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# BRIEF COMMUNICATION

# Does proximity to aquatic pollution affect reproductive traits in a wild-caught intertidal fish?

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How chronic exposure to aquatic pollution affects reproductive traits was assessed in nesting wild-caught plainfin midshipman *Porichthys notatus* in areas with low and high contaminant exposure on Vancouver Island, British Columbia. Males in high-exposure areas had a greater degree of testicular asymmetry, sperm with shorter heads and fewer live eggs in their nests. The results of this study provide important insights into the potential consequences of contaminant exposure on the reproductive physiology of wild-caught fishes.


Key words: contaminants; egg survival; *Porichthys notatus*; sperm; testicular asymmetry.

Contaminants present in aquatic environments are of major concern as they can compromise reproductive success of water-dwelling organisms by severely affecting gonadal (Kime, 1995) and gamete structure (sperm: Au *et al*., 2000; Rurangwa *et al*., 2002; McAllister & Kime, 2003; Lahnsteiner *et al*., 2004; Fitzpatrick *et al*., 2008; Hatef *et al*., 2010; egg: Khan & Weis, 1993). While there is abundant laboratory evidence for the effects of aquatic contaminants on reproductive traits, few studies have examined how contaminants affect reproductive traits of wild-caught, naturally exposed fishes experiencing real-world combinations of pollutants. Here, the effects of contaminant exposure on gonadal and gametic characteristics were examined in wild-caught plainfin midshipman *Porichthys notatus* Girard 1854. *Porichthys notatus* could serve as a suitable model to study the effects of contaminants on reproductive traits because nest-tending males guard developing embryos under rocks in intertidal areas for up to three consecutive months during the reproductive season (Arora,

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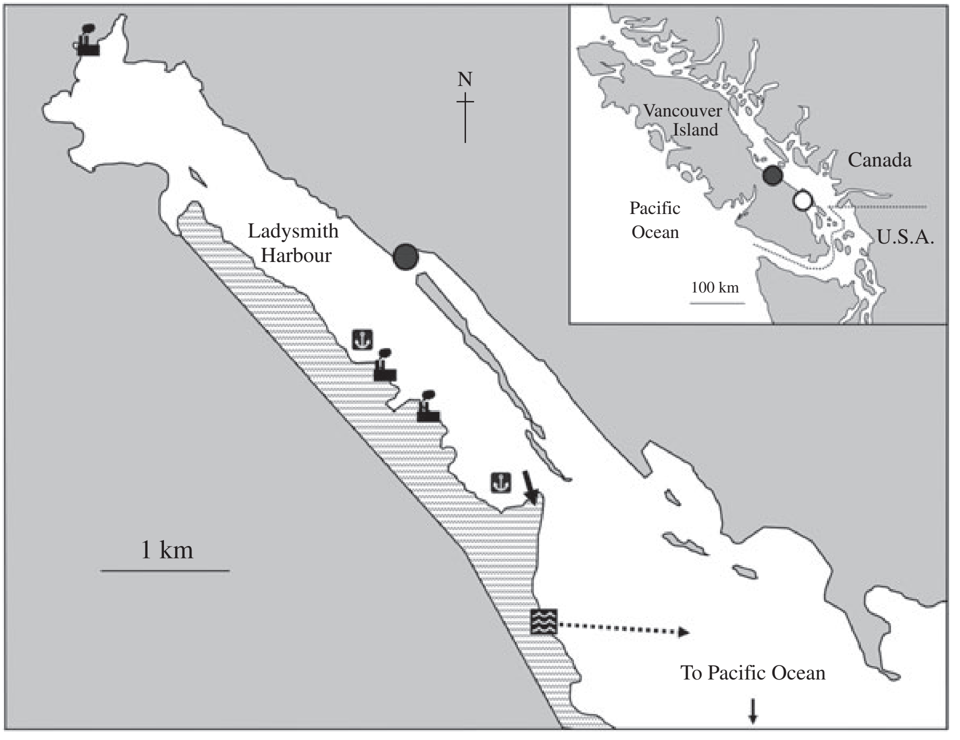


