Sperm pHertility: male gamete responses to ocean acidification and other stressors

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"Spermatozoa are among the most remarkable of cells and have excited wonder and fascination ever since their discovery in the 1670s."

Tim R. Birkhead and Robert Montgomerie (Three centuries of sperm research, Sperm Biology An Evolutionary Perspective edited by T.R. Birkhead, D.J. Hosken, S. Pitnick, Elsevier, 1st Edition, 2009).

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ABSTRACT

Ocean acidification (OA) together with other anthropogenic perturbations is projected to dramatically alter marine environments over the coming centuries. The vast majority of marine species reproduce by freely spawning sperm directly into the water column, where fertilisation can then either be external or a female can draw sperm into a burrow, brooding chamber or onto her external surface. Hence, sperm are now being released into rapidly changing seawater conditions. In this thesis, I firstly assess what is currently known on the potential for OA and other anthropogenic stressors to influence freely spawned sperm in marine invertebrate taxa. I then present a series of experimental chapters investigating the influence of OA, as a single stressor or in conjunction with a second stressor, copper, on sperm function, physiology and competitive fertilisation performance in a range of invertebrate taxa.

My research demonstrates that sperm are vulnerable to the projected changes in seawater carbonate chemistry under OA, with responses observed at all biological levels from sperm physiology, swimming performance, fertilisation ecology and sperm competitiveness. In a multi-stressor experiment on polychaete gametes and larvae, I provide empirical evidence that changes to seawater pH under OA can alter the susceptibility of early life stages including sperm, to the common coastal pollutant copper. Sperm DNA damage increased by 150 % and larval survivorship was reduced by 44 % in combined exposures, than when exposed to copper alone. As a single stressor OA also acted to significantly reduce *Arenicola marina* sperm swimming speeds and fertilisation success. This work was followed up with a mechanistic investigation of *A. marina* sperm swimming performance under OA conditions. I found that the length of time between spawning and fertilisation can strongly influence the impact of OA on sperm performance. Key fitness-related aspects of sperm functioning declined after

several hours under OA conditions, and these declines could not be explained by changes in sperm ATP content, oxygen consumption or viability.

In a final set of experiments, I ran a set of paired competitive fertilisation trials in the sea urchin, *Paracentrotus lividus*. In addition to reducing fundamental sperm performance parameters, OA conditions affected competitive interactions between males during fertilisation, with potential implications for the proportion of offspring contributed by each male under the new conditions. This work suggests that the 'best' males currently may not be the most competitive under OA. Overall this body of work reveals a series of significant changes to sperm performance under OA that might act to perturb sperm functioning in future oceans.

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LIST OF ACRONYMS

$[K^+]e$ *external potassium ion concentration*

Δ_i(AICc) *difference between the AICc value of the* i^{th} *model in the selection and the minimum AICc value.*

ΔpH *difference in pH values*

ADP *adenosine diphosphate*

AICc *Akaike information criterion corrected for small sample sizes*

AK *adenylate kinase*

AR *the acrosome reaction*

ASW *artificial seawater*

ATP *adenosine triphosphate*

BPA *bisphenol-A*

Ca^{2+} *calcium ion*

cAMP *cyclic adenosine monophosphate*

CASA *computer assisted sperm analysis*

cGMP *cyclic guanosine monophosphate*

*C*I *confidence interval*

CK *creatine kinase*

CKc *flagellar creatine kinase*

CKmit *mitochondrial creatine kinase*

CO_2 *carbon dioxide*

CO2SYS *program for making carbonate system calculations in seawater*

Cr *creatine*

Cu^{2+} *copper ion*

DBT *dibutyltin*

DIC *dissolved inorganic carbon*

DO *dissolved oxygen*

DWH *Deepwater Horizon*

EC_{50} *concentration of a compound that gives half the maximal response*

EDC *endocrine disrupting chemical*

Em *membrane potential*

ENM *engineered nanomaterial*

EQS *environmental quality standard*

GI *gonad index*

GLMM *generalized linear mixed-effects model*

H^+ *hydrogen ion*

HCO_3^- *bicarbonate ion*

ID *inner dynein arm*

IPCC WGI AR5 *Intergovernmental Panel on Climate Change working group one fifth assessment report*

K^+ *potassium ion*

LIN *average sperm path linearity*

MAM *minimum adequate model*

MMP *mitochondrial membrane potential*

NBC *sodium ion-bicarbonate ion co-transporter*

NHE *sodium ion-hydrogen ion exchanger*

NKA *sodium ion-potassium ion ATPase*

OA *ocean acidification*

OD *outer dynein arm*

OECD *organisation for economic co-operation and development*

OXPHOS *oxidative phosphorylation*

PC *phosphaditylcholine*

PCB *polychlorinated biphenol*

pCO_2 *partial pressure of carbon dioxide*

P-Cr *phosphocreatine*

PCR *polymerase chain reaction*

pHi *internal pH*

pH_{NBS} *pH measured on the National Bureau of Standards scale*

PKA *protein kinase A*

POP *persistent organic pollutant*

PVC *polyvinylchloride*

RCP *Representative Concentration Pathway*

ROS *reactive oxygen species*

SAP *sperm activating peptide*

SE *standard error*

SST *sea surface temperature*

STR *average sperm path straightness.*

suNBC *sea urchin sodium ion-bicarbonate ion co-transporter*

susAC *sea urchin soluable adenylyl cyclase*

TA *total alkalinity*

TBT *tributyltin*

TG *triglyceride*

VAP *average path velocity*

VCL *curvilinear velocity*

VSL *straight line velocity*

w_i(AICc) *maximised rounded Akaike weight*

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AUTHOR DECLARATION

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Presentations and conferences attended:

- Poster presentation at the 'Exe-BioCon' meeting, University of Exeter, UK, June 2016.

  "Will climate change alter the marine 'dating scene'?"

- Poster presentation at the 'Oceans in a high CO_2 world' meeting, Tasmania, Australia, May 2016.

  "Ocean acidification changes the male fitness landscape"

- Poster presentation at a CeMEB advanced course 'Marine evolution under climate change', The Sven Lovén Centre for Marine Sciences, Kristineberg, Sweden, December 2015.

  "Sperm performance and fitness under ocean acidification"

- Oral presentation at 'Advancing the Science of Limnology and Oceanography (ASLO) Aquatic Science' meeting, Granada, Spain, March 2015.

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Introduction 1

Male fertility in a high CO_2 ocean: how will ocean acidification influence the performance and function of freely spawned sperm?

Male fertility in a high CO_2 ocean: how will ocean acidification influence the performance and function of freely spawned sperm?

