Male Characteristics on Female Mud Snails Caused by Antifouling Bottom Paints

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This study continues an investigation of an anatomical abnormality, named 'imposex', which consists of a superimposition of male characteristics on to a functionally normal female reproductive anatomy of the dioecious snail *Nassarius obsoletus* Say. Imposex is prevalent in natural populations living near yacht basins and rarely found distant from them. In the current study caged snails were transferred between a yacht basin and a distant 'clean' locality where the natural population of snails was normal. Imposex was induced in some normal snails kept at the marina and suppressed, but not lost in abnormal snails kept at the clean locality. A similar positive result was obtained in the laboratory by exposing normal snails to organotin-containing antifouling paints and abnormal snails to clean sea water. Results were negative in parallel tests of various marina-associated materials which did not contain organotin. The laboratory studies have thus identified a causative factor of the anatomical abnormalities common near yacht basins in the natural environment. They also provide a rare, if not unique, example of a chemical agent which causes the appearance of superfluous anatomical features in an animal.

INTRODUCTION

Females of more than 30 species of dioecious snail display pseudohermaphroditism.[1,2] Depending on the species and on the individual animal the aberrant characteristics may be a penis, a vas deferens, convolution of the normally straight oviduct or a combination thereof. The abnormality has been reported in species collected from the San Francisco Bay region of California,[3] Scotland,[4] the English Channel,[4] the Bay of Biscay, France[5] and the French Mediterranean[6] and the east coast of the USA.[1,2] The most extensive examination centered on the common intertidal mud snail, *Nassarius obsoletus (Ilyanassa obsoleta)* (Mollusca: Gastropoda: Stenoglossa = Neogastropoda). The term 'imposex' was coined to describe the pseudohermaphroditism in *N. obsoletus*. All three male characteristics, the penis, the vas deferens and the convoluted oviduct, were found in some females of this species. A scheme was developed for the evaluation of the frequency of occurrence of imposex and the intensity of expression in bearer snails.[2] The frequency of occurrence in snails collected from localities a few kilometers apart often varied greatly[1,3,7,8] as did the intensity of expression.[3,7,8] It was also reported that imposex can be induced or suppressed when snails were transferred between localities where imposex was minimal or absent and those where it was prevalent.[1] The pattern of expression of imposex at localities along a transect ranging from Wickford, Rhode Island, to Savannah, Georgia, strongly suggested a correlation with proximity to yacht basins or marinas.[8] This report examines the hypothesis that imposex is caused by a substance associated with marinas. Snails were transferred between a marina and a distant clean area and changes in frequency and intensity of imposex were recorded. A number of substances used at a marina were tested to determine the capacity to induce imposex. Those substances showing signs of activity were tested to determine how they affected snails collected from different localities.

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EXPERIMENTAL

Localities studied

All snails used in this study were collected from three localities bordering on Long Island Sound in Westport and Southport, Connecticut. One locality, a salt marsh landward to Burial Hill Beach in Westport (41° 07′ 01″ N 73° 19′ 08″ W) was over 3.5 km distant from the nearest marina, and virtually all female snails from this locality were normal. Another was Ye Yacht Yard, a marina in Southport Harbor (41° 07′ 44″ N 73° 17′ 13″ W) where virtually all the female snails were abnormal and the intensity of imposex was greatest. The third was a rocky tidal pool near the mouth of Southport Harbor (41° 07′ 35″ N 73° 17′ 23″ W) where almost all of the snails were abnormal but the intensity of expression of imposex was intermediate. These localities were labeled respectively as 'clean', 'dirty' and 'intermediate'.

Criteria for evaluation of snails

A female with imposex is a female with one or more male characteristics, such as a penis, a vas deferens or convolution of the normally straight gonadal oviduct. Imposex is measured by its frequency of occurrence in the adult female population, R, and by the intensity of expression of all male characteristics in bearer females, I, as described by

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Table 1. Intensity of penis expression in females[a]

Level	Criteria
5	No visible protuberance on head mass: cyst, often filled with brown particulate material, in position normally occupied by base of penis
	Proximal portion of penis only, measuring (in length):
10	⩽0.35 mm (= bump)
15	>0.35 mm < 0.80 mm
20	>0.80 mm < 1.70 mm
25	>1.70 mm
	Distal portions of penis also visible, measuring (in length):
30	<0.35 mm
32	>0.35 mm ⩽ 0.70 mm
35	>0.70 mm ⩽ 1.25 mm
38	>1.25 mm ⩽ 1.70 mm
40	>1.70 mm

[a] All measurements were made when the penis was contracted, often in response to prodding with a probe. The limits of the dimensions for each category were chosen so as to capitalize on observed nodes in a penis size frequency distribution. Further details of reproductive anatomy are provided in Ref. 2.