FIG. 1. Field collection sites. Inset: Sites with low contaminant exposure (○, Mill Bay) and high contaminant exposure (●, Ladysmith Harbour) on Vancouver Island, British Columbia, Canada. Full: The site with high contaminant exposure is located in Ladysmith Harbour on the eastern coast of Vancouver Island. The ▤ represents areas with industrial activity (saw mills, ), recreational marinas () and agricultural and urban sewage discharge locations. Within this hatched area is Slag Point (⟶), a coal fill with areas containing buried refuse, dredged wood waste, sand and gravel. Ladysmith wastewater treatment plant (, sewage outfall indicated by ····▸) is located just south of Slag Point.

1948). Following breeding, *P. notatus*, which are common along the Pacific coast of North America, migrate to deeper waters (75–150 m; DeMartini, 1988). Thus, nest-tending males can be exposed to contaminated sediments and water during the entire reproductive season and perhaps even throughout the year as they migrate to only moderate depths.

How living in areas near and distant to sources of pollution impacts reproductive traits was investigated in *P. notatus* on the east coast of Vancouver Island in British Columbia, Canada. During low tides, *P. notatus* nests were surveyed at two sites: Ladysmith Harbour (49° 01′ N; 123° 83′ W) and Mill Bay (48° 63′ N; 123° 53′ W). On the basis of previous environmental studies, these two sites were chosen to represent locations with higher (Ladysmith Harbour) and lower (Mill Bay) contaminant levels. Ladysmith Harbour is characterized by historical and current saw-mill industry, recreational marinas and is an endpoint for agricultural and urban sewage effluent (Golder Associates Ltd, unpubl. data). At the mouth of Ladysmith Harbour is Slag Point, an area of concern identified by the provincial government of British Columbia (Fig. 1) due to sediment primarily composed of coal fill containing polycyclic aromatic hydrocarbons (PAH), petroleum hydrocarbons and metals (copper, up to 478 μg $g^{-1}$, zinc up to 5300 μg $g^{-1}$; Golder Associates Ltd, unpubl. data). These compounds are known to impair reproduction in male fishes (Lahnsteiner *et al*., 2004; Fitzpatrick *et al*., 2008). In contrast, Mill Bay is a relatively unexposed openwater site with fewer sources of pollution (Fig. 1) and is adjacent to undeveloped forest (EVS Environmental Consultants, 1996). Levels of metals in sediment collected from John's Creek, which runs through this undeveloped forest, are significantly lower (copper <20 μg $g^{-1}$, zinc <33 μg $g^{-1}$; Drinnan *et al*., 1995). than those observed at Slag Point. The analyses focused on these two sites because *P. notatus* nests were readily available, as was prior literature about the sediment contaminant levels. Importantly, quantification of dioxins, furans and full congener PCB body burdens in *P. notatus* tissues is currently being pursued following the methodologies described by Ikonomou *et al*. (2007: gas chromatography high-resolution mass spectrometry or GC–HRMS). Preliminary observations based on composite liver samples from a small number of nest-tending males showed that lipid normalized dioxin–furan (183 pg $g^{-1}$) and PCB (1420 ng $g^{-1}$) levels in *P. notatus* from the high-exposure site were somewhat higher than levels measured in *P. notatus* collected at the low-exposure site (dioxin–furan: 79–83 pg $g^{-1}$; PCB: 1000–1089 ng $g^{-1}$). Although these apparent site differences are preliminary and are based on a sample size too small to perform robust statistical comparisons, the differences indeed correspond with site differences in sediment contamination.