Spermatozoa are extremely morphologically diverse cells (Birkhead and Montgomerie, 2009), which once ejaculated essentially spend their pre-fertilisation lives as free living organisms (Pitnick *et al.*, 2009). The vast majority of marine species reproduce by freely spawning sperm directly into the water column (Lewis and Ford, 2012), where fertilisation can then be external or a female can draw sperm into a burrow, brooding chamber or onto her external surface. Single celled spermatozoa are faced with an important task: securing a male's reproductive success, with competition between males for females played out by their gametes in the water column. On their journey towards an egg, sperm may face adverse environmental conditions and competition from rival male ejaculates. The fertilisation success of male and female gametes is a key determinant of the reproductive success and population persistence of many marine species (Marshall, 2002) and sperm are therefore under intense selective pressure.

Atmospheric concentrations of carbon dioxide (CO_2) are presently 40 % greater than pre-industrial levels and set to double by the end of this century (Feely *et al.*, 2009). Since the industrial revolution approximately one third of all CO_2 emissions have been absorbed by the oceans (Sabine *et al.*, 2004) and this is driving fundamental changes to seawater chemistry. Oceanic CO_2 uptake inflates seawater pCO_2 and subsequent acid base reactions lead to increases in hydrogen and bicarbonate ions (H^+ and HCO_3^-), whilst reducing the saturation state of calcium carbonate (Feely *et al.*, 2009). Ocean surface pH has already reduced by approximately 0.1 pH units since the industrial revolution (a 30 % increase in H^+) in a phenomenon termed ocean acidification (OA; Caldeira and Wickett, 2003), and climate models predict a further decrease of approximately 0.33 pH units by 2100 unless emissions are dramatically cut (Bopp *et al.*, 2013). These projected changes in ocean chemistry may have a wide range of effects on marine organisms, some of which may be mediated through a disturbance to the acid-base status of affected animals. The regulatory mechanisms maintaining an organism's extracellular and/or intracellular pH may be overwhelmed under the new conditions, leading to a change in

the physiological pH, which can influence many processes including ion transport, protein function and enzyme activity. Many marine species form skeletons or shells out of calcium carbonate, and the reduced saturation state projected under OA conditions will likely increase the energetic costs of calcification and maintenance of these calcium carbonate structures (Gattuso, 2011). The rate and scale of OA are now widely considered to represent a major threat to global marine biodiversity (Halpern *et al.*, 2008; Wittmann and Pörtner, 2013; Kroeker *et al.*, 2010; Dupont *et al.*, 2010).

Seawater conditions are currently changing at a rate faster than at any other time for the past 300 million years (Hönisch *et al.*, 2012), fundamentally altering the fertilisation environments in which freely spawned sperm operate. Whilst the absolute values of atmospheric pCO_2 are not thought to be unique in the Earth's history (Pearson and Palmer, 2000; Zeebe, 2012), the rate at which these changes are taking place has not been previously observed in the geological record leading to concerns that we are entering unknown territory of marine ecosystem change (Hönisch *et al.*, 2012). Spermatozoa may be particularly vulnerable to the changing seawater chemistry, as shifts in their external pCO_2 are likely to cause a greater relative change in internal pCO_2 than cells of later ontogenic stages (Melzner *et al.*, 2009). Mature sperm are considered translationally inactive (Schlegel *et al.*, 2012) and as a result cannot respond to environmental conditions through the synthesis of proteins; limiting their ability to respond to adverse environmental conditions through regulation. Given the large numbers of marine species that reproduce by freely spawned sperm it is important to determine the impacts of the projected changes to seawater chemistry on their fertilisation ecology in order to understand how population dynamics and ultimately marine biodiversity may be affected.

A number of scientific studies have found fertilisation success to be reduced under OA conditions across broad free spawning taxa (Albright and Mason, 2013; Gonzalez-Bernat *et al.*, 2013b; Barros *et al.*, 2013; Albright *et al.*, 2010). However, most studies have reported external fertilisation to be tolerant of experimental OA (Ho *et al.*, 2013; Byrne *et al.*, 2009; Chua *et al.*, 2013; Martin *et al.*, 2011; Havenhand and Schlegel, 2009) including a suite of marine invertebrates from South-East Australia (Byrne *et al.*, 2010b). Contradictory responses, reported for the same species by different research groups,

highlight the potential for population-specific gamete sensitivities that may be driven by adaptation to local environmental conditions. Or they may simply result from different methodologies employed by studies.

It is widely acknowledged that experimental methodology (e.g. gamete handling, sperm-egg contact time, gamete age, seawater volume, test vessel type, stage at which fertilisation scoring takes place and whether gametes are pooled or single male-female crosses are used) can influence fertilisation assay results (Byrne, 2011; Styan, 1998; Palumbi, 1999; Byrne *et al.*, 2010b; Byrne *et al.*, 2010a; Evans and Marshall, 2005). There is also a tendency for assays of external fertilisation success to select unrealistically high sperm concentrations that may saturate the assay- such that any impact of OA on sperm function is masked due to the sheer numbers of sperm in the experimental vessel. Sperm concentration is arguably the most important determinant of external fertilisation success (Claereboudt, 1999), and when a range of sperm concentrations has been tested several assays have found the influence of OA on fertilisation success to be sperm concentration dependent (Gonzalez-Bernat *et al.*, 2013a; Ericson *et al.*, 2010) i.e. the magnitude and/or presence of effects depended on the sperm to egg ratio. There is currently very little field data on sperm concentrations during natural spawning events as it can be difficult to predict the location and timing of these rare events. Hence, there may not be a sufficient understanding of the ecological conditions during natural fertilisation events to appropriately design and interpret the results of OA-fertilisation assays. It may be more pertinent to explore the impacts of climate change stressors on male fertility parameters as these are less likely to be influenced by experimental design and might allow inter- and intra-specific differences in sperm functional response to CO_2-induced OA to be pulled apart and identified with a greater clarity when present (Lewis *et al.*, 2012).

Studies investigating free spawned sperm performance at elevated seawater pCO_2 have generally employed computer assisted sperm analysis (CASA) to examine motility parameters. Any potential impacts of OA upon other aspects of sperm performance (e.g. longevity, chemotaxis and chemokinesis) have received very little research attention, but may also play an important role in the overall impact of OA on marine invertebrate fertilisation ecology. When parameters of sperm swimming have been directly examined,

most investigations have reported significant reductions in sperm swimming speed and/or the percentage of motile sperm in an ejaculate for at least one OA treatment level (Havenhand *et al.*, 2008; Morita *et al.*, 2010; Schlegel *et al.*, 2012; Vihtakari *et al.*, 2013; Uthicke *et al.*, 2013; Schlegel *et al.*, 2014), implying a common sensitivity. Some studies however, have found no effect for either swimming endpoint (Havenhand and Schlegel, 2009; Nakamura and Morita, 2012; Sung *et al.*, 2014) and one reported enhanced sperm swimming performance under OA conditions (Caldwell *et al.*, 2011) adding to mounting evidence of species-specific sperm responses. In addition to inter-species variation in OA-sperm response, significant variation between males has been reported in free spawning species (Schlegel *et al.*, 2014; Schlegel *et al.*, 2015). The influence of OA on the fertilisation success of male-female pairs can also vary (Sewell *et al.*, 2013; Schlegel *et al.*, 2012), and in one study this variation could be partially explained by a significant relationship between the decreases in percentage sperm motility and fertilisation success at one OA treatment level (Schlegel *et al.*, 2012).

