$1.39_{(3,80)}$	0.23
7	42.6	$0.85_{(3,61)}$	0.54
8	50.0	$1.39_{(3,43)}$	0.24
9	59.7	$0.07_{(3,41)}$	0.99
10	61.3	$0.46_{(3,41)}$	0.84

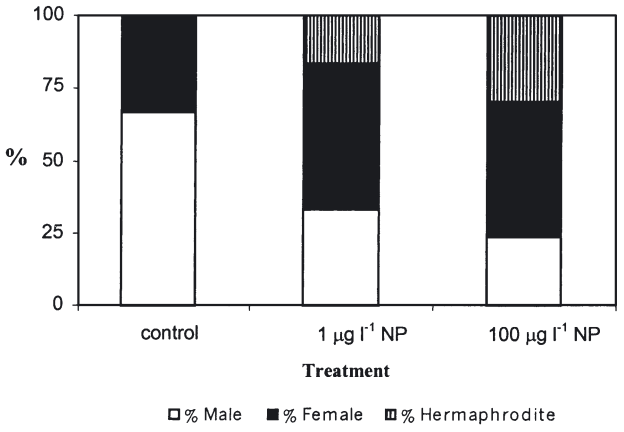

Fig. 1. *Crassostrea gigas.* Proportion of male, female and hermaphrodite adults after nonylphenol (NP) exposure during Days 7 to 8 post-fertilization (pf). Each bar represents percentage of male, female and hermaphrodite adult oysters resulting from each treatment regime at 10 mo pf

significantly larger than males of the same age (Needler 1932a, Menzel 1951, Kennedy & Battle 1964, Kennedy et al. 1996), there was no difference in size between male, female or hermaphrodite oysters in the current investigation. There is no information in the literature concerning the relative body size of hermaphrodites, perhaps because so few have been found.

Sexual differentiation: Up to 30% hermaphroditism was seen in oysters exposed to nonylphenol for a 48 h period during early larval development (Days 7 to 8 pf). However, no hermaphrodites were present in the controls. Historically, the global incidence of hermaphroditism in oviparous oysters is very low. Over the last century, numerous studies of the closely related eastern oyster *Crassosstrea virginica* have shown the incidence of hermaphroditism to range between 0 and 0.96% (Needler 1932a,b, Coe 1934, 1943, Burkenroad 1937, Kennedy & Battle 1964, Kennedy 1983, Kennedy et al. 1996). Similarly, in studies of other species from Pakistan (*C. rivularis*, *C. madrasensis*, *Saccostrea glomerata* and *S. cucculata*), only 0.06% true hermaphroditism has been recorded (Asif 1979). In *C. glomerata*, 0.7% hermaphroditism has been recorded (Dinamani 1974), in *S. commercialis* 0.32% (Cox et al. 1996), and in *C. rhizophorae* 0.5% (Nascimento et al. 1980). Likewise, studies of the Pacific oyster *C. gigas have* also shown a relatively low incidence of hermaphroditism for yearling oysters, ranging between 0 and 1.1% recorded at a variety of different sites in Japan, the USA and Canada (Amemiya 1929, Berg 1969).

The majority of the studies cited did not discriminate between fully functional hermaphroditism and morphological hermaphroditism. Although still extremely rare, morphological hermaphrodites are more likely to occur within a population of oysters than functional hermaphrodites, and typically these are individuals that contain both sperm and eggs. However, because either only one or neither type of gamete is in a mature, fertile state, self-fertilization is not possible (Berg 1969). Conversely, fully functional or true hermaphrodites contain mature sperm and eggs and are capable of self-fertilization (Asif 1979). Although *Crassostrea gigas* has the capability to change sex between seasons, usually there is a clear period during which the gonad remains undifferentiated between reproductive seasons; and once gametogenesis has been initiated the oyster loses the ability to change sex for that season (Kennedy et al. 1996). Eggs usually only begin to develop after the sperm have been extruded—usually with a winter (period of sexual undifferentiation) between the 2 sexual phases (Needler 1932b). Hence it is extremely unusual to find evidence of both male and female gametes in the same individual simultaneously. All the hermaphrodites in the current study were fully functional or true hermaphrodites. Thus, successful fertilization took place between gametes from the same individuals.