Forty-nine high-exposure nests and 24 low-exposure nests were surveyed in May and June 2009. Eggs in nests were enumerated from digital photographs and scored as either live (golden-orange coloured, spherical eggs) or dead (opaquely white coloured eggs, white egg fragments that had ripped or ruptured indicating that eggs were once in the nests but were no longer present, and egg scars where a leftover ring is present on the rock). *Porichthys notatus* occupying nests were measured for body mass ($M_B$) to the nearest 0·001 g and standard length ($L_S$) to the nearest 0·01 cm. Following DeMartini (1988), *P. notatus* were sexed using a combination of ventral colouration, shape of urogenital papilla and the presence of testes upon dissection in *P. notatus* sampled for sperm ($n = 14$ males from the low-exposure site, $n = 16$ males from the high-exposure site). *Porichthys notatus* sampled for sperm were given a lethal overdose of benzocaine, and then the mass of the left ($M_{LT}$) and right ($M_{RT}$) testes was measured to the nearest 0·001 g. A measure of testicular asymmetry was calculated using the formula: testicular asymmetry $= |M_{LT} - M_{RT}|$. The mass of the sonic muscle ($M_{SM}$) was also measured to the nearest 0·001 g. Videos of swimming sperm were captured from 30 nest-tending males following Fitzpatrick *et al*. (2009). Videos were recorded at 60 frames $s^{-1}$ with an Olympus CX41 microscope (Olympus; www.olympus.com), mounted with a Prosilica EC-650 digital camera (Prosilica; www.alliedvisiontec.com) and Astro IIDC (v. 4.04.00) software (www.outcastsoft.com/ASCASTROIIDC.html). Sperm swimming speed was measured using NIH ImageJ software (v. 1.42q; rsb.info.nih.gov/ij/) and the CASA plugin (rsbweb.nih.gov/ij/plugins/casa.html). Smooth path velocity ($V_{AP}$) and curvilinear velocity ($V_{CL}$) were calculated for each male at five different 1 s intervals; 45, 60, 120, 240 and 360 s after sperm began swimming. In external fertilizers, $V_{AP}$ and $V_{CL}$ are highly correlated and both are positively related to fertilization success (Au *et al*., 2002), so a principal components analysis (PCA) score was obtained based on these two measures. The eigenvalue of PC1 was >1 (1·98), explained 99·1%

of the variation in sperm speed and was used as the independent variable of sperm speed in subsequent analyses. To analyse sperm morphology, an image was taken of spermatozoa (15 images per male) under ×400 magnification. Length of sperm head, midpiece and flagellum were measured to the nearest 0·1 μm using ImageJ. Measurements were calculated by drawing a freehand line over each sperm section using an Intuos graphic tablet (Wacom Co. Ltd; www.wacom.com). Two males were excluded from analyses related to testicular investment, as testes of these males were accidently not measured. Six males were excluded from the sperm morphology dataset because it was not possible to find any sperm in their fixed samples to photograph, and six males were excluded from the sperm swimming speed dataset due to a very high sperm density in these videos. Body mass and seasonal effects were controlled for in all of the analyses, as these factors are known to influence reproductive traits (Montgomerie & Fitzpatrick, 2009).