Here, I adopted a sperm cellular life history approach to identify and synthesise key aspects of sperm functioning that may be affected by projected changes in seawater chemistry driven by the oceanic uptake of atmospheric CO_2 (Fig. 1). Each stage of a typical free spawned sperm life history is discussed in terms of current scientific knowledge of the cellular processes involved**.** Potential roles for pH, CO_2 and HCO_3^- in sperm functioning are identified in order to gain a mechanistic understanding of how near-future OA may impact upon sperm function and how species-specific differences in sperm OA response might arise at a biochemical level. By linking physiology and biochemistry with the ecology of sperm performance, a framework for predicting the impacts of elevated CO_2 on male fertility is developed.

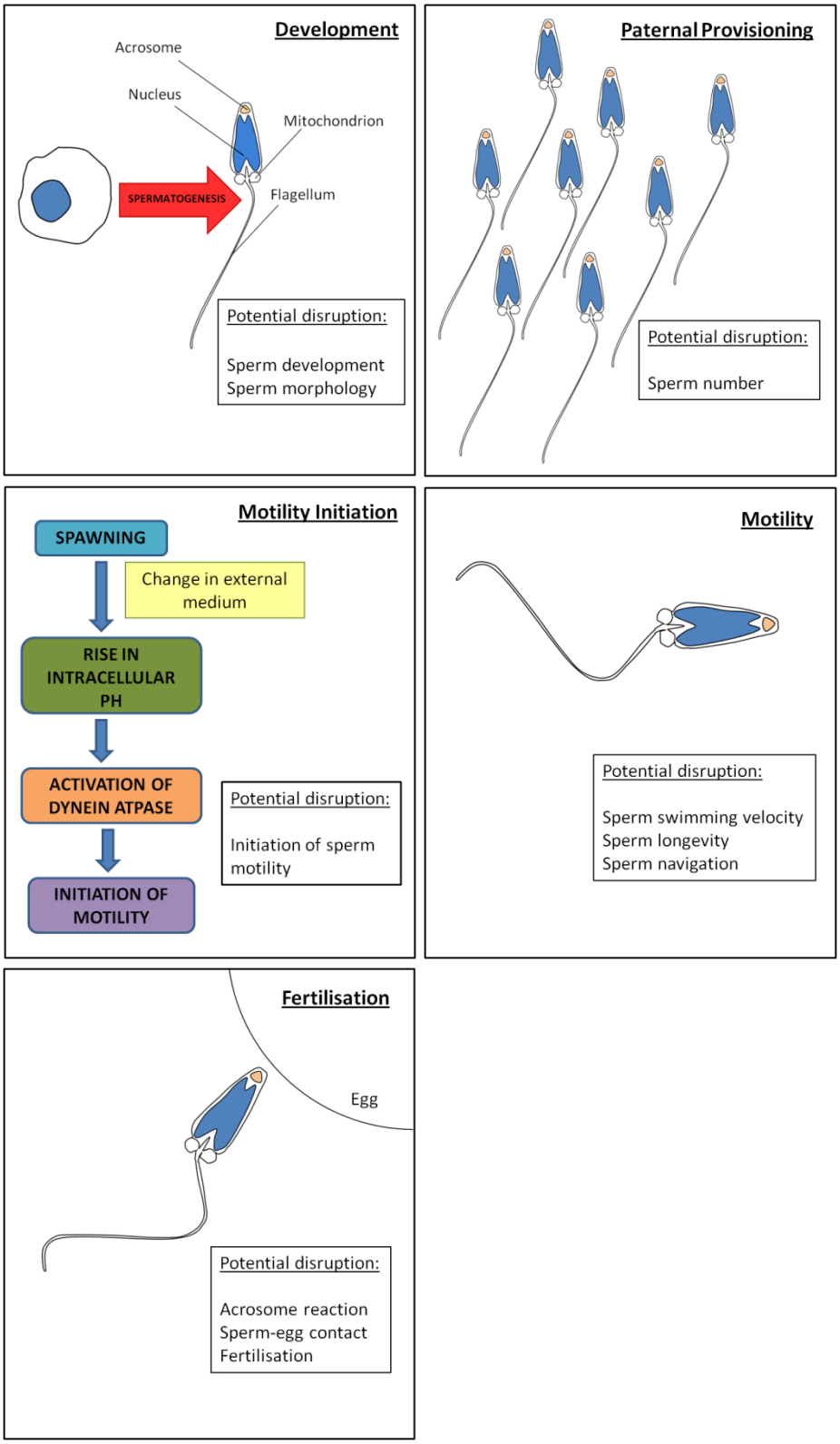

Figure 1. Key aspects of free spawned sperm performance with the potential to be impacted by CO_2 induced seawater acidification.

1.1 OA INFLUENCES ON SPERM DEVELOPMENT

There is evidence in the ecotoxicology literature of the sensitivity of free spawned sperm production to environmental stressors (e.g. defects in sperm morphology in the sea urchin *Anthocidaris crassispina after chronic cadmium exposure; Au et al., 2001).* Whilst there is currently no evidence for a direct mechanism by which near-future OA might impact sperm development in a similar manner, changes to seawater pH can influence the speciation of metals and increase their toxicity and/or bioavailability to marine organisms, thus providing an indirect route of disruption (Millero *et al.*, 2009). Ecologically relevant multiple stressor studies are required in order to identify such negative synergies and assess the reproductive consequences in free spawning species.

