Unusual male size *vs* sperm count relationships in a coastal marine amphipod indicate reproductive impairment by unknown toxicants

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Abstract:

Sperm quantity/quality are significant reproductive endpoints with clear links to population level dynamics. Amphipods are important model organisms in environmental toxicology. Despite this, field monitoring of male fertility in invertebrates has rarely been used in monitoring programs. The aim of this study was to compare sperm quality/quantity in an amphipod collected at six UK locations with differing water quality. Due to low sperm counts and an observed lack of relationship between sperm count and weight in amphipods collected from a nationally protected conservation area (Langstone Harbour, England), we also compared datasets from this site over a decade to determine the temporal significance of this finding. One collection to evaluate a female reproductive endpoint was also performed at this site. Interestingly, this harbour consistently presented some of the lowest sperm counts comparable to highly industrial sites and low eggs number from females. Amphipods collected from all the sites, except from Langstone Harbour, presented strong positive correlations between sperm count and weight. Given Langstone Harbour has several international and national protected statutes primarily for marine life and birds, our results indicate that *E. marinus*, one important food component for wading birds, might be impacted by unknown reproductive stressors. These unknown stressors maybe related to agricultural runoff, leachate from historical landfills and effluent from storm water overflows. This study highlights the importance of exploring new reproductive endpoints such as sperm quantity/quality in marine monitoring programs.

Keywords: sperm quality; reproduction; invertebrates; ecotoxicology; pollution

Capsule: Unusual crustacean sperm counts and sperm count/weight parameters in a marine conservation area highlight impacts of unknown reproductive toxicants

Introduction

Healthy reproduction is a vital process to maintain the integrity of populations and ecosystems and gamete fertilization is a fundamental step to achieve successful reproduction. Spermatozoa are specialized cells used to transfer genetic information from males to eggs and their quality depends on a number of factors, like nutrition and other environmental conditions (Lewis and Ford, 2012). Sperm quality is essential for successful fertilization and has been measured by several metrics including sperm number, sperm viability, sperm motility, spermatophore size, acrosome reaction, melanization and spermatophore absence rate and energy content of spermatophores (Harlıoğlu et al., 2018). The most commonly used methods to determine sperm quality are sperm count, sperm viability and sperm motility (Harlıoğlu et al., 2018).

Sperm count and sperm concentration are the main parameters to assess sperm quality in mammals (Ravanos et al., 2018), insects (Strobl et al., 2019), and marine invertebrates (Harlıoğlu et al., 2018). Positive relationships between body size and sperm counts are already known in insects, with larger males having more sperm cells; however, these relationships may vary depending on the environment that the organisms live in (Strobl et al., 2019). In many crustaceans, larger males tend to produce significantly more quantities of sperm than smaller males (Rodríguez et al., 2007). This significant positive correlation between sperm counts and male weight was observed in many species (Peralta-Martínez et al., 2019; Rodríguez et al., 2007). It has been noted that larger amphipods have an advantage to pair with larger females in a process known as mate guarding (Elwood and Dick, 1990), so it is important to study whether this advantage is only because of the size or if sperm quality of larger males is also higher than sperm quality of smaller males. Positive correlations between adult male size and sperm counts have also been reported in amphipods, for example, in *Echinogammarus marinus* (Yang et al., 2008), *Gammarus pulex* (Galipaud et al., 2011) and *Gammarus duebeni* (Arundell et al., 2014). However, no relationship between these parameters were reported in *G. duebeni* (Dunn et al., 2006), *G. pulex* (Lemaître et al., 2009) and *Gammarus roeseli* (Couchoux et al., 2018). To date, no studies have looked carefully into the relationship between sperm counts and sperm viability with male size in the context of environmental pollution.

Sperm viability is related to the ability of sperm cells to survive and for enough time to reach the place of fertilization (Holman, 2009). It is usually measured with a combination of two fluorescent dyes, SYBR-14, which stains live cells in green, and propidium iodide, which stains dead cells in red, and the cells can be observed by flow cytometry or fluorescence microscopy (Lewis and Ford, 2012). This method is relatively quick, it has been used for more than 50 years in reproduction and fertility studies of human and domestic animals and only in the last decade it has been increasingly applied to ecology (Holman, 2009). Holman (2009) and Gress and Kelly (2011) recommended to always associate sperm viability with sperm count to avoid spurious results, since the sperm viability assay could kill cells and reduce viability. According to these authors, a non-linear positive correlation between sperm count and viability is usually associated with problems in the technique that kill sperm cells and in this case sperm count should be used as a covariate for the analysis. A positive relationship between sperm count and sperm viability was found in insect house cricket (Gress and Kelly, 2011).

Crustaceans, specifically amphipods, are ideal candidates as model organisms having been successfully used in ecotoxicology for decades due to their short life-cycles and capacity to be maintain and reproduce in the laboratory (Podlesińska and Dąbrowska, 2019). They are essential components of aquatic ecosystems because they occupy several trophic niches and serve as food for fish and birds (Glazier, 2014). *Echinogammarus marinus* is one of the most abundant amphipod species in coastal communities in the northeast Atlantic (Martins et al., 2014), and lives mainly associated with assemblages of macro algae *Fucus* spp. These amphipods have great importance in the structure and functioning of intertidal communities, as an active predator of other invertebrates and an important prey for wading birds (Martins et al., 2014). This species has been a potential model organism in many fields of study, like ecology (Maranhão et al., 2001), reproductive biology (Ford et al., 2003a) and ecotoxicology (Yang et al., 2008).

General observations in gammarid amphipods from temperate zones show that females typically produce several broods in succession in warmer months (Hyne, 2011). *E. marinus* population density usually have a clear seasonal variation, with peaks during summer months in Southern England (Guler, 2012), Portugal (Maranhão et al., 2001) and Southwest Netherlands (Vlasblom, 1969). Environmental parameters, specifically temperature, seems to impact reproductive process of the amphipods (Maranhão et al., 2001). Sexual activity and recruitment happen during all year in *E. marinus* population from Southern England (Guler, 2012) and Portugal; however, in Portugal the recruitment was minimum by the end of winter (Maranhão et al., 2001). There is limited information about the effects of contaminants on male amphipod reproduction (Lewis and Ford, 2012; Yang et al., 2008). Decreases in amphipod sperm counts have been observed following exposures to contaminants, like cyproterone acetate (Gismondi et al., 2017), methoxyfenozide, pyriproxyfen and cadmium (Trapp et al., 2014) in laboratory studies. In field studies in Scotland, amphipods collected from industrially polluted sites had been reported to have about 20% significantly fewer sperm compared to the ones from reference sites (Yang et al., 2008). Studies assessing sperm viability have rarely been conducted in amphipods, although a reduction in viability of sperm cells has already been observed in amphipods exposed to ionizing radiation (Fuller et al., 2019). Low sperm counts and sperm viability in amphipods have been linked to fewer fertilized eggs and brood success (Dunn et al., 2006; Fuller et al., 2019), which highlights the potential impact that reduced sperm counts could cause at the population level. This has been modelled for different species of amphipods, indicating that a decrease in sperm counts below certain thresholds could lead to population level impacts (Ford et al., 2012).