Males collected from high-exposure nests were heavier (two-way ANOVA, site: $F_{1,87} = 17{\cdot}62$, $P < 0{\cdot}001$; month: $F_{1,87} = 7{\cdot}04$, $P < 0{\cdot}01$) and longer (site: $F_{1,93} = 23{\cdot}57$, $P < 0{\cdot}001$; month: $F_{1,93} = 8{\cdot}10$, $P < 0{\cdot}01$) than males collected from low-exposure nests. Body size corrected investment in sonic muscle and testes (sum of right and left testes) did not vary between males from high and low-exposure areas (ANCOVAs, $P > 0{\cdot}05$). Sperm from males collected in high-exposure nests, however, had shorter heads [ANCOVA, site: $F_{1,17} = 10{\cdot}22$, $P < 0{\cdot}05$; month: $F_{1,17} = 0{\cdot}32$, $P > 0{\cdot}05$; body mass: $F_{1,17} = 0{\cdot}93$, $P > 0{\cdot}05$; site × body mass: $F_{1,17} = 4{\cdot}54$, $P < 0{\cdot}05$; month × body mass: $F_{1,17} = 5{\cdot}37$, $P < 0{\cdot}05$; Fig. 2(a)] and overall were smaller in size [sum of head, midpiece and flagellum length, site: $F_{1,17} = 7{\cdot}39$, $P < 0{\cdot}05$; month: $F_{1,17} = 6{\cdot}96$, $P < 0{\cdot}05$; site × body mass: $F_{1,17} = 5{\cdot}20$, $P < 0{\cdot}05$; month × body mass: $F_{1,17} = 5{\cdot}85$, $P < 0{\cdot}05$; Fig. 2(b)]. Sperm midpiece and flagellum length did not vary between males from high and low-exposure nests (site: $P > 0{\cdot}05$, midpiece, month: $F_{1,19} = 31{\cdot}26$, $P < 0{\cdot}001$; flagellum length, month: $F_{1,19} = 2{\cdot}22$, $P > 0{\cdot}05$; body mass: $P > 0{\cdot}05$), indicating that the difference detected in total sperm length between sites was probably driven by differences in sperm head length. Sperm swimming speed did not differ between males from high and low-exposure areas (repeated measures ANOVA, site: $F_{1,13} = 0{\cdot}44$, $P > 0{\cdot}05$; month: $F_{1,13} = 3{\cdot}72$, $P > 0{\cdot}05$; time: $F_{4,13} = 42{\cdot}46$, $P < 0{\cdot}001$; month × time: $F_{4,13} = 3{\cdot}55$, $P < 0{\cdot}05$; body mass: $F_{4,13} = 0{\cdot}10$, $P > 0{\cdot}05$). No correlations were detected between sperm morphological traits and sperm swimming speed ($P > 0{\cdot}05$).

Fluctuating asymmetry (deviations from bilateral symmetry) is thought to arise as a result of exposure to environmental and genetic perturbations during development (Leary & Allendorf, 1989). Fluctuating asymmetry, a biomarker for exposure to environmental contaminants (Valentine & Soulé, 1973; Clarke, 1993; Bonada & Williams, 2002; Chang *et al.*, 2007; Al-Shami *et al.*, 2011), was assessed between the right and left testes. The degree of testicular asymmetry was greater in *P. notatus* collected from the high contaminant exposure site [ANCOVA, site: $F_{1,23} = 7{\cdot}11$, $P < 0{\cdot}05$; month: $F_{1,23} = 1{\cdot}09$, $P > 0{\cdot}05$; soma mass: $F_{1,23} = 2{\cdot}20$, $P > 0{\cdot}05$; Fig. 2(c)]. Such testicular asymmetry may also signal male quality, given the negative relationship between the degree of asymmetry in reproductive traits and the quality of secondary sexual traits that are important in mate acquisition (Møller, 1994). Hence, the relationship between testicular asymmetry and sonic muscle mass, a secondary sexual trait used by nest-tending male *P. notatus* to attract females