1.2 OA INFLUENCES ON PATERNAL PROVISIONING

A departure from evolutionary optimal conditions may result in adverse impacts upon energy homeostasis (Lannig *et al.*, 2010) and lead to a reduced investment in reproduction under OA (Fabry *et al.*, 2008). The provisioning of an ejaculate comprised of sperm in high numbers and with adequate endogenous energy reserves is an adaptive strategy adopted by free spawners to overcome the rapid dilution of sperm in seawater (Levitan *et al.*, 1991) lacking substrates for spermatozoal energy metabolism (Mita and Nakamura, 1998). Reduced male investment in reproduction under OA has the potential to compromise the success of this strategy. Short term exposures to OA conditions in sea urchins have resulted in reduced gonad growth (pH reduction of 0.47 units; Stumpp *et al.*, 2012) and reduced male spawnability i.e. a lower ejaculate volume and quality (pH reduction of 0.20 pH units; Uthicke *et al.*, 2012). Whilst, another short-term study in urchins observed no effect of OA conditions on male gonad index (pH reduction of 0.20 pH units; Uthicke *et al.*, 2014). Longer OA exposures have also revealed mixed results for reproductive investment. There was no effect of rearing for 11 months under OA (ΔpH -0.4 units) on male gonad index in the urchin *Echinometra sp. EE* (Hazan *et al*., 2014). Whilst, 9 months at a pH reduction of 0.27 units delayed reproductive development and

spawning dates by one month in the urchin *Hemicentrotus pulcherrimus* and resulted in significantly higher gonad indices in OA conditions later on in the experiment (animals had not spawned yet in this treatment; Kurihara *et al.*, 2013). Dupont *et al.* (2013) found that a long-term exposure to elevated pCO_2 mitigated reductions in female fecundity observed in their short-term study. There is clearly further work required to better understand the potential for species-specific responses, compare the sensitivity of endpoints and assess adaptation potential. There is currently a paucity of investigations into the response of species in other free spawning taxa beyond echinoderms, which needs to be addressed to better understand the wider reproductive consequences.

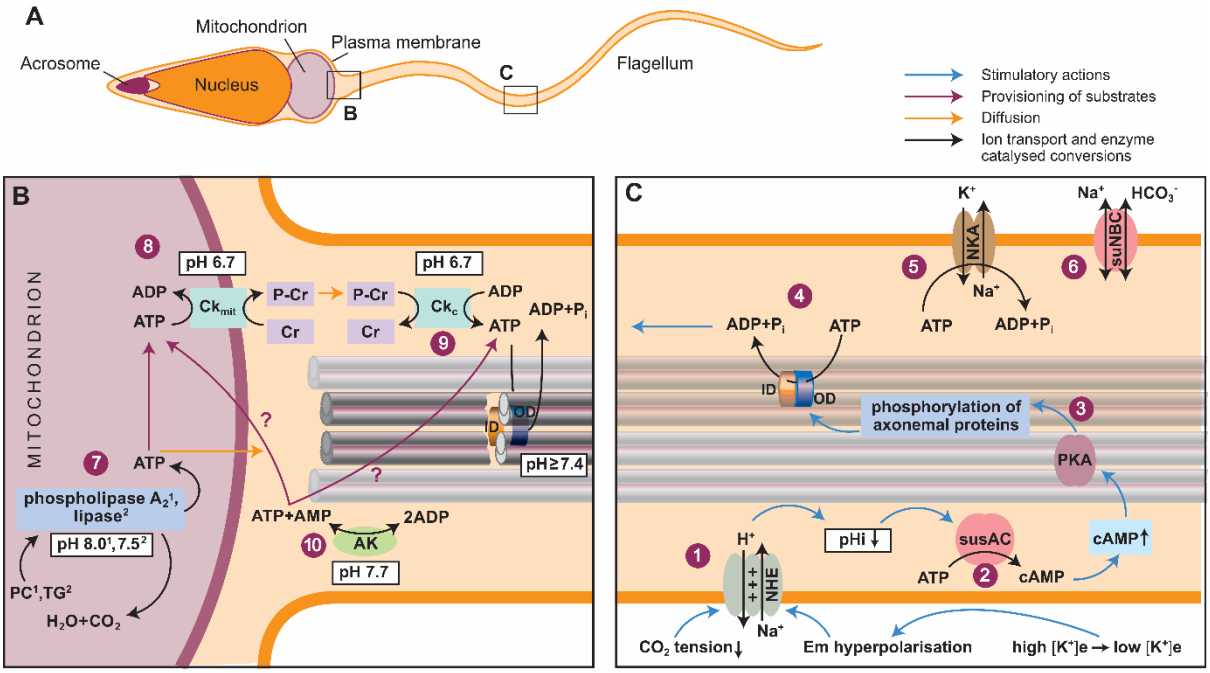

Figure 2. Schematic representation of the sperm structure of a generalised sea urchin (A), energy generation and mechanisms to deliver adenosine triphosphate (ATP) along the sea urchin sperm flagellum (B) and the series of events that trigger the initiation of sea urchin spermatozoal motility upon spawning (C). Where known the optimum pH for enzyme activity is included [Em= membrane potential, $[K^+]e$= external potassium ion concentration, NHE= Na^+/H^+ exchanger, pHi= internal sperm pH, susAC= sea urchin soluable adenylyl cyclase, cAMP= cyclic AMP, PKA= protein kinase A, ID= inner dynein arm, OD= outer dynein arm, NKA= Na^+/K^+-ATPase, suNBC= sea urchin Na^+/HCO_3^- co-transporter, PC= phosphaditylcholine, TG= triglyceride, CKmit= mitochondrial creatine kinase, Cr= creatine, P-Cr= phosphocreatine, CKc= flagellar creatine kinase, AK= adenylate kinase, [1] refers to urchins of the order Echinoida, [2] refers to urchins of the orders Arabacioida, Diademotoida and Clypeasteroida]. *This schematic representation is author's own.*

1.3 POTENTIAL BIOCHEMICAL PATHWAYS FOR OA IMPACTS ON SPERM MOTILITY

Sperm biochemistry has been best studied within free spawning species in sea urchins due to the morphological simplicity of their sperm (Fig. 2A) and their evolutionary position as basal deuterostomes (Gunaratne *et al.*, 2006). Many of the concepts discussed are applicable across other taxa because several aspects of sperm physiology are highly conserved across free spawning species. The cytoplasm of the sperm head is greatly reduced allowing the nucleus containing highly condensed DNA to occupy the majority of the head. The apex of the sperm head contains a specialised exocytotic vesicle; the acrosome (Vacquier and Moy, 1997). Mitochondria are contained within the sperm midpiece from which the flagellum (or sperm tail) extends out. The axoneme is a highly organised internal cytoskeletal structure core to flagellum-powered motility and has been well conserved through evolution (Gagnon and de Lamirande, 2006; Nomura and Vacquier, 2006). The nine peripheral microtubule doublets and two central microtubules forming the axoneme run the entire length of the flagellum with dynein arms projecting from one outer microtubule doublet to the adjacent doublet (Hayashi and Shingyoji, 2009).