The aim of this study was to compare sperm counts and animal weight, and sperm count and sperm viability relationships in *E. marinus* collected from England and Scotland using pre-published and previously unpublished field and laboratory data. Following an unusual observation that amphipods collected in one location (Langstone Harbour) in Southern England over a decade ago, had what appeared to be no or very little relationship between sperm count and weight, we compared datasets over a decade to determine the temporal significance of this finding. To complement this data we also recorded egg/embryo numbers from females from Langstone Harbour and compared to the published literature.

Methods

Site selection and sampling

E. marinus were manually collected from seaweed in the intertidal zone at five different sites along the shores of the United Kingdom during different years and seasons (Table 1). In Scotland, the organisms were collected at one industrially contaminated site (Inverkeithing, 56.025637, -3.385377), and two references sites (Loch Fleet, 57.933809, -4.010696, and Thurso, 58.597759, -3.512685). In England, the organisms were collected at Portsmouth Harbour (Tipner, 50.827035, -1.095151) and Langstone Harbour (50.789624, -1.042419). Inverkeithing is characterized by high levels of PCBs, heavy metals and paper fibres in the sediment. It is a semi-enclosed bay nearby to a shipbreaker's yard and a paper mill (SEPA, 2000). Loch Fleet and Thurso are classified as Class A (excellent) under the coastal classification scheme of water quality. Tipner has elevated levels of TBT and Irgarol 1051 (antifouling biocide) due to historical activity of antifouling painting on boats (Zhou, 2008). Langstone Harbour had water quality classified as "excellent" by Havant Borough Council during the period of sampling; however, it has a legacy of pollution with chemicals such as TBT and other biocides (Cole et al., 2018) and it also regularly receives sewage effluents as storm water overflows in periods of heavy rain (Langstone Harbour Board, 2020), which could increase nitrogen compounds concentrations. According to an Environmental Agency report, Langstone Harbour is considered a eutrophic area since 1994, and the surveys from 2009, 2011 and 2014 showed improvements in the water classification, but opportunistic macroalgae cover in the intertidal area was still above than recommend (Environmental Agency, 2016). Although nitrogen is considered good and oxygen high, the overall water body from Langstone Harbour is classified as moderate and the water quality had failed in the chemical classification in 2013, 2014 and 2019 due to priority hazardous substances, like mercury and its compounds, and polybrominated diphenyl ethers (PBDE) (Environmental Agency, 2021). Thurso (Thurso/Lab 1 and Thurso/Lab 2) and Langstone Harbour also had specimens maintained in laboratory to compare with field amphipods. This period of acclimation in the laboratory without females was thought might provide time to restore their spermatozoa (Table 1). In laboratory, the sex of the organisms was determined using a stereomicroscope and classified as males through the presence of genital papillae. Amphipods were visually inspected for parasites (trematode) infections and the sampling procedure was performed if they were from field groups, otherwise they were maintained in the laboratory away from the females (Table 1).

Table 1: *Echinogammarus marinus* collections used in this study.

Site	Collection date	Sample number	Condition	Time maintained in the laboratory (days)	Analysis	Anaesthetic	Medium form sperm cells
Loch Fleet	April/May2007	47	Field sample	-	Sperm count	Carbonated seawater	Distilled water
Thurso	April/May2007	58	Field sample	-	Sperm count	Carbonated seawater	Distilled water
Thurso/Lab 1	April/May 2009	15	Laboratory	20	Sperm count	Carbonated seawater	Distilled water
Thurso/Lab 2	November 2008	34	Laboratory		Sperm count	Carbonated seawater	Distilled water
Inverkeithing	April/May2007	47	Field sample	-	Sperm count	Carbonated seawater	Distilled water
Tipner	December 2009	30	Field sample	-	Sperm count	Carbonated seawater	Distilled water
Langstone Harbour	October/December 2009	58	Field sample	-	Sperm count	Carbonated seawater	Distilled water
Langstone Harbour/Lab	March/April 2012	11	Laboratory	14	Sperm count	Carbonated seawater	Distilled water
Langstone Harbour/Lab	October 2015	20	Laboratory	28	Sperm count and viability	Clove oil	Leibovitz L-15 and HEPES
Langstone Harbour/Lab	November 2016	21	Laboratory	28	Sperm count and viability	Clove oil	Leibovitz L-15 and HEPES

Langstone Harbour/Lab	June 2017	11	Laboratory	9	Sperm count and viability	Clove oil	Leibovitz L-15 and HEPES
Langstone Harbour/Lab	July 2017	10	Laboratory	33	Sperm count and viability	Clove oil	Leibovitz L-15 and HEPES
Langstone Harbour/Lab	October 2017	6	Laboratory	9	Sperm count and viability	Clove oil	Leibovitz L-15 and HEPES
Langstone Harbour/Lab	November 2017	7	Laboratory	33	Sperm count and viability	Clove oil	Leibovitz L-15 and HEPES
Langstone Harbour	October 2020	116	Field sample	-	Egg numbers	Clove oil	-

Sperm counts and sperm viability

Sperm counts of amphipods collected in Scotland (Thurso, Loch Fleet and Inverkeithing) in 2007 have already been published in Yang et al. (2008). The methods used for sperm counts and sperm viability are described in Yang et al. (2008) and Fuller et al. (2019). Organisms were anaesthetized using carbonated water or clove oil (Table 1) and the organisms were weighted (mg). Testes were dissected onto a cavity slide under stereo microscope, using micro-dissecting scissors and tweezers. Spermatozoa in seminal vesicles were smeared onto the cavity slide and mixed with distilled water or a medium made with Leibovitz L-15 Medium (Sigma-Aldrich, UK) and HEPES (Fisher Scientific, UK) (Table 1), then transferred to an Eppendorf tube with the same solution. Distilled water and L-15/HEPES probably have different osmotic concentrations than marine sperm cells. The modifications in sampling procedure were necessary to preserve cell integrity through the whole procedure for sperm viability, which was assessed using LIVE/DEAD viability kit (Molecular Probes Inc) and a fluorescence microscope with filters of 340–480nm and 450–490nm for SYBR-14 and PI staining, respectively. The cell suspension was mixed, added to a Neubauer haemocytometer and number of sperm cells were counted as three technical replicates for each organism. For sperm viability analysis, number of live and dead cells were counted separately. Total sperm count and sperm viability (% of live sperm) were calculated as the average of three technical replicates. Sperm count data was grouped according to the condition (field or maintained in laboratory) and collection site, normalized by organism weight and means were calculated to compare the different datasets.