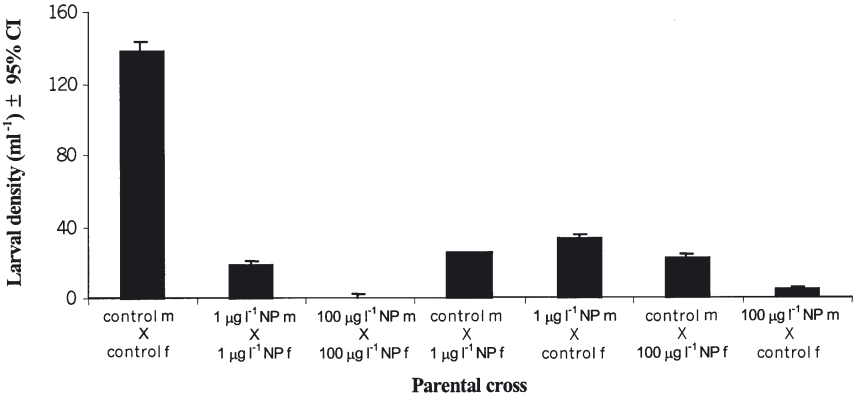

Fig. 2. *Crassostrea gigas.* Density (mean no. ml^{-1}; ± 95% CI) at 48 h pf of surviving embryos and larvae from different combinations of parents. Parents were exposed to different treatment regimes during their own larval development at Days 7 to 8 pf. m: male; f: female; NP: nonylphenol

For the purpose of analysis, in the current study values for both experimental and control treatments were compared with values taken from the literature for each category (male, female and hermaphrodite). Even when the highest recorded hermaphrodite value for *Crassostrea gigas* from the literature (1.1%) was used (Guo et al. 1998) as the *expected* value in the chi-squared analysis, the incidence of her-

Table 3. Nonylphenol concentrations (µg l^{-1}) in the water column over time during each exposure period according to GC-MS analysis

Treatment	Time (h)		
	Nominal 0	Measured 6	Measured 48
Control	0	0	0
1 µg l^{-1} nonylphenol	1	<1	<1
100 µg l^{-1} nonylphenol	100	9	2

maphroditism in the current study was still significantly higher. The *expected* male and female values were taken as the mean of the range of values recorded in the literature for *C. gigas* (range 62.9 to 70% males; Amemiya 1929, Paniagua-Chavez & Acosta-Ruiz 1995, Guo et al. 1998).

Within the genus *Crassostrea*, there is typically a preponderance of male oysters in the first year of a cohort (protandry) (Amemiya 1929, Needler 1932a,b, Coe 1934, 1938, 1943, Menzel 1951, Kennedy & Battle 1964, Dinamani 1974, Andrews 1979, Mackie 1984, Paniagua-Chavez & Acosta-Ruiz 1995, Guo et al. 1998). This may be because sperm are energetically cheaper to produce than eggs, which have substantial yolk reserves (Russell-Hunter 1979). Egg production in oysters requires 50% more energy than sperm production (Kennedy et al. 1996). However, in this investigation, whilst no difference from the norm was observed in control oysters, those that had been exposed to nonylphenol (Days 7 to 8 pf) showed a clear skew towards females; that is, the majority of oysters from both exposure concentrations during this period were females.

It has been suggested that if energy diversion, tested by means of starvation (Bahr & Hilman 1967), shell repair (Davis & Hilman 1971) and parasitism (Cox & Mann 1992) can influence sexual phase, then the presence of some wide-scale environmental stressor could be reflected in a regional modification of sex ratios, with a shift towards increased proportions of males (Kennedy 1983). From the current investigation, it can be seen that this hypothesis does not hold for an environmental stressor such as nonylphenol, since an increase in females rather than males resulted.

Although it is widely accepted that the sex of oysters from the genus *Crassostrea* can be affected by environmental conditions such as temperature, nutrient availability and parasitic stress (Kennedy et al. 1996), the design of the current investigation took these factors into account by keeping conditions constant and optimal between treatments and controls at all times. The oysters in the current study also did not appear to be infected with parasites.