1.3.1 Motility initiation

In free spawning marine invertebrates sperm are generally stored immotile in the gonad, and it is the changes to ionic conditions in their surrounding medium that occur upon spawning, which trigger a chain of events leading to activation and the initiation of vigorous motility (Gagnon and de Lamirande, 2006). The reduced CO_2 tension (~pCO_2) of seawater with respect to the gonad and the change in external potassium ion concentration from high to low (gonad to seawater) hyperpolarise sperm membrane potential (Em) and activate sodium (Na^+)/H^+ exchange across the sperm plasma membrane (Lee, 1984; Christen *et al.*, 1983) [Fig. $2C_1$]. The efflux of H^+ out of the sperm

cell via a voltage dependent Na^+/H^+ exchanger (NHE) alkalinises internal sperm pH (pHi), and the low intracellular Na^+ concentration, which favours this exchange, is maintained through the activity of a Na^+-K^+-ATPase (Morisawa *et al.*, 1999; Gatti and Christen, 1985) [Fig. $2C_5$]. The elevation of seawater pCO_2 levels projected under OA could directly impact this early stage of motility initiation through an alteration to the pCO_2 gradient between the gonad and seawater. However, our lack of knowledge on the importance of the magnitude of this gradient and of the effects seawater acidification will have on marine invertebrate gonad CO_2 tension limits our ability to predict any impacts.

The membrane potential sensitive soluble adenylyl cyclase (sAC) is activated through pHi alkalinisation and a sea urchin orthalog (susAC) has been identified (Nomura *et al.*, 2005; Bookbinder *et al.*, 1990). The ubiquitous secondary messenger 3',5'-cyclic adenosine monophosphate (cAMP) is synthesised by susAC and the resultant elevation of cellular cAMP levels activates protein kinase A (PKA) to phosphorylate specific axonemal proteins, which in turn activates dynein ATPase (Nomura and Vacquier, 2006) [Fig. $2c_{2,3}$]. susAC exhibits a strong pH dependency and activity increases two fold at pH 7.5 compared to pH 7.0 (Nomura *et al.*, 2005) [Fig. 3A]. Upon spawning into ambient seawater, sea urchin sperm pHi approaches a pH of approximately 7.5 (Clapper *et al.*, 1985). Given the steep relationship between pH and susAC activity any reduction to pHi could affect intracellular cAMP synthesis by susAC which may delay PKA and ultimately dynein ATPase activation. Early work undertaken by Christen *et al.* (1982) highlighted the potential for external seawater pH to influence sperm pHi, although our scientific understanding of this area remains limited. Identifying any link between external seawater pH and sperm pHi is clearly important to understanding potential OA impacts on sperm function, but is currently a knowledge gap in need of attention.

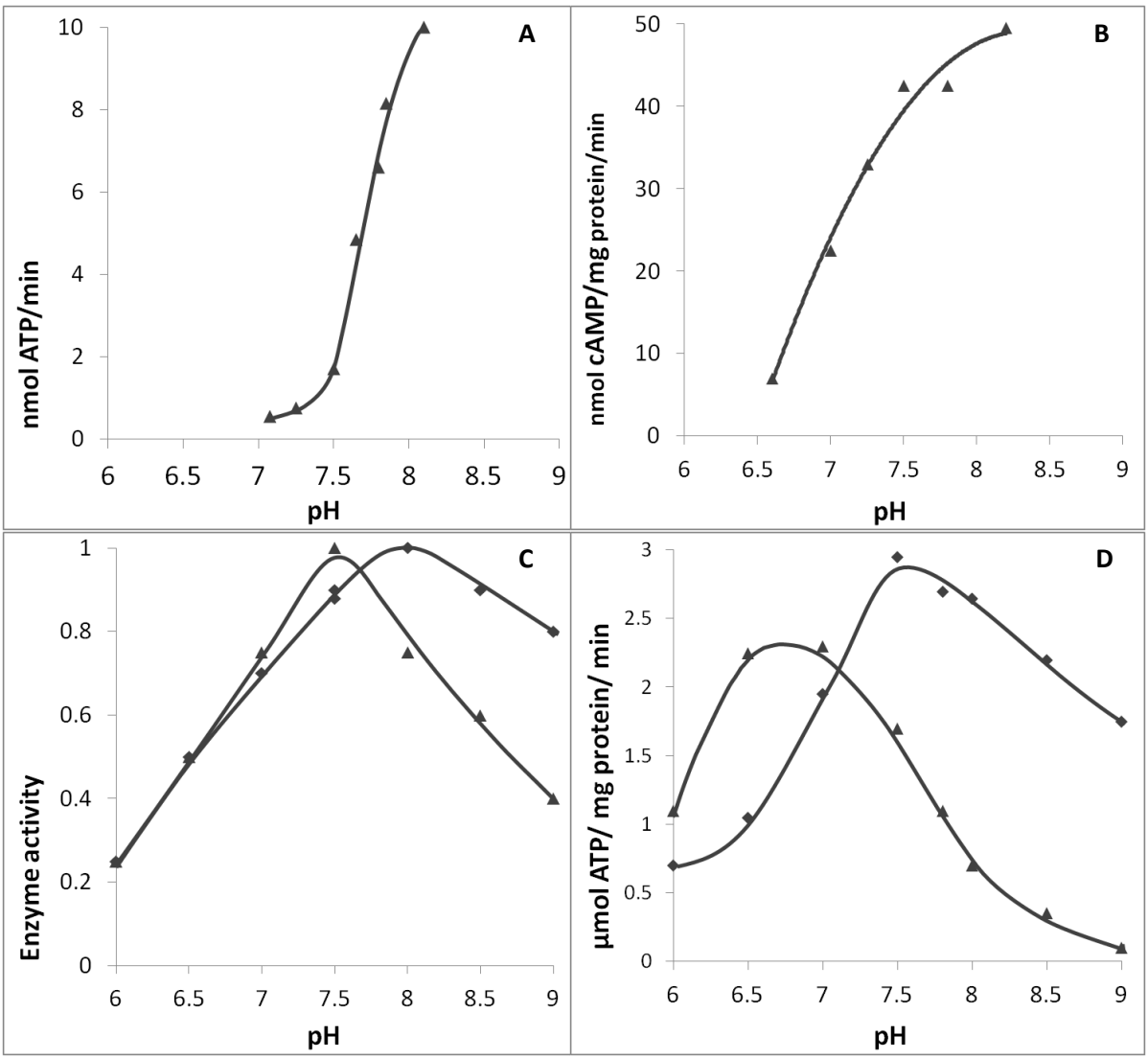

Figure 3. Spermatozoal enzyme activities at various pH values. Dynein ATPase (A); susAC (B); phospholipase A_2 [diamonds] and lipase [triangles] (C); adenylate kinase [diamonds] and creatine kinase [triangles] (D). Data redrawn from Christen *et al.* (1983) (A), Nomura *et al.* (2005) (B), Mita and Nakamura (1998) (C) and Kinukawa *et al.* (2007) (D).