Egg numbers

Females collected at Langstone Harbour in 2020 (n = 116) were anaesthetized with clove oil, weighted and embryos were removed from gravid females using a fine pipette. The embryos were counted and classified as their stage as described by Sheader and Chia (1970). Embryo stages were grouped as early (stages 1 to 3) and late (stages 4 and 5) as recommended by Ford et al. (2003a). All embryos were classified within two days of collection to avoid further development. Egg numbers were normalized by organism weight.

Statistical analysis

Sperm count data was normalized by fourth root transformation. One-way ANCOVA was employed to compare different sites and controls from laboratory, using weight as covariate to account for the effect of weight on sperm count results. Pearson’s correlation analysis was also applied to evaluate the relationship between sperm count and animal weight for each collection site. Data from Langstone Harbour was separately compared using a one-way ANCOVA to evaluate the differences in the sperm counts among collection years using animal weight as covariate. One-way ANCOVA was also used to evaluate differences of sperm counts among different collection months in 2009 and 2016, also using animal weight as covariate. Weight was used as covariate for statistical analysis and to normalized data to calculate means, because it is considered a good proxy for length (Ford et al., 2003a). Sperm viability was evaluated with one-way ANCOVA to compare differences among period of collection using sperm count as covariate. For all tests, residuals were tested for normality with Shapiro-Wilk test. Residuals were not normal only when data from all sites was grouped together ($p = 0.009$); however, ANCOVA is robust across non-normal distributions (Rheinheimer and Penfield, 2001).

Results

Sperm counts and relationships between sperm counts and animal weight of *E. marinus* collected at different sites from Scotland and England were compared (Figure 1A). Significant differences were detected among sites and covariate animal weight (ANCOVA, F = 32.992, DFn = 7, DFd = 366, $p = 1.75 \times 10^{-35}$ and F = 55.758, DFn = 1, DFd = 366, $p = 6.06 \times 10^{-13}$, respectively) indicating a strong positive relationship between amphipod weight and sperm counts. Multiple comparison analysis (Bonferroni method) indicated that the sites were separated into three distinct groups that were significantly different from each other. The first group included Inverkeithing (Scotland) and Tipner (England), which are both industrially polluted sites and boat breakers yards. Oddly, Langstone Harbour and Langstone Harbour/Lab also fell into this group with the lowest sperm counts. The second group included Loch Fleet and Thurso which are reference sites in Scotland, with intermediate sperm count values. The third group included the two groups of organisms maintained in laboratory collected in Thurso, with the highest sperm counts. Langstone Harbour sperm counts normalized by weight were 70% lower in field amphipods compared to field amphipods from Thurso (Figure 2). Comparing amphipods maintained in laboratory, sperm counts from Langstone Harbour were between 67% and 74% lower compared to Thurso lab specimens (Figure 2).

Sperm counts of amphipods at Langstone Harbour sampled from the field or maintained in laboratory also did not have a clear relationship with animal weight (Figure 1A); however, the other sites had a significant positive correlation between these two parameters, with large amphipods presenting higher sperm counts (Table 2). Thurso/Lab 1 also did not have a significant positive correlation between sperm count and male weight, however, this is possibly due to the smaller number of amphipods sampled (n = 15) compared to the other sites (Loch Fleet n = 47, Thurso n = 58, Inverkeithing n = 47, Langstone Harbour n = 58, Langstone Harbour/Lab = 84, Tipner n = 30, Thurso/Lab 2 n = 34).

Sperm counts from amphipods collected at Langstone Harbour were compared among years (Figure 1B). Significant differences were observed among different years (ANCOVA, F = 4.273, DFn = 1, DFd = 141, p = 0.041); however, animal weight was not significant covariate (ANCOVA, F = 3.739, DFn = 1, DFd = 141, p = 0.055) in any year of collection. The sample years clustered roughly into three groups. The first group included data from 2009 and 2015 and it had the lowest values of sperm count. The second group included only data from 2012 and it had the highest sperm counts, however, the values were still lower than sperm counts from amphipods from reference sites in Scotland. And the third group included data from 2016 and 2017 with intermediate sperm count values. Sperm counts from amphipods collected were also compared using the different periods that they were maintained in the laboratory as covariate. Significant differences among the years of collection were the same as previous analysis comparing amphipods collected in Langstone Harbour in different years using weight as covariate and without considering the time in the laboratory (ANCOVA, F = 5.473, DFn = 1, DFd = 141, p = 0.021) and period in laboratory was not a significant covariate (ANCOVA, F = 0.593, DFn = 1, DFd = 141, p = 0.442).

Table 2: Pearson's correlation between sperm count and weight of *Echinogammarus marinus* collected at different sites from England and Scotland.

Collection site	Pearson's correlation		
	t.test value	df	p value
Loch Fleet	5.3115	45	3.24×10^{-6}
Thurso	4.3325	56	6.18×10^{-5}
Inverkeithing	5.5274	45	1.56×10^{-6}
Thurso/Lab. 1	0.72724	13	0.48
Thurso/Lab. 2	4.6323	32	5.77×10^{-5}
Tipner	4.2062	28	0.000241
Langstone Harbour	-0.75066	56	0.456
Langstone Harbour/Lab.	0.96308	84	0.3383