Smith,[2] and by the intensity of penis expression, *P*. The latter was scored as follows. The mature penis is a highly contractile organ protruding from the right side of the head mass. When contracted it looks like a hatchet, with a rod-like proximal portion attached to the head mass, and a blade-like distal portion articulated on the proximal portion. Each penis is classified into one of ten categories based on the presence and size of these anatomical features (Table 1). The intensity of penis expression in a sample, *P*, is the average expression in bearer female snails. Although all three imposex characteristics were scored when the snails were dissected, detailed analyses were done only on the overall frequency of occurrence of imposex (*R*) and the intensity of penis expression, *P*.

Transfer in cages

In the first experiment, snails were transferred between the clean locality and the dirty locality. Seventy adult females (shell height no less than 12 mm) from the clean site and another 70 from the dirty site were dissected at the start of the experiment. Intact animals from each site were placed in groups of ten in cages made of polyethylene screening. During early April 1975 half of the cages were placed in a subtidal area at the site of origin, and the others were transferred to the alternate site. Food was naturally available in the form of algae growing on the cage surfaces and detritus moving through the screening. Cages were recovered 19 weeks later, and about 75 females from cages at each receiving site were dissected. At that time more than 70 adult females were collected from the natural population at each site and were immediately dissected.

Screening of chemicals

Eight chemical products used at marinas were screened searching for an agent which would cause imposex. The products were: (1) a chemical toilet disinfectant, Parchem Waste System Concentrate (ITT Jabsco Products); (2) a spray detergent, Deck Hand, All Purpose Boat Cleaner (Western Products) was sprayed in to a beaker to allow the froth to subside before being used; (3) an antifouling paint, 502C Red (Gloucester Paints, Inc.) containing 25.2% cuprous oxide. The paint was applied to filter paper in spots of 5-7 mm diameter (4 mg average dry weight) and was added to chambers after drying for the period specified on the directions for use of the paint; (4) an antifouling paint, 711 copper (C. A. Woolsey Paint and Color Co., Inc.) containing 33% copper-bronze powder was prepared as specified above for 502C; (5) an antifouling paint, T755 Red (Woolsey) containing 18.8% tri-*n*-butyltin resinate was prepared as specified for 502C; (6) an antifouling paint, Alumacide Green (Petit Paint Co., a discontinued product containing a mixture of bis(tributyltin) oxide (TBTO) and Paris Green (lead arsenate). It was also prepared as specified for 502C; (7) a leaded gasoline (Atlantic Richfield Co.); (8) an aqueous dispersion of internal combustion emissions. This was made by vigorously bubbling exhaust from a four cycle gasoline engine through a beaker of sea water for 5 min.

Based on the results of a preliminary screening study which lasted seven days, appropriate conditions of exposure were selected for the screening test of induction of imposex, which lasted 35 days. The conditions were 10% of the lowest lethal concentration or, in the event that a lethal condition had not been identified, the highest level used in the preliminary study. The concentration of products (1) and (2) employed in this test were 7 and 10 ppm respectively. Five spots of the paint 502C were added to chambers testing product (3), and ten spots of 711 copper, T755 and Alumacide were added to respective chambers testing products (4), (5) and (6). The leaded gasoline, product (7), was added to each chamber testing product (7) to make a mixture equivalent to 0.013% gasoline. This was added on each of 27 days during the experiment to compensate for volatility. Three milliliters of combustion emissions, product (8), were added on 27 occasions to chambers testing product (8) to compensate for possible volatility and instability. On each occasion these emissions were added to each chamber within an hour of preparation.

Pre-exposure values for imposex were obtained by dissecting 72 females at the start of the experiment. Final values were obtained after exposing groups of 35 snails to each product and to a control regimen for 35 days.