Once activated dynein ATPase orchestrates a highly coordinated sequence of force generation that results in axonemal microtubule sliding and the propagation of undulating waves along the length of the flagellum to provide the propulsive thrust required for spermatozoal swimming (Gagnon and de Lamirande, 2006). Dynein driven adenosine triphosphate (ATP) hydrolysis produces adenosine diphosphate (ADP) which stimulates mitochondrial respiration and spermatozoal energy production (Gagnon and de Lamirande, 2006) [Fig. $2C_4$]. A 50-fold activation of sperm respiration has been observed upon spawning (Christen *et al.*, 1982). Dynein ATPase displays a steep pH activation curve (Christen *et al.*, 1983) and activity could be strongly impacted through alterations to pHi (Fig. 3B). A decrease in pHi below 7.5 would reduce dynein ATPase activity resulting in not only changes to sperm swimming behaviour but a reduced stimulation of mitochondrial respiration and thus ATP supply for subsequent motility.

In many cellular systems HCO_3^- plays a crucial role in pHi control. A regulatory role for a Na^+/HCO_3^- co-transporter (NBC) in events leading to capacitation and involving Em hyperpolarisation has been identified in mouse sperm (Demarco *et al.*, 2003). A sea urchin NBC (suNBC) has been identified. suNBC is localised predominantly in the flagellar plasma membrane and shares many of the features common to the mammalian NBC family (Gunaratne *et al.*, 2006). Em hyperpolarisation is an important step in sea urchin sperm motility initiation and suNBC might undertake a regulatory role in this process or in other aspects of sperm motility (Fig. $2C_6$). The direction of Na^+/HCO_3^- flux has yet to be established alongside the potential mechanisms through which HCO_3^- motility regulation might take place. The projected increase in seawater HCO_3^- is likely to alter the concentration gradient of this ion across the sperm plasma membrane, which could influence suNBC transportation with unknown consequences for the potential regulatory roles.

1.3.2 Energy metabolism and delivery

The oxidative phosphorylation of endogenous lipids by the singular sea urchin sperm mitochondrion is the sole ATP providing pathway supplying the energy required by dynein ATPase to achieve flagellar motility (Dorsten *et al.*, 1997) [Fig. $2B_7$]. Differences

between the enzymes and substrates utilised for oxidation have been identified between orders within Echinoidea. Phosphaditylcholine (PC) is hydrolysed by phospholipase A_2 in urchins of the order Echinoida (Mita and Nakamura, 1993; Mita *et al.*, 1994), whilst urchins of the orders Arbacioida, Diadematoida and Clypeaseteroida use triglyceride (TG) as a substrate for energy metabolism in reactions catalysed by lipase (Mita *et al.*, 1994). Phospholipase A_2 and lipase display optimal activity at different pH values; 7.5 for lipase and 8.0 for phospholipase A_2 (Fig. 3C). This may have driven adaptation in sperm biochemistry or be a source of species-specific responses in sperm energy metabolism to OA conditions if they result in a change to sperm pHi. A reduction in sperm pHi below 7.5 would reduce the activities of both enzymes, which would presumably decrease ATP production.

Aerobic ATP generation in the mitochondrion requires coordination between the two main components of oxidative phosphorylation (OXPHOS); the respiratory chain and ATP synthase (Piomboni *et al.*, 2012). Oxygen is used in the mitochondrial respiratory chain as the final electron acceptor in a series of redox reactions and in the process is converted into water. During this process protons are translocated across the inner mitochondrial membrane creating a proton gradient termed the mitochondrial membrane potential (MMP; Piomboni *et al.*, 2012). ATP synthase uses the MMP to generate ATP. Schlegel *et al.* (2015) identified significant reductions in sperm MMP under OA conditions (pH 7.80, 950 µatm) in the sea urchin *Centrostephanus rodgersii* which strongly suggested a reduced OXPHOS activity in this species.

The sea urchin sperm flagellum extends 40-50 µm from the mitochondrion creating a requirement for an efficient means of delivering ATP generated by the mitochondrion to sites of use by dynein along the entire flagellum length (Kinukawa *et al.*, 2007). In order to overcome the diffusional limitation of ADP along these polar and elongated cells, sea urchin sperm employ a phosphocreatine shuttle which plays a central role in sperm energy metabolism (Wallimann *et al.*, 1998). Creatine kinase (CK) catalyses the reversible transfer of the N-phosphoryl group from phosphocreatine (P-Cr) to ADP to regenerate ATP (Wallimann *et al.*, 1998). Two CK isozymes have been identified, a mitochondrial isozyme (CKmit) and a cytoplasmic isozyme (CKc) which is located along the entire flagellum length as a component of the axoneme (Tombes *et al.*, 1987). At the

mitochondrion creatine (Cr) accepts the phosphate group from ATP in reactions catalysed by CKmit (Fig. $2B_8$). P-Cr freely diffuses along the flagellum, where CKc rephosphorylates ADP (Fig. $2B_9$) to provide dynein ATPase with the ATP required to fuel microtubule sliding (Tombes and Shapiro, 1985; Tombes *et al.*, 1987). CK displays optimal activity at pH 6.7, intriguingly a pHi at which all sperm are immotile (Kinukawa *et al.*, 2007) [Fig. 3D]. Any reduction to pHi has the potential to enhance the efficiency of the phosphocreatine shuttle through increased CK activity given sufficient quantities of shuttle molecules and ATP produced by the mitochondrion.

A second enzymatic system with a functional role in sperm energy homeostasis has been proposed. Adenylate kinase (AK) catalyses the reversible conversion of 2ADP to AMP and ATP and is found along the entire sperm flagellum tightly bound to the axoneme (Kinukawa *et al.*, 2007). The ATP produced by AK may be used directly by dynein ATPase for motility and/or in the rephosphorylation of Cr for use in the phosphocreatine shuttle (Tombes *et al.*, 1987; Kinukawa *et al.*, 2007). AKs may be important in the removal of ADP within the flagellum where it may act as a potent inhibitor of dynein ATPase and would otherwise restrict motility (Okuno and Brokaw, 1979) [Fig $2B_{10}$]. The optimal pH for AK activity is approximately 7.7 suggesting that reductions to sperm pHi below 7.5 would negatively impact the activity of this enzyme (Kinukawa *et al.*, 2007). Both flagellar beat frequency and the velocity at which microtubule sliding takes place (Kamimura and Takahashi, 1981) are dependent upon ATP concentrations (Okuno and Brokaw, 1979). Reductions in AK activity could act to decrease the concentration of ATP available to dynein ATPase with negative implications for sperm swimming velocity.