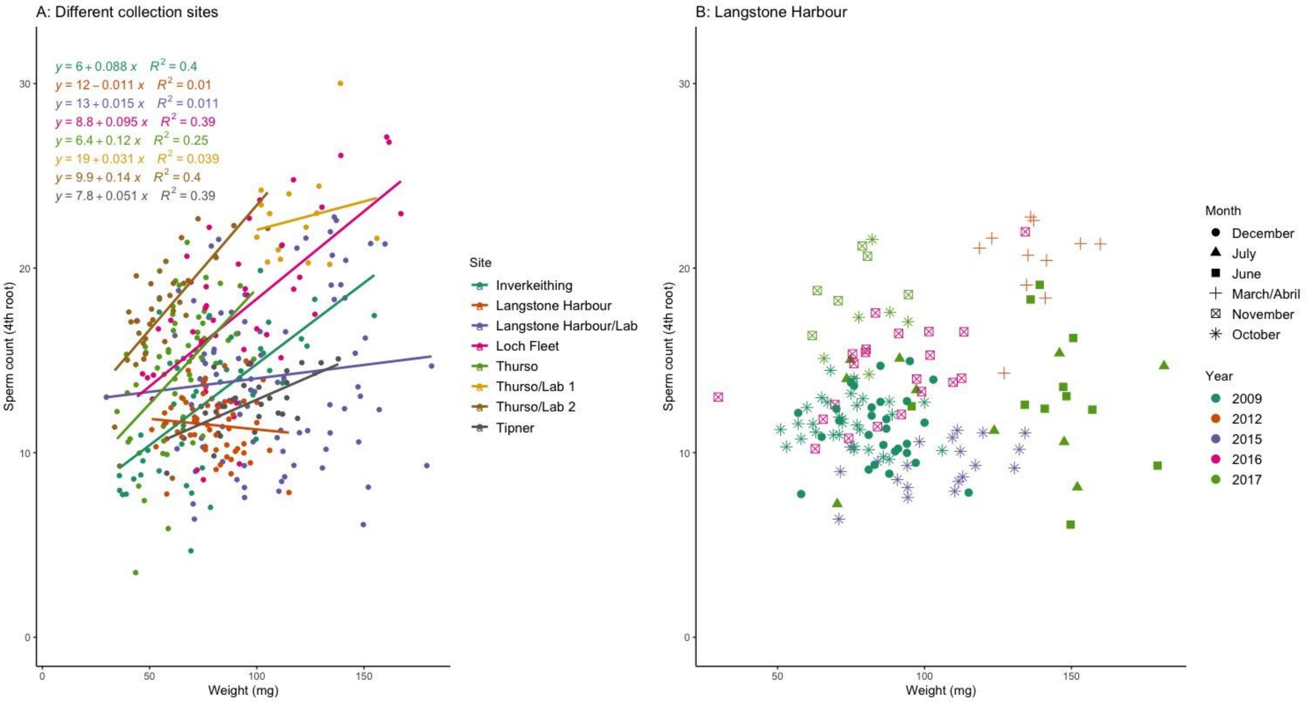

Figure 1: Relationship between sperm counts (normalized by the fourth root) and weight (mg). A: *Echinogammarus marinus* collected at different sites from Scotland and England (for linear Pearson correlation results see Table 2). B: Sperm count from *Echinogammarus marinus* collected at Langstone Harbour in different periods. Amphipods from 2009 were sampled from the field and amphipods from 2012, 2015, 2016 and 2017 were maintained in the laboratory before sampling.

The time of the year also seemed to influence sperm counts. Therefore, another comparison was done in the years of 2009 and 2017, as these had sufficient data available for comparisons among months. In 2009, animal weight did not have significant effect on sperm count (ANCOVA, F = 0.237, DFn = 1, DFd = 55, p = 0.628) and October and December sperm counts had similar values with no significant difference (ANCOVA, F = 0.245, DFn = 1, DFd = 55, p = 0.622). In 2017, animal weight had no significant effect on sperm count (ANCOVA, F = 0.069, DFn = 1, DFd = 29, p = 0.794); however, there was a significant difference among months (ANCOVA, F = 3.464, DFn = 3, DFd = 29, p = 0.029). November had a significantly higher sperm count than July. The other months, June and October, had no significant differences between them.

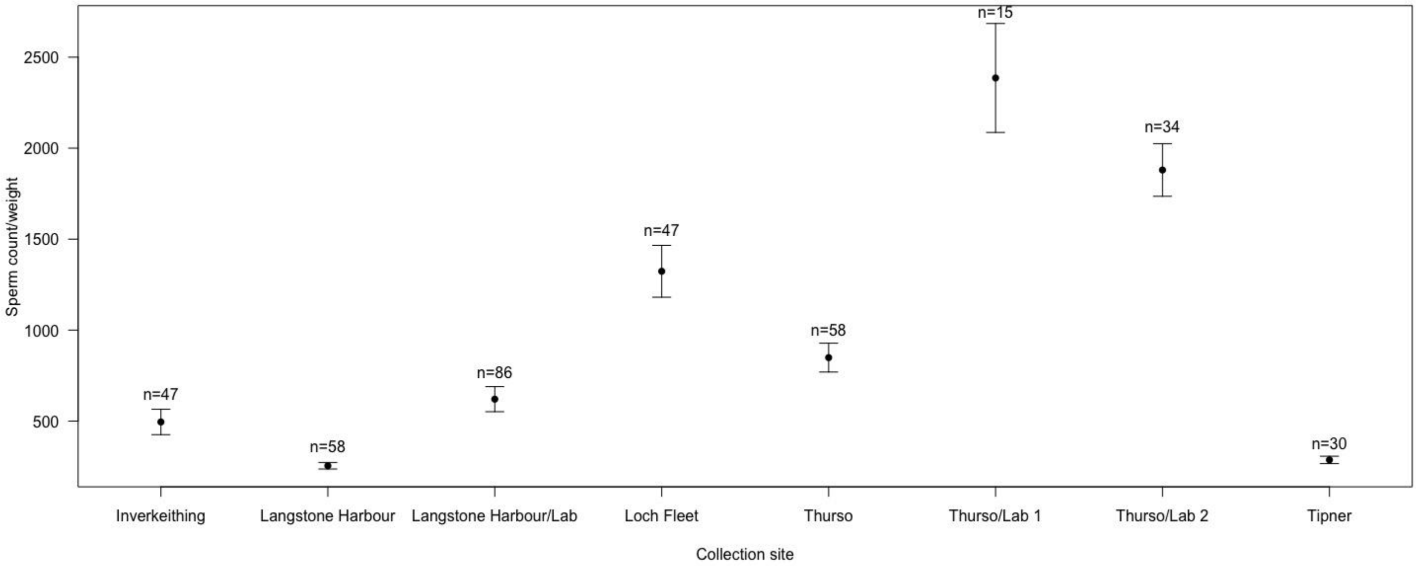

Figure 2: Mean ± SEM of *Echinogammarus marinus* sperm counts normalized by weight collected in different sites from the United Kingdom. Groups called Lab were collected from the respective site and maintained in laboratory for a period (for more information about this period see Table 1).

Sperm viability was evaluated only in amphipods from Langstone Harbour collected in 2015, 2016 and 2017 (Figure 3). A significant reduction in sperm viability was observed in 2017 compared to 2015 and 2016 (ANCOVA, F = 65.372, DFn = 1, DFd = 71, $p<0.05$). However, no significant effect of the covariate sperm count was observed on sperm viability (ANCOVA, F = 3.969, DFn = 1, DFd = 71, $p>0.05$). Interestingly, even with low sperm counts, amphipods from Langstone Harbour had high sperm viability.