The reproductive physiology of oysters is also known to be under ectocrine or pheromonal influence (Kennedy 1983). It is likely that one or more pheromones are involved in sex determination, but the nature of these compounds remains to be determined (Kennedy et al. 1996). Several studies have shown oestrogens to be involved in sexual maturation following an undifferentiated phase in older (2 to 3 yr) *Crassostrea gigas* (Mori 1968a,b, Matsumoto et al. 1997). However, there are no reports that link sexual development with hormonal mechanisms during the larval phase of *C. gigas*. Oestrone (E_1), 17β-oestradiol (E_2) and oestriol (E_3) have been localized in the ovary of *C. gigas* using immunohistochemical techniques (Matsumoto et al. 1997). E_2 was also found in the testis of male *C. gigas*, but at lower levels than in females, and in males it showed no distinct variation during the spawning period, indicating that its physiological function is different from that in females. Matsumoto et al. (1997) showed that the enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD) is involved in steroid biosynthesis and conversion of E_1 to E_2 and vice versa. The levels of these oestrogens in the gonad vary with stage of the reproductive cycle, and they are also considered to have a role in the development of gametes. Glucose-6-phosphate dehydrogenase, another enzyme involved in steroid metabolism in *C. gigas*, increases in activity as sexual maturation proceeds during a season, with a rapid decline in activity after spawning (Mori 1967).

In studies where E_2 was administered to adult (2 to 3 yr) *Crassostrea gigas*, sex reversal from male to female was induced when administration began at early stages of sexual maturation between reproductive seasons (Mori et al. 1969). However, at a later stage, i.e. once gonad development had begun, the addition of E_2 had no effect on sex ratio (Mori et al. 1969). Exposure to E_2 was also found to accelerate sexual maturation in female *C. gigas* (Mori 1969).

There is evidence to suggest that the reproductive physiology of an oyster can be affected by water-borne pheromones from another oyster (Kennedy 1983). Therefore, it follows that this system may also be sensitive to other chemicals, hormonal or otherwise, present in the local environment during particular stages of development. Oestrogens are known to be involved in the development of *Crassostrea gigas* ovaries and gametes (Matsumoto et al. 1997), and nonylphenol has been shown to bind with the oestrogen receptor *in vitro* using the DNA sequence of the human oestrogen receptor integrated into the yeast genome (Routledge & Sumpter 1996), using rat and rabbit oestrogen receptors (Mueller & Kim 1978), and using trout oestrogen receptors (Jobling & Sumpter 1993). The data currently presented suggest that nonylphenol influences the

reproductive physiology of *C. gigas* by an oestrogenic mechanism during a critical period that includes Days 7 to 8 pf. The specific hormonal mechanisms acting in *C. gigas* during this period are currently unknown. However, evidence from the present study suggests that nonylphenol interferes with one or more of the mechanisms involved with sexual differentiation during the subsequent reproductive season in *C. gigas.* It may act in the same way that has been shown for elevated E_2 to do (Mori 1969, Mori et al.1969). This possibly explains the female-skewed sex ratio of the adult offspring of the oysters exposed on Days 7 to 8 pf.

With respect to the hermaphroditism observed in *Crassostrea gigas* during this study, nonylphenol has caused similar effects in other aquatic organisms. The hermaphroditic condition described as imposex (whereby a female animal develops a penis and vas deferens that ultimately blocks the oviduct and renders the animal infertile) has been described for the dog whelk *Nucella lapillus* exposed to nonylphenol (Evans et al. 2000). However, the levels of exposure that induced this condition were unrealistically high, i.e. 8.95 g l^{-1}; and the method of exposure was nonylphenol 8.95 g l^{-1} paint applied directly to the shells of juveniles and adult dog whelks for a period of 6 mo. In the current study, the exposure concentrations were 3 orders of magnitude lower, the duration of exposure was 48 h only, and the exposure medium was the water column in which the larvae were swimming.

Conversely, the intersex condition (whereby the organism first develops as a male and then starts to develop as a female) has been reported for *Oryzias latipes* (Gray & Metcalfe 1997), where nonylphenol exposure during development led to 86% of the fish developing the intersex condition. The closely related compounds 4-tert-octylphenol and 4-tert-pentylphenol have been shown to have the same effects in medaka and in the common carp *Cyprinus carpio* (Gimeno et al. 1997, Gray et al. 1999a,b). Octylphenol has also been shown to induce the development of 'super-females' (enhancement of oocyte production and enlarged accessory pallial sex glands) in *Nucella lapillus* and the freshwater snail *Marisa cornuarietis* by Oehlmann et al. (2000). The same study reported male *N. lapillus* as exhibiting a reduced length of penis and prostate gland on exposure to octylphenol. In all the above studies, although the mechanisms were generally considered to be oestrogenic, the exact mechanism remained unknown.