1.3.3 Response to sperm activating peptides (sperm navigation)

Interaction between male and female gametes can take place at some distance apart due to the presence of small diffusible peptides known as sperm activating peptides (SAPs) in echinoderms. SAPs interact with specific sperm receptors to cause a cellular activation of sperm which either enhances sperm movement, respiration and phospholipid metabolism (chemokinesis) or aids sperm navigation towards the egg (chemotaxis;

Beltrán *et al.*, 2007; Neill and Vacquier, 2004; Darszon *et al.*, 2008). Speract was the first SAP to be purified and characterised (Hansbrough and Garbers, 1981; Suzuki *et al.*, 1981) and the signalling pathway of the speract response has been well studied in *Strongylocentrotus purpuratus* sperm. The binding of speract to its flagellar receptor transiently activates guanylyl cyclase to synthesise cyclic guanosine monophosphate (cGMP; Bentley *et al.*, 1986; Beltrán *et al.*, 2007). The elevation of cellular cGMP stimulates Em hyperpolarisation via a K^+ efflux (Strünker *et al.*, 2006) which activates several components, one of which is Na^+/H^+ exchange through a NHE resulting in pHi alkalinisation (Lee and Garbers, 1986). Feedback involves pH-sensitive enzymes (Ramarao and Garbers, 1985) suggesting that sperm response to SAPs could be impacted through potential OA-induced changes to sperm pHi (Fig. 1D). HCO_3^- and a NBC are involved in the modulation of pHi during the SAP response potentially leaving this regulatory role vulnerable to elevations in HCO_3^-concentration as OA progresses. There are no known studies that have investigated sperm response to SAPs or sperm navigational efficiency under OA conditions leaving a significant gap in our understanding of fertilisation in future seas.

1.3.4 The acrosome reaction

Once a sperm has arrived in the close vicinity of a conspecific egg a key exocytotic event crucial to fertilisation in a number of species must take place: the sperm acrosome reaction (AR; Vacquier and Moy, 1997). The AR has been well studied in sea urchin sperm (Beltrán *et al.*, 2007) although some mechanisms remain unclear. There are two important steps in the AR; exocytosis of the acrosomal vesicle releasing the protein bindin and the pHi-dependent polymerisation of actin to form the acrosomal process. The acrosomal process extends out from the apex of the sperm head approximately 1 µm and is covered by a new bindin coated membrane which will interact with the egg via the bindin egg membrane receptor (Barré *et al.*, 2003).

The AR is initiated through the interaction of species-specific egg jelly derived sulphated polysaccharides with sperm plasma membrane receptors (Alves *et al.*, 1997). A net influx

of calcium ions (Ca^{2+}) and Na^{+} and a net efflux of K^{+} and H^{+} occur within seconds (Su *et al.*, 2005) resulting in changes to Em, an increase in pHi and an increase in cellular Ca^{2+}. Alongside these changes a number of physiological alterations take place including the Ca^{2+}-dependent activation of adenylate cyclase (Watkins *et al.*, 1978). This leads to an elevation in cAMP and a subsequent increase in PKA activity (Garbers *et al.*, 1980). The involvement of pHi in yet another important sperm event highlights the large number of biochemical processes that could be affected if a sperm's ability to regulate pHi is negatively influenced by CO_2-induced changes to seawater conditions. SAPs may also be involved in facilitating the AR (Hirohashi and Vacquier, 2002) and aiding sperm penetration of the egg jelly layer (Suzuki and Garbers, 1984), and thus alterations to a sperm's ability to response to SAPs could impact upon these later processes alongside events bringing a sperm into the close vicinity of an egg.

1.3.5 The end to progressive motility

Reported longevities of free spawned sperm are highly variable ranging from minutes to several days (Powell *et al.*, 2001; Benzie and Dixon, 1994; Williams and Bentley, 2002). The physiological sperm traits that determine swim duration and the events involved in the cessation of swimming are poorly understood, making predictions of OA impacts on longevity problematic. Ohtake *et al.* (1996) observed that the activity of NADH-cytochrome c reductase decreased in proportion to the decline in sperm respiration rate in *Hemicentrotus pulcherrimus*. This suggests that the reduction in activity of this enzyme might be responsible for the degeneration of the sperm respiratory system in this species. It has been widely suggested that a sperm's life span is based on its consumption of energy reserves (usually phospholipids; Harumi *et al.*, 1990), which is a function of the amount of energy consumed for motility. However, Suquet *et al.* (2010) found that sperm from the oyster *Crassostrea gigas* contained 94 % of their initial ATP stores following a 24 hour seawater incubation suggesting that the cessation of sperm motility was unlikely to result from an exhaustion of ATP in this species. The termination of spermatozoal swimming may have resulted from the changes in sperm morphology observed by the authors following the motile phase such as damage to chromatin and

the plasma membrane. Alternatively, sperm may have stopped swimming due to a lack of ATP shuttle molecules (e.g. CR in sea urchins).

A small number of studies have investigated the impacts of seawater temperature elevation on freely spawned sperm longevity, with the majority identifying significant reductions in the duration of fertilising ability at elevated temperature (Binet and Doyle, 2013; Rahman *et al.*, 2009; Johnson and Yund, 2004). Rahman *et al.* (2009) identified species-specific sperm sensitivity to warming. Given the variation in sperm motility responses to near-future OA amongst free spawning species, it is perhaps surprising that there are no known studies investigating sperm longevity in elevated seawater pCO_2 treatments. Future work should address this knowledge gap and investigate potential interactions with the projected increases in sea surface temperature.

1.4 ECOLOGICAL IMPACTS OF SPERM DISRUPTION

Fertilisation success in free spawning marine species is a complex process influenced by a range of parameters. Sperm concentration (Powell *et al.*, 2001), sperm age (Benzie and Dixon, 1994; Manríquez *et al.*, 2001), compatibility with eggs (Evans and Marshall, 2005) and swimming velocity (Levitan, 2000) are all thought to be important male gamete parameters that influence fertilisation success. A reduction in the number of sperm initiating motility under OA is likely to reduce effective sperm concentrations surrounding a given ova, and this could be exacerbated by a reduced male reproductive investment at elevated seawater pCO_2. Sperm concentration is arguably the most important determinant of fertilisation success in marine free spawners (Claereboudt, 1999). Selective pressure for behavioural strategies that enhance gamete concentrations during spawning events may be strengthened under OA; for example the aggregation of mobile individuals prior to spawning, the synchronisation of spawning (Levitan, 1998) and the selection of favourable environmental conditions for spawning events such as periods of reduced water movement. Potential reductions in effective sperm concentrations are likely to be exacerbated in species where sperm swimming speeds are also reduced under OA. This will likely decrease the number of gamete collisions with negative implications for population fertilisation success (Styan, 1998; Vogel *et al.*, 1982).