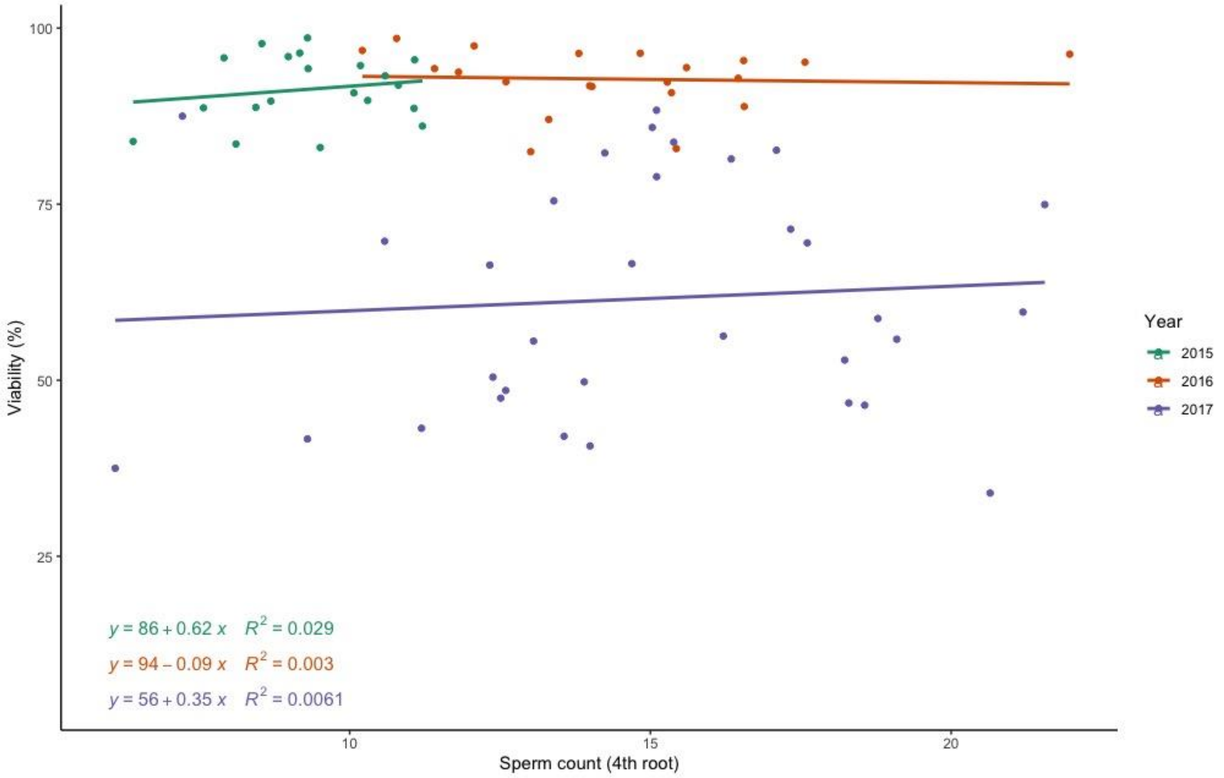

Figure 3: Sperm viability and sperm count (transformed by the forth root) of *Echinogammarus marinus* collected in Langstone Harbour, England, in different periods.

Egg numbers from females collected in Langstone Harbour were were 18.55±7.98 for early stages, 14.77±5.98 for late stages and 17.04±7.42 for total eggs (Table 3). Egg numbers normalized by organism weight (mg) were 0.39±0.21 for early stages, 0.28±0.10 for late stages and 0.35±0.18 for total eggs (Table 3).

Table 3: Egg numbers from *Echinogammarus marinus* females collected in Langstone Harbour, Southern England.

Stage	Egg numbers		Egg numbers/organism weight (mg)	
	Mean	St. deviation	Mean	St. deviation
Early	18.55	7.98	0.39	0.21
Late	14.77	5.98	0.28	0.10
Total (early + late)	17.04	7.42	0.35	0.18
Hatched	6.15	5.61	0.13	0.12

Discussion

In this study, we evaluated the relationship between sperm counts and weight in a marine amphipod collected from different regions of Scotland and England. Furthermore, the relationships between sperm counts and animal weight, and sperm count and sperm viability were analysed, since there is limited understanding of these relationships in these organisms.

A positive correlation between sperm counts and animal weight in *E. marinus* was found at all sampling sites and organisms maintained in laboratory, except Langstone Harbour and Thurso/Lab1. The most contaminated sites presented organisms with the lowest sperm counts, as previously published by Yang et al. (2008), with exception of Langstone Harbour, which also presented specimens with low sperm counts despite being an area with national and international conservation protection. As expected, those specimens kept in the laboratory for a period of time presented the higher sperm counts compared to those collected directly from the field due to a period of abstinence from females. It is also important to highlight that at Langstone Harbour, no relationship between sperm counts and male size (weight) was observed, regardless of the year and month when organisms were collected, which we believe is unusual since significant and positive relationships occurred at all other collection sites. Although one cannot rule out that the lack of this relationship and low sperm counts in *E. marinus* from Langstone Harbour could be related to ecological factors, such as population more actively reproductive, or genetic differences among different populations, we propose another explanation could be related to chemical and/or physiochemical variables. The comparison between collection sites corroborates this and potentially rules our reproductive activity, since amphipods from Langstone Harbour sampled from field and maintained in laboratory (i.e. separated from females) had similar sperm counts to places known to be polluted (Inverkeithing and Tipner) and significantly lower sperm counts compared to Scotland's reference sites. Egg numbers from females collected in Langstone Harbour were also low compared to reference locations and similar to those previously recorded at the polluted site, Inverkeithing (Ford et al., 2003b).

Organisms from Tipner in Southern England also had low sperm counts, which are possibly related to a variety of legacy pollutants, since this location has a history of contamination due to boating activity (Zhou, 2008). Interestingly, Inverkeithing and Tipner are both boat breakers yards. Specimens kept in the laboratory from both England and Scotland had approximately 30% higher sperm counts than those from their respective field collected locations. Organisms maintained in the laboratory were kept away from females, so they had enough time to restore spermatozoa, which could explain their higher sperm counts. Amphipods take between 6 to 12 days to replenish the sperm (Couchoux et al., 2018; Lemaître et al., 2009).