Induction with Alumacide

This experiment was an adaptation to laboratory tanks of the transfer experiments previously done in cages in the field. The water conditions of the dirty locality were modeled by adding Alumacide to sea water which did not induce imposex by itself. Snails were collected from the clean locality, the dirty locality and the intermediate locality. The experiment was conducted in six tanks, each containing 110 l of aerated sea water maintained at 22.5 °C and 23‰ salinity, with *Ulva* sp. algae for food. A piece of filter paper coated with 1.5-1.8 g of dried paint was put in each of three experimental tanks and an untreated piece of filter paper was put with sham-treated controls. Snails were collected from the three localities in the middle of July 1975. A portion of the snails from each locality were desig-

nated as untreated controls and were dissected immediately. Out of the remainder, 350 from each locality were divided equally between experimental and sham-treated tanks. Halfway through the experiment the coated filter paper was removed from the experimental tanks because some of the snails became lethargic. After a period of 75 days the expermental groups, the control groups, and samples freshly collected from the natural environment were dissected.

RESULTS

Transfer in cages

Imposex remained relatively constant in local populations both in terms of frequency of occurrence (R) and intensity of penis expression (P) (Table 2). Confinement of the snails in cages at the same sites did not result in marked changes in imposex. Abnormal females were found in the natural populations at the clean locality with an average frequency of 2.6%, and the few females showing a penis never had an intensity of expression which was greater than 10. At the dirty locality, practically all females were abnormal, and the intensity of expression averaged well over 20. In contrast to local snails, imposex was induced in those snails which were transferred from the clean to the dirty locality and was suppressed in intensity in those transferred from the dirty to the clean locality.

Together with the previously established geographic relationship of imposex with marinas, this result confirmed the hypothesis that something related to marinas both induced imposex in snails from the clean locality and was responsible for maintenance of advanced expression in abnormal snails from the dirty locality.

Table 2. Transfer of snails between localities[a]

Transfer condition		Sampled population		Caged snails
Sampled site	Site of cages	At start	At finish	At finish
Clean	Clean	R = 4.2% P = 10 n = 71	R = 1.0% P = 0 n = 101	R = 10.3% P = 6.7 n = 39
Clean	Dirty			R = 14.3%** P = 22.9* n = 49
Dirty	Dirty	R = 98.6% P = 29.9 n = 73	R = 98.6% P = 28.4 n = 73	R = 96.1% P = 22.9 n = 26
Dirty	Clean			R = 97.3% P = 10** n = 37

[a] Sites and duration of experiment are specified in the Experimental section. R = frequency of occurrence, P = intensity of penis expression, n = sample size. The pair of R and P = 0 values for the population in a 'clean-clean at finish' condition occurred because the one snail with imposex displayed a vas deferens, but not a penis. * Denotes a significant response to transfer ($p < 0.025$), and ** denotes a highly significant response to transfer ($p < 0.01$). Significance was tested by chi square for R and by analysis of variance for P.

Screening of chemicals

The preliminary screening studies yielded lethal concentrations for the chemical toilet disinfectant, the detergent and the 502C copper-based paint. The other preparations were not acutely toxic at the highest concentration tested. Exposure conditions for the test of induction of imposex were derived from acute toxicity data. The screening test revealed females with a high level of penis expression ($P \geqslant 20$) in the two groups exposed to the organotin-containing paints, Alumacide and T755. In all other groups this aberrant structure was expressed only at a low level ($P \leqslant 10$) characteristic of the natural population. This test was not intended to identify substances incapable of inducing imposex, and was not intended to identify all possible causes of imposex. The sole purpose of this test was rather to suggest one possible inducer which could be tested further. Since Alumacide was a slightly more effective inducer of imposex than T755 under these conditions of exposure, it was tested further using the experiment on transfer of snails between clean and dirty localities as a general model.

Induction with Alumacide

The results of exposures to Alumacide in the laboratory replicated the effects seen in the field transfer experiment and confirmed Alumacide as an agent which both induced imposex and was sufficient for its maintenance. When exposed to Alumacide, snails from all three localities