It is important to consider sperm disruption within an ecological framework as the impacts are likely to be highly context and habitat-specific. Reductions in effective sperm concentrations under OA conditions may have no noticeable effect on the fertilisation success of populations characterised by high sperm concentrations. There may even be positive effects through a reduction in the occurrence of lethal polyspermy, where more than one spermatozoa fuses with the egg membrane (Hunter, 1998). However, most populations of free spawners are thought to be characterised by sperm limitation (Farley and Levitan, 2001) i.e. incomplete fertilisation success due to limited sperm numbers. Here, reductions in effective sperm concentrations resulting from OA are likely to exacerbate the problems of sperm limitation and act to further reduce population fertilisation success. Reductions in sperm performance may have more acute implications for organisms at their range edge and could decrease an organism's tolerance to other environmental drivers (Widdicombe and Spicer, 2008). OA-induced sperm motility disruption might strongly influence the fertilisation success of populations inhabiting turbulent environments; where sperm swimming may play an important role bridging the gap between gametes before they slip past one another (Mead and Denny, 1995). Reductions in freely spawned sperm longevity, if present under OA, may have more acute implications for populations characterised by diffuse eggs where sperm may need to be capable of swimming for a longer duration in search of the dispersed eggs (Levitan, 2000).

Potential carry-over effects from the male gamete stage to subsequent life history stages have so far received relatively little attention. Recent work by Ritchie and Marshall (2013) demonstrated a link between sperm and offspring phenotypes in the polychaete *Galeolaria geminoa* providing evidence that the environment sperm experience can have far reaching consequences and affect offspring developmental success. Marshall *et al.* (2002) found that the concentration of sperm at fertilisation partly determined offspring size (and therefore quality) in the free spawning ascidian *Pyura stolonifera.* Their work suggests that any OA-induced perturbation of sperm concentration could have far reaching consequences for later life history stages.

1.5 CONCLUSIONS

OA is progressing at an unprecedented pace, increasing seawater pCO_2, reducing sea surface pH and lowering carbonate saturation states around the globe. This review has highlighted numerous roles for pH, potential regulatory roles for HCO_3^- and an important role for seawater pCO_2 in various key aspects of sperm behaviour integral to their ability to function. Elucidating the relationship between external seawater pH and sperm pHi within the projected range of pH reductions for near-future and longer-term OA will be crucial for attempts to gain a mechanistic understanding of physiological sperm responses. This will also be essential in disentangling inter-male and inter-species variation in OA-sperm response, which may underpin differential fertilisation success in high CO_2 oceans. Future experimental work should adopt a holistic view of free spawned sperm functioning encompassing numerous sperm life history stages and include potential indirect and direct routes of OA-induced sperm disruption. By examining multiple sperm stages, carry-over effects with potentially far reaching consequences may be identified and an overall picture of male fertility and fertilisation ecology in future acidified oceans can begin to be pieced together.

1.6 HYPOTHESIS DEVELOPMENT

This review has highlighted numerous potential roles for pH, CO_2 and HCO_3^- in sperm functioning and hence, aspects of sperm life history that may theoretically be affected by the projected changes in seawater chemistry driven by oceanic uptake of atmospheric CO_2. To analyse what has been empirically demonstrated to date, I conducted a systematic search of the peer reviewed scientific literature, which forms Chapter 2 of this thesis. This systematic literature search aimed to synthesise the potential for OA and other anthropogenic drivers of marine environmental change to influence any aspect of freely spawned sperm morphology, physiology and performance. Unfortunately, the search revealed that there was not enough data for each individual driver or combination of drivers for species at similar treatment levels to conduct a meta-analysis and compare sensitivities between free spawning taxa, hence a systematic mapping approach was adopted. Systematic maps identify and collect literature relevant to a particular research question using robust, transparent and repeatable methodology. I conducted my search using a sperm cellular life history approach, which enabled the inclusion of effects on male reproductive investment and impacts on sperm during development and subsequent storage in the adult male, alongside impacts on sperm during the period between spawning and fertilisation. The systematic map highlighted key knowledge gaps, which in conjunction with the information reviewed here, resulted in the development of several experimental hypotheses, which were then tested in this PhD thesis.

One of the key knowledge gaps highlighted by the systematic literature search was a paucity of data on the potential for multiple anthropogenic drivers to interact and influence freely spawned sperm, with only a small number of species and potential driver combinations investigated to date. I therefore selected two potential stressors that are highly likely to co-occur for coastal marine invertebrates; OA and copper contamination. I designed a set of experiments to investigate their potential to interactively impact early life stages. The behaviour, speciation and therefore bioavailability of many heavy metals in seawater is strongly dependent on seawater chemistry, with a number of metals known to be sensitive to speciation changes within the pH range projected for near-

future OA (Millero *et al.*, 2009). Copper is a common coastal contaminant and OA is predicted to increase the toxic free ion concentration of copper (Cu^{2+}) in coastal waters by as much as 115 % in the next 100 years (Richards *et al.*, 2011), which may enhance toxicity responses in marine species. I decided to approach this research question in the polychaete worm *Arenicola marina* using the rationale that as a group polychaetes have been poorly studied and *A. marina* inhabits soft-sediment coastal zones where copper contamination may be prevalent. I tested the following hypothesis:

Hypothesis 1: Copper toxicity (measured as sperm DNA damage, fewer motile sperm, slower swimming sperm, reduced fertilisation success and decreased larval survival) will be enhanced at reduced seawater pH for early life stages of *Arenicola marina*.

Another key knowledge gap highlighted by the systematic literature search was our understanding of the biochemical and physiological mechanisms through which OA conditions perturb freely spawned sperm motility. Numerous roles for pH in sperm motility were highlighted in this introductory review, yet the systematic literature search revealed that there has been very little research attention dedicated towards a better understanding of the mechanisms underlying OA-induced sperm motility perturbation. This knowledge could be key to an understanding of sensitivity and tolerance, and could aid the identification of vulnerable populations or species beyond the small number tested to date.

In another set of experiments, I developed a novel exposure technique to control the pH and oxygen content of sperm incubations, without mechanical disruption from direct air bubbling. This technique enabled me to undertake a mechanistic investigation of sperm swimming performance over an extended exposure to OA conditions. I monitored sperm ATP content, oxygen consumption and viability in addition to sperm swimming behaviour in an attempt to uncover some of the physiological mechanisms underpinning changes to swimming performance resulting from OA. I explored these questions in the coastal polychaete *A. marina. A. marina* has an unusual reproductive strategy where interaction between sperm and eggs may take place several hours after spawning and dilution in seawater (Williams and Bentley, 2002). Under future ocean conditions this may result in a prolonged sperm exposure to OA in this species, hence providing a really interesting

model to explore OA impacts on sperm biochemistry over an extended exposure and investigate effects on longevity. The following hypotheses were tested:

Hypothesis 2: Sperm motility perturbation will develop or intensify over an exposure to OA conditions in *Arenicola marina*.

Hypothesis 3: Changes to sperm ATP content, oxygen consumption and/or viability under OA conditions significantly influence sperm swimming behaviour in *Arenicola marina*.

Finally, I decided to explore the reproductive consequences of OA-induced changes to sperm motility in a competitive context. Most fertilisation in the sea takes place under sperm competition; where multiple male ejaculates compete to