Langstone Harbour is a protected area, being a site of special scientific interest (SSSI) and special area of conservation (SAC) and is also a Ramsar Convention of Wetlands of International Importance (also known as Convention on Wetlands) for bird observation (Baily et al., 2002). However, it has a legacy of pollutants, like TBT and other biocides (Cole et al., 2018), due to antifouling painting boat activity (El-Shenawy et al., 2010), and nowadays it frequently receives discharges from storm water overflows due to heavy rains (Langstone Harbour Board, 2020) and may still be impacted by a legacy of leachate from old landfills (Walton and Higgins, 1998). For example, the site where *E. marinus* were collected is situated close to a historic landfill for naval waste containing asbestos and heavy metals, such as lead, mercury, zinc and cadmium (Walton and Higgins, 1998). The extent to which this impacts coastal waters is unknown. Eutrophic conditions were already observed in Langstone Harbour in 1981 (Montgomery and Soulsby, 1981) and this area had received several alerts classifications due to high levels of nutrients and eutrophic conditions (Leaf and Chatterjee, 1999; Maier et al., 2009). Surveys from 2009, 2011 and 2014 in Langstone Harbour showed improvements in the water classification and in opportunistic macroalgae cover in the intertidal area. However, overall the data indicate that Langstone Harbour still had elevated concentrations of nutrients especially in winter months (Environmental Agency, 2016). Sewage effluent is usually considered responsible for the excess of nitrogen and phosphate compounds in coastal areas (Taylor, 1999), which could lead to algal blooms and algal mats, impacting the O_2 availability and, consequently, the local organisms (Baily et al., 2002; Maier et al., 2009; Taylor, 1999). Langstone Harbour area includes the major conurbation of Portsmouth, Hayling Island and Havant, the upper catchment is mainly arable land, with areas of woodland, agricultural and cultivated lands, and the lower catchment is significantly more urbanised, with few industrial and commercial areas (Environmental Agency, 2016). During 2013 to 2019, overall water body from Langstone Harbour was classified as "moderate" and the water quality has failed in chemical classification in 2013, 2014 and 2019. due to priority hazardous substances, like polybrominated diphenyl ethers (PBDE), and mercury and its compounds (Environmental Agency, 2021). Therefore, organisms from Langstone Harbour are exposed throughout their life stages to different types of contamination.

These conditions are a potential cause for the low sperm counts observed at Langstone Harbour *E. marinus* similar to Inverkeithing and Tipner, although the absence of a relationship between sperm count and animal weight is worth highlighting and not observed in previous recorded locations. In aquatic wildlife a variety of contaminants have been shown to adversely affect sperm counts or viability. For example, Carp fish (*Cyprinus carpio*) exposed to high concentrations of nitrite and nitrate had a significant decreased of sperm motility (Epler et al., 2000) and lower sperm counts when cultivated in eutrophic ponds (Bieniarz et al., 1996). Mosquitofish (*Gambusia holbrooki*) had a decrease of total sperm counts per spermatozeugmatum as nitrate concentration increased, which could be related to the increased apoptosis of sperm cells due to potential conversion of nitrate to nitrite and ultimately toxic nitric oxide *in vivo* (Edwards and Guillette, 2007).The prawn (*Machobrachium nipponense*) exposed to hypoxia showed significant decrease of testicular weight and sperm membrane integrity, and a significant increase of apoptosis rate in spermatids (Sun et al., 2020). In amphipods, decreases in sperm counts have been observed following exposures to pesticides and metals in laboratory studies (Trapp et al., 2014; Gismondi et al., 2017). Therefore, it would be difficult to pinpoint a particular stressor at this stage or rule out other ecological factors at this stage.

The data from this work also demonstrated a seasonal variability of sperm counts in Langstone Harbour. Amphipods collected in July had significantly lower sperm counts than the ones from November. Therefore, the period of collection should be considered for future monitoring studies. This variability on sperm counts among the months is possibly related to breeding periods. Gammarid amphipods in temperate zones usually produce several successive broods during warmer months (Hyne, 2011), thus males collected in the summer would likely have lower sperm counts compared to those collected in the winter, due to the increased use of sperm for breeding. Also, in *E. marinus* populations, males dominate from August to November and females dominate from April to July (Guler et al., 2012). Photoperiod appear to be an influential factor in determining sex of *E. marinus* under laboratory conditions, since male bias was observed in broods over a long-day photoperiod regime and female bias was observed in broods during short-day photoperiod regime (Guler et al., 2012), therefore, with fewer males in July, they would have less time to replenish sperm compared to November, when the male population is larger than the female population. Horseshoe crabs also suffer the influence of season on sperm concentrations, which were more elevate in spring than autumn (Sasson et al., 2012).

Sperm viability is also important to reproduction, since sperm cells have to be viable to fertilize the eggs (Holman, 2009). Sperm viability is commonly evaluated in studies with mammals, however, it is not very commonly used in marine invertebrates (Lewis and Ford, 2012). A relationship between sperm viability and egg numbers was already recorded in *E. marinus*, indicating that low sperm quality could reduce the fecundity (Fuller et al., 2019). No relationship between sperm counts and sperm quality was observed in Langstone Harbour and sperm viability appeared relatively high but variable between years. Further studies on the variability between sites would be useful to understand what might be considered ‘good’ baseline levels of sperm viability Sperm counts and sperm viability are relevant to evaluate impacts that males can cause on reproduction. Reductions in sperm counts have already been associated with a decrease in eggs production and embryos viability (Lemaître et al., 2009). Also, sperm viability has a positive relationship with eggs number produced by the females (Fuller et al., 2019). Moreover, modelling has predicted that a reduction in sperm counts would decrease the female brood size, causing effects on population size and, if perpetuated, the population could collapse (Ford et al., 2012). Amphipods from Langstone Harbour maintained in laboratory for a period of time were not able to reach similar sperm counts to the ones from reference sites. Low sperm counts and eggs number of *E. marinus* from Langstone Harbour should be monitored, because in a long-term time it could affect the densities and overall rigor of these population. If this was the case, it is likely that the impacts would be passed on wading birds and marine life for which amphipods are an important food source (Martins et al., 2014).In Langstone Harbour population density of *E. marinus* was estimated between 5.8 to 97 individuals/m^2 depending on the month of collection (Guler, 2012), however more southerly populations in Mondego estuary (Portugal) the population density of this species is considerably higher (100 to 600 individual/m^2 depending on the season of collection) (Maranhão et al., 2001). Whether the low sperm counts and low population densities are currently linked is currently unknown, but an important research question for further enquiry. While the causal agents for these unusual sperm indices have not been identified, this study has highlighted the importance of evaluating sperm quantity/quality in environmental monitoring.

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REDPOL project - Reduction of Pollution by endocrine disrupting compounds at source. Graphical abstract created with BioRender.com.