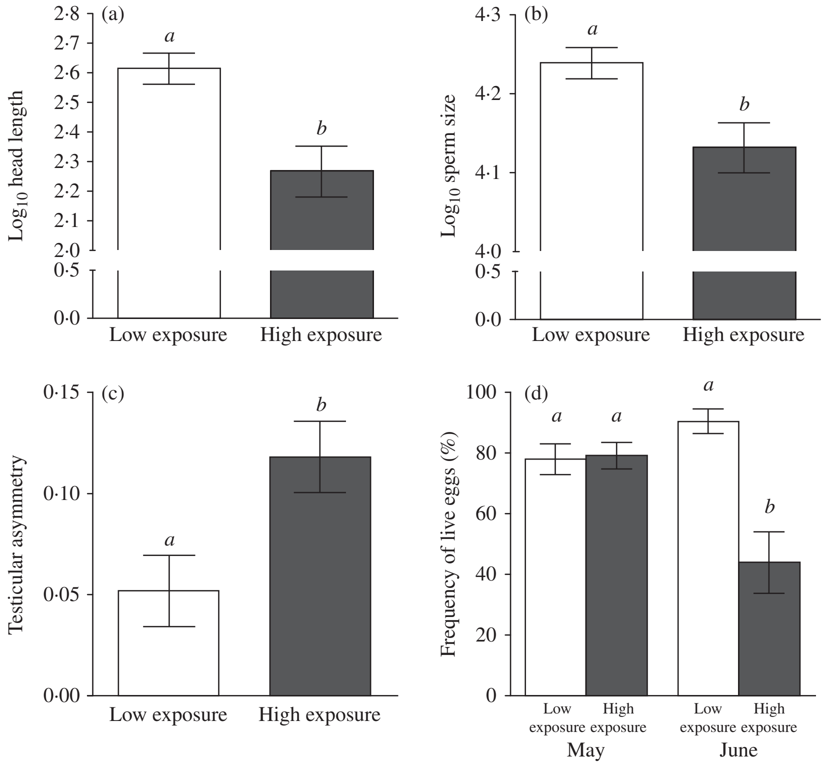


FIG. 2. (a) Sperm head and (b) midpiece length from nest-tending male *Porichthys notatus* from low contaminant (□, $n = 12$) and high contaminant (■, $n = 12$) exposure sites. Data shown are $\log_{10}$-transformed $L_S$ means ± S.E. (μm) controlling for body mass and month. (c) Testicular asymmetry of nest-tending *P. notatus* collected from low contaminant ($n = 14$) and high contaminant ($n = 14$) exposure sites. Data shown are $L_S$ mean ± S.E. controlling for month, soma and sonic muscle mass. (d) Frequency of live eggs (mean ± S.E.; %) in nests surveyed in May (low exposure, $n = 12$; high exposure, $n = 34$) and June (low exposure, $n = 12$; high exposure, $n = 15$). (c), (d) Non-transformed data are presented for visual purposes only (statistical analyses were performed using linear models on $\log_{10}$-transformed data) and Tukey's honestly significant difference (HSD) tests were used to determine *post hoc* differences ($P < 0.05$), which are denoted by different lowercase letters.

acoustically (Ibara *et al.*, 1983), was also tested. Males with larger sonic muscles had greater testicular asymmetry (sonic muscle mass: $F_{1,23} = 7.14$, $P < 0.05$), suggesting that nest-tending males may face trade-offs between investment in courtship and reproduction and that the effects of this trade-off may be more apparent when males are exposed to environmental contaminants.

Nests in the high-exposure sites had similar numbers of eggs (sum of live and dead eggs) as nests in the low-exposure site (ANCOVA, site: $F_{1,47} = 2.10$, $P > 0.05$; month: $F_{1,47} = 1.31$, $P > 0.05$; body mass: $F_{1,47} = 22.04$, $P < 0.001$; site × body mass: $F_{1,47} = 8.45$, $P < 0.01$). After controlling for total egg number, however, nests in the high-exposure site had fewer live eggs than nests in the low-exposure site but this was true only in June and not May [site: $F_{1,68} = 9.99$, $P < 0.01$; month: $F_{1,68} = 6.73$, $P < 0.05$; site × month: $F_{1,68} = 10.23$, $P < 0.01$; total egg number: $F_{1,68} = 34.41$, $P < 0.001$; Fig. 2(d)].

 

This study addresses the effects of living near multiple sources of aquatic pollution (*i.e.* urban, industrial and agricultural) on testicular and gametic characteristics in wild-caught *P. notatus*, a species thought to be sensitive to contaminants (Bard, 1998). Males from areas in close proximity to sources of pollution had sperm with shorter heads and more testicular asymmetry, a previously undocumented potential effect of contaminant exposure. Additionally, in June, fewer live eggs were found in nests from areas with high contaminant exposure, which may be due to several possible factors. Seasonal effects on reproductive traits were also detected. In line with previous studies [Atlantic cod *Gadus morhua* L. 1758 (Rouxel *et al.*, 2008) and Brazilian flounder *Paralichthys orbignyanus* (Valenciennes, 1839) (Lanes *et al.*, 2010)], males collected later in the reproductive season (June) had slower swimming sperm. Sperm head length is known to reduce in *G. morhua* as the reproductive season progresses (Butts *et al.*, 2010). In this study, sperm midpiece was reduced in males collected in June. These results offer some of the first insights into several biologically relevant assays that quantify testicular and gametic impairment potentially mediated by real-world exposure to contaminants. Rarely are such traits looked at in naturally exposed animals, and these results suggest that there are likely to be substantial issues associated with contaminants in other naturally exposed aquatic species.