Transgenerational effects: Although fertilization was successful, by 48 h pf very few of the larvae from crosses other than those where both parents were untreated remained alive. This suggests that a single 48 h nonylphenol exposure at Days 7 to 8 pf causes the resulting adults, 10 mo later, to produce gametes of poor quality. As noted earlier, oestrogens such as oestrone and 17β-oestradiol are involved in the development of the gonad and gametes (Matsumoto et al. 1997). The current data suggest that nonylphenol disrupts the oestrogenic (or other steroidally regulated) mechanisms involved in normal gonad and gamete development, resulting in gametes of poor quality. Even when the time of exposure is too early to coincide with the development of gametes themselves, nonylphenol may be accumulated in the developing oyster and be transferred at a later stage to developing gametes. Nonylphenol readily accumulates in the lipids of aquatic animals (Lewis & Lech 1996), and parental transfer of chemicals to developing gametes is a well-known phenomenon in fishes (Pierson 1981, Landner et al. 1985, Naggler & Cyr 1997). Male American plaice *Hippoglossoides platessoides* exposed to marine sediments contaminated with organic compounds such as polycyclic aromatic hydrocarbons (PAHs) and polychlolrinated biphenyls (PCBs) were shown to produce less viable sperm than those from control sites, with up to 48% fewer larvae when sperm from exposed males were crossed with eggs from a non-exposed female (Naggler & Cyr 1997). Other studies have shown zinc to be passed to gametes and embryos of the rainbow trout *Oncorhynchus mykiss* (Zeitoun et al. 1976) and the guppy *Poecilia reticulata* (Pierson 1981).

Examples of nonylphenol exposure causing transgenerational effects in other systems include a reduction in egg viability in the polychaete worm *Dinophilus gyrociliatus* (Price & Depledge 1998) and deformed egg masses combined with a reduction in the number of hatchlings in the pond snail *Lymnaea stagnalis* (Smith & Skingsley pers. comm.). Exposure of parental medaka to the closely related alkylphenol, 4-tert-octylphenol, caused developmental problems in the resultant eggs, such as incomplete eye development (anisophthalmia), circulatory problems in the resultant larvae, and failure to inflate swim bladders upon hatching (Gray et al.1999b). Anisophthalmia was also in medaka offspring whose fathers alone had been exposed to 4-tert-octylphenol (Gronen & Brouwer 1998).

In the current study, when self-crosses were performed with gametes from the same hermaphrodite, fertilization did occur, indicating true or fully functional hermaphroditism (Asif 1979). However, all resulting embryos died within the early stages of larval development i.e. within 12 h pf. This low success rate may not be due to exposure of the parent to nonylphenol. When an unexposed hermaphrodite mussel, *Mytilus galloprovincialis*, was self-crossed, high proportions of morphological abnormalities were seen in addition to a significantly lower survival rate compared

with larvae from a cross-fertilization (Beaumont & Abdulmatin 1994). It is a well-established fact across many animal groups that the rare or artificial event of self-fertilization does not yield individuals with a high survival rate, often because of poorly complementary gametes and the limitation of 'good' genes (Charlesworth & Charlesworth 1987).

The exposures described here (100 and 1 µg l^{-1} nonylphenol) were those present at the beginning of the exposure periods. Measurement of actual concentrations by GC-MS showed a substantial reduction of real levels with time. Thus, after 6 h, nonylphenol levels were 9 and <1 µg l^{-1} respectively (detection limit 1 µg l^{-1}). Possible reasons for the loss of nonylphenol from the water column include bioaccumulation in the larvae, or the more likely adsorption of nonylphenol to the test-vessel surfaces. This suggests that the effects seen were likely to have been induced by lower levels of nonylphenol than the nominal values of 1 and 100 µg l^{-1}.

The present study is the first to show that a single exposure to a pollutant at environmentally realistic levels, administered through the water during a key stage of larval development can cause transgenerational effects in oysters. This study has also highlighted that Days 7 to 8 pf in larval development represent a labile period for sexual differentiation in *Crassostrea gigas*, an observation not previously reported. Whilst the exact mechanisms for such findings remain unknown at this stage, it seems likely that nonylphenol acts in an endocrine-disrupting manner, at least in the case of the sex ratio and hermaphroditism effects observed herein. Nonylphenol is clearly an extremely potent chemical that is effective enough (even with a single 48 h exposure) to cause a variety of long-term effects in *C. gigas*, some of which are extremely deleterious to its survival. This may result in severe consequences, not only for natural populations, but also for commercial hatcheries situated in areas where nonylphenol is present in the water.

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