References

Arundell, K.L., Wedell, N., Dunn, A.M., 2014. Perceived risk of sperm competition affects sperm investment in a mate-guarding amphipod. Anim. Behav. 87, 231–238. https://doi.org/10.1016/j.anbehav.2013.11.005

Baily, B., Pearson, A., Collier, P., Fontana, D., 2002. Mapping the intertidal vegetation of the harbours of southern England for water quality management. J. Coast. Conserv. 8, 77–86. https://doi.org/10.1652/1400-0350(2002)008[0077:mtivot]2.0.co;2

Bieniarz, K., Epler, P., Sokolowska-Mikolajczyk, M., Chyb, J., Popek, W., 1996. Carp reproduction in highly eutrophic pond conditions. Bull. Environ. Contam. Toxicol. 57, 842–848. https://doi.org/10.1007/s001289900266

Cole, R.F., Mills, G.A., Hale, M.S., Parker, R., Bolam, T., Teasdale, P.R., Bennett, W.W., Fones, G.R., 2018. Development and evaluation of a new diffusive gradients in thin-films technique for measuring organotin compounds in coastal sediment pore water. Talanta 178, 670–678. https://doi.org/10.1016/j.talanta.2017.09.081

Couchoux, C., Dechaume-Moncharmont, F.X., Rigaud, T., Bollache, L., 2018. Male *Gammarus roeseli* provide smaller ejaculates to females infected with vertically transmitted microsporidian parasites. Anim. Behav. 137, 179–185. https://doi.org/10.1016/j.anbehav.2018.01.008

Dunn, A.M., Andrews, T., Ingrey, H., Riley, J., Wedell, N., 2006. Strategic sperm allocation under parasitic sex-ratio distortion. Biol. Lett. 2, 78–80. https://doi.org/10.1098/rsbl.2005.0402

Edwards, T.M., Guillette, L.J., 2007. Reproductive characteristics of male mosquitofish (*Gambusia holbrooki*) from nitrate-contaminated springs in Florida. Aquat. Toxicol. 85, 40–47. https://doi.org/10.1016/j.aquatox.2007.07.014

El-Shenawy, N., Nabil, Z., Abdel-Nabi, I., Greenwood, R., 2010. Comparing the passive and active sampling devices with biomonitoring of pollutants in Langstone and Portsmouth Harbour, UK. J. Environ. Sci. Technol. 3, 1–17.

Elwood, R.W., Dick, J.T.A., 1990. The amorous *Gammarus*: the relationship between precopula duration and size-assortative mating in *G. pulex*. Anim. Behav. 39, 828–833. https://doi.org/10.1016/S0003-3472(05)80946-7

Environmental Agency, UK, 2021. Catchment data explorer - Langstone Harbour [WWW Document].https://environment.data.gov.uk/catchment-planning/WaterBody/GB580705130000

Environmental Agency, UK, 2016. Nitrate vulnerable zone (NVZ) designation 2017 – Eutrophic Waters (Estuaries and Coastal Waters) - Portsmouth Harbour, Langstone Harour and Chichester Harbour.http://apps.environment-agency.gov.uk/static/documents/nvz/NVZ2017_ET2_Chichester_Langstone_Portsmouth_Datasheet.pdf

Epler, P., Chyb, J., Kime, D.E., Sokolowska-Mikolajczyk, M., 2000. The effects of nitritites (NO_2^-) and nitrates (NO_3^-) on sperm motility of common carp *in vitro*. Arch. Polish Fish. 8, 15–24.

Ford, A.T., Fernandes, T.F., Rider, S.A., Read, P.A., Robinson, C.D., Davies, I.M., 2003a. Reproduction in the amphipod, *Echinogammarus marinus*: A comparison between normal and intersex specimens. J. Mar. Biol. Assoc. United Kingdom 83, 937–940. https://doi.org/10.1017/S0025315403008099h

Ford, A.T., Fernandes, T.F., Rider, S.A., Read, P.A., Robinson, C.D., Davies, I.M., 2003b. Measuring sublethal impacts of pollution on reproductive output of marine Crustacea. Mar. Ecol. Prog. Ser. 265, 303–309. https://doi.org/10.3354/meps265303

Ford, A.T., Martins, I., Dunn, A.M., 2012. Insights into sperm-fertilisation relationships in the Arthropoda with ecological significance modelled in an amphipod. Invertebr. Reprod. Dev. 56, 50–56. https://doi.org/10.1080/07924259.2011.606176

Fuller, N., Smith, J.T., Ford, A.T., 2019. Impacts of ionising radiation on sperm quality, DNA integrity and post-fertilisation development in marine and freshwater crustaceans. Ecotoxicol. Environ. Saf. 186, 109764. https://doi.org/10.1016/J.ECOENV.2019.109764

Galipaud, M., Gauthey, Z., Bollache, L., 2011. Pairing success and sperm reserve of male *Gammarus pulex* infected by *Cyathocephalus truncatus* (Cestoda: Spathebothriidea). Parasitology 138, 1429–1435. https://doi.org/10.1017/S0031182011001247

Gismondi, E., Fivet, A., Joaquim-Justo, C., 2017. Effects of cyproterone acetate and vertically transmitted microsporidia parasite on *Gammarus pulex* sperm production. Environ. Sci. Pollut. Res. 24, 23417–23421. https://doi.org/10.1007/s11356-017-0162-4

Glazier, D.S., 2014. Amphipoda, in: Elias, S.A. (Ed.), Reference Module in Earth Systems and Environmental Sciences. Elsevier, pp. 1–49. https://doi.org/10.1016/b978-0-12-409548-9.09437-9

Guler, Y., Short, S., Kile, P., Ford, A.T., 2012. Integrating field and laboratory evidence for environmental sex determination in the amphipod, *Echinogammarus marinus*. Mar. Biol. 159, 2885–2890. https://doi.org/10.1007/s00227-012-2042-2

Guler, Y.Z., 2012. Population dynamics and sex-determining mechanisms in the marine amphipod, *Echinogammarus marinus*. University of Portsmouth.

Harlıoğlu, M.M., Farhadi, A., Gür, S., 2018. Determination of sperm quality in decapod crustaceans. Aquaculture 490, 185–193. https://doi.org/10.1016/j.aquaculture.2018.02.031

Holman, L., 2009. Sperm viability staining in ecology and evolution: potential pitfalls. Behav. Ecol. Sociobiol. 63, 1679–1688. https://doi.org/10.1007/s00265-009-0816-4

Hyne, R. V., 2011. Review of the reproductive biology of amphipods and their endocrine regulation: Identification of mechanistic pathways for reproductive toxicants. Environ. Toxicol. Chem. 30, 2647–2657. https://doi.org/10.1002/etc.673

Langstone Harbour Board, 2020. Water Quality [WWW Document]. http://www.langstoneharbour.org.uk/environment-water-quality.php.