Potential effects of proximity to aquatic pollution on testicular and sperm structure were detected. To date, testicular asymmetry is documented in only one other fish species [lake whitefish *Coregonus clupeaformis* (Mitchill 1818) (Burness *et al.*, 2008)]. It is possible that greater testicular asymmetry in males from the high-exposure area is a result of contaminant exposure during gonadal development and maturation, and increased asymmetry could have important fitness consequences (Thornhill & Sauer, 1992). This idea can be tested in experiments that expose juvenile and adult *P. notatus* to contaminants and monitor subsequent gonadal growth and reproductive success. Smaller sperm heads (where the cell's genetic material is located; Kunz, 2004) observed in males collected from the high-exposure site could decrease fitness, particularly if smaller sperm heads are a result of DNA damage (Labbe *et al.*, 2001) and degradation (Selevan *et al.*, 2000). Further work linking sperm head morphology with sperm viability or levels of DNA fragmentation (Ruiz-Lopez *et al.*, 2010), and also quantifying how sperm head morphology influences fertilization, would improve understanding of how contaminants influence male fertilization success and offspring viability. Contrary to expectations, sperm velocity did not vary between males collected from low and high-exposure areas. Differences may not have been detected because: (1) natural exposure to contaminants did not influence sperm swimming speed (although others found that it did: McMaster *et al.*, 1992; Aravindakshan *et al.*, 2004; Marchand *et al.*, 2008), (2) there were no site differences detected in sperm flagellum length and sperm flagellum length correlates with sperm swimming speed (Fitzpatrick *et al.*, 2010) and (3) sperm was obtained from dissected testes rather than stripped milt (accessory gland fluids + sperm) and motility of sperm can be altered by the presence of accessory-gland fluids (Fitzpatrick *et al.*, 2005). Distinguishing between these potential hypotheses should be the focus of future research.

Egg survival could be reduced because males produced poor quality sperm or because of female-mediated effects. Female *P. notatus* from high-exposure sites could have deposited eggs laden with a high burden of contaminants (Rudolph *et al.*, 2008). In addition, aquatic contaminants could directly influence eggs, reducing fertilization success (Khan & Weis, 1987) probably by blocking of the micropyle (the opening on an egg's surface through which a sperm enters), which prevents sperm from entering and fertilizing the egg (Khan & Weis, 1993). Finally, contaminant exposure may impair parental care causing lower offspring survival (Pedersen & Saether, 1999). Future studies should aim to: (1) parse out the relative contribution of male and female gametic impairment to reproductive success (*i.e.* examination of the structure of eggs and *in vitro* fertilizations) and (2) explore how egg survival is influenced by direct exposure to contaminants, reduced paternal care or nest desertion (potentially affected by contaminant exposure), maternal effects (shunting of contaminants into gametes) or a combination of these factors.

Controlled laboratory studies investigating how exposure to aquatic contaminants affects gonads and gametes continue to be the prominent methodology in ecotoxicological research. In contrast, research investigating how contaminants affect gametes, in particular sperm, in naturally exposed fishes remains extremely limited ($<10$ studies). Despite the limitations in this study (a single pair-wise comparison between a high and low-exposure sites), these results are a first step in understanding how combinations of contaminants influence animals in the wild and suggest that real-world exposure to contaminants can influence male reproductive physiology and embryo survival. There is a pressing need to determine the fitness effects of real-world contaminants and reduce the growing gap between laboratory studies using exposures to single contaminants and field studies that explore effects of cocktails of contaminants in wild populations.

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