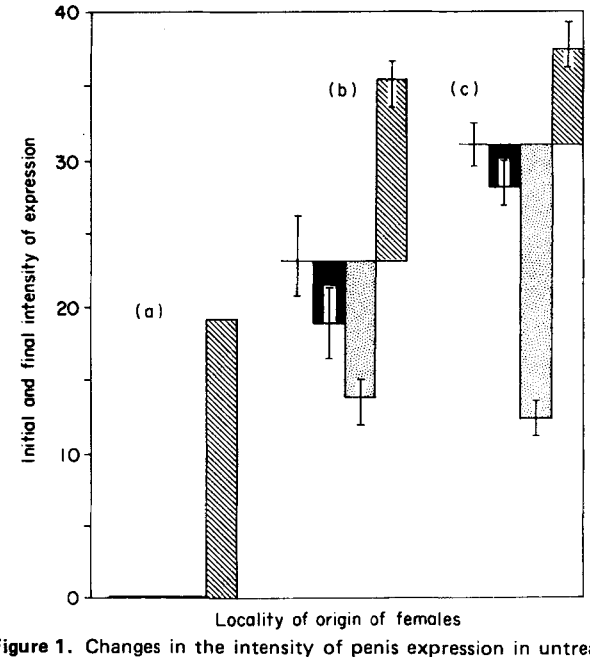

Figure 1. Changes in the intensity of penis expression in untreated females and in those exposed to Alumacide Green. Snails were collected from three localities: (a) the clean locality where snails had virtually no imposex; (b) the intermediate locality where snails had a moderate amount of imposex, and (c) a dirty locality where snails were markedly affected. The baseline of each bar graph marks the intensity of expression in the natural population at the start of the experiment and the bars show the change in intensity of expression at the end of the experiment. Black bar: snails from a second collection at that locality. Point-shaded bar: snails from that locality which were kept as sham-treated controls. Line-shaded bar: those exposed to Alumacide Green. Vertical lines show ± 1 S.D.

developed a significantly more intense penis expression ($p < 0.01$; Fig. 1). Also the frequency of occurrence of imposex increased from $R = 0\%$ to $R = 13.4\%$ in exposed snails from the clean locality ($p < 0.01$) but remained relatively constant in snails from the intermediate locality (from $R = 95.0\%$ to $R = 96.0\%$) and in snails from the dirty locality (from $R = 98.3\%$ to $R = 98.8\%$). In control tanks the intensity of penis expression in snails from the intermediate and dirty localities regressed significantly below the levels in the field ($p < 0.01$; Fig. 1) as did the frequency of occurrence in snails from the intermediate locality (from $R = 97.5\%$ to $R = 91.0\%$, $p < 0.05$). In field samples, snails from the clean locality had no imposex either at the start or at the end of the experiment. At the contaminated sites imposex persisted with no significant changes: while a minor loss of penis expression was evident between the start and the end (Fig. 1), it was balanced by minor increases in frequency of occurrence (from $R = 98\%$ to $R = 100\%$ at the intermediate locality and from $R = 98.3\%$ to $R = 100\%$ at the dirty locality).

DISCUSSION

An anatomical abnormality consisting of superimposition of male characteristics on female snails has been reported at many sites in North America and Europe.[1,3,8] Although it has been found in many species of normally dioecious snails its cause or significance was not known. In this report a convenient method of quantifying the abnormality was introduced, the existence of an environmental cause was confirmed, and a cause was traced to pollution of water by some antifouling paints containing organotin compounds.

In previous studies the abnormality was named 'imposex' and, as evaluated by R, the frequency of occurrence of female snails possessing any of the characteristics comprising imposex: the penis, the vas deferens and convolution of the gonadal oviduct. The intensity of expression in the sample, I, was the average of these sums.[2] The current report retained the use of R, but simplified the evaluation of intensity of expression by considering only the data for the most common characteristic, the penis. This change was prompted by experience with dissection of over 10 000 females[2,3,7,8] which suggested that the intensity of penis expression, P, was the easiest characteristic to evaluate and was possibly the most sensitive to the influence of a factor emanating from marinas.

The existence of an environmental cause postulated on the basis of geographic distribution[8] was confirmed in field tests by an induction of imposex in normal snails kept at a marina and reduction in imposex in initially afflicted snails transferred to an unpolluted site.

Screening of a variety of suspected pollutants from marinas led to the identification of two materials with imposex-inducing activity. Both were antifouling paints and both contained tributyltin compounds similar or identical to those used in tropical climates to kill snail vectors of schistosomiasis.[9] The potency of one of the paints, Alumacide, was then tested in the laboratory not only on normal snails, but also on groups of snails initially afflicted with an intermediate or a high level of abnormality. Not only was imposex induced in some normal snails exposed to Alumacide, but it was also suppressed in abnormal snails not exposed to the paint. This shows that something in Alumacide is responsible for the appearance and the maintenance of imposex. This is a rare, if not unique, example of a biocidal chemical formulation which causes the appearance of superfluous anatomical features in an animal. Further toxicological information is needed to relate imposex to a specific compound, to help predict any ecological impact which may be related to antifouling paints and to help identify courses of action, if any, which may be appropriate. These subjects will be considered in a future article.[10]

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