Leaf, S.S., Chatterjee, R., 1999. Developing a strategy on eutrophication. Water Sci. Technol. 39, 307–314. https://doi.org/10.1016/S0273-1223(99)00348-0

Lemaître, J.-F., Rigaud, T., Cornet, S., Bollache, L., 2009. Sperm depletion, male mating behaviour and reproductive 'time-out' in *Gammarus pulex* (Crustacea, Amphipoda). Anim. Behav. 77, 49–54. https://doi.org/10.1016/J.ANBEHAV.2008.08.028

Lewis, C., Ford, A.T., 2012. Infertility in male aquatic invertebrates : a review. Aquat. Toxicol. 120–121, 79–89. https://doi.org/10.1016/j.aquatox.2012.05.002

Maier, G., Nimmo-Smith, R.J., Glegg, G.A., Tappin, A.D., Worsfold, P.J., 2009. Estuarine eutrophication in the UK: current incidence and future trends. Aquat. Conserv. Mar. Freshw. Ecosyst. 19, 43–56. https://doi.org/10.1002/aqc

Maranhão, P., Bengala, N., Pardal, M., Marques, J.C., 2001. The influence of environmental factors on the population dynamics, reproductive biology and productivity of *Echinogammarus marinus* Leach (Amphipoda, Gammaridae) in the Mondego estuary (Portugal). Acta Oecologica 22, 139–152. https://doi.org/10.1016/S1146-609X(01)01112-2

Martins, I., Leite, N., Constantino, E., 2014. Consumption and feeding preference of *Echinogammarus marinus* on two different algae: *Fucus vesiculosus* and *Ulva intestinalis*. J. Sea Res. 85, 443–446. https://doi.org/10.1016/J.SEARES.2013.07.017

Montgomery, H.A.C., Soulsby, P.G., 1981. Effects of eutrophication of the intertidal ecology of Langstone Harbour, U.K., and proposed control measures. Water Sci. Technol. 13, 287–294.

Peralta-Martínez, M. de L.A., Unzueta-Bustamante, M.L., Montaldo, H.H., Caballero-Zamora, A., Castillo-Juárez, H., 2019. Morphometric relationships among spermatophore structures and their association with female fertility in the Pacific white shrimp *Litopenaeus vannamei*. J. Appl. Aquac. 31, 301–308. https://doi.org/10.1080/10454438.2019.1586614

Podlesińska, W., Dąbrowska, H., 2019. Amphipods in estuarine and marine quality assessment – a review. Oceanologia 61, 179–196. https://doi.org/10.1016/J.OCEANO.2018.09.002

Ravanos, K., Petousis, S., Margioula-Siarkou, C., Papatheodorou, A., Panagiotidis, Y., Prapas, N., Prapas, Y., 2018. Declining sperm counts... or rather not? A mini review. Obstet. Ginecol. Surv. 73, 595–605. https://doi.org/10.1097/01.pec.0000526609.89886.37

Rheinheimer, D.C., Penfield, D.A., 2001. The effects of type I error rate and power of the ANCOVA F test and selected alternatives under nonnormality and variance heterogeneity. J. Exp. Educ. 69, 373–391. https://doi.org/10.1080/00220970109599493

Rodríguez, S.R., Regalado, E.M., Pérez, J.A.C., Pastén, A.N., Ibarra, R.S., 2007. Comparison of some reproductive characteristics of farmed and wild white shrimp males *Litopenaeus vannamei* (Decapoda: Penaeidae). Rev. Biol. Trop. 55, 199–206.

Sasson, D.A., Johnson, S.L., Brockmann, H.J., 2012. The role of age on sperm traits in the American horseshoe crab, *Limulus polyphemus*. Anim. Behav. 84, 975–981. https://doi.org/10.1016/j.anbehav.2012.07.023

SEPA, 2000. Scottish Environment Protection Agency. Inverkeithing Bay Sediment Quality. Report TW 2/00.

Sheader, M., Chia, F.S., 1970. Development, fecundity and brooding behaviour of the amphipod, *Marinogammarus obtusatus*. J. Mar. Biol. Assoc. United Kingdom 50, 1079–1099. https://doi.org/10.1017/S0025315400005968

Strobl, V., Straub, L., Bruckner, S., Albrecht, M., Maitip, J., Kolari, E., Chantawannakul, P., Williams, G.R., Neumann, P., 2019. Not every sperm counts: Male fertility in solitary bees, Osmia cornuta. PLoS One 14, 1–17. https://doi.org/10.1371/journal.pone.0214597

Sun, S., Chen, Y., Hu, R., 2020. Aquatic hypoxia disturbs oriental river prawn (*Macrobrachium nipponense*) testicular development : A cross-generational study *. Environ. Pollut. 266, 115093. https://doi.org/10.1016/j.envpol.2020.115093

Taylor, R., 1999. The green tide threat in the UK — a brief overview with particular reference to Langstone Harbour, south coast of England and the Ythan Estuary, east coast of Scotland. Bot. J. Scotl. 51, 195–203. https://doi.org/10.1080/03746609908684935

Trapp, J., Armengaud, J., Pible, O., Gaillard, J.C., Abbaci, K., Habtoul, Y., Chaumot, A., Geffard, O., 2014. Proteomic investigation of male *Gammarus fossarum*, a freshwater crustacean, in response to endocrine disruptors. J. Proteome Res. 14, 292–303. https://doi.org/10.1021/pr500984z

Vlasblom, A.G., 1969. A study of a population of *Marinogammarus marinus* in the Oosterschelde. Netherlands J. Sea Res. 4, 317–338.

Walton, N.R.G., Higgins, A., 1998. The legacy of contaminated land in Portsmouth: Its identification and remediation within a socio-political context, in: Lerner, D.N., Walton, N.R.G. (Eds.), Contaminated Land and Groundwater: Future Directions. Geological Society Engineering Geology Special Publications, London, pp. 29–36. https://doi.org/10.1144/GSL.ENG.1998.014.01.04

Yang, G., Kille, P., Ford, A.T., 2008. Infertility in a marine crustacean: Have we been ignoring pollution impacts on male invertebrates? Aquat. Toxicol. 88, 81–87. https://doi.org/10.1016/j.aquatox.2008.03.008

Zhou, J.L., 2008. Occurrence and persistence of antifouling biocide Irgarol 1051 and its main metabolite in the coastal waters of Southern England. Sci. Total Environ. 406, 239–246. https://doi.org/10.1016/j.scitotenv.2008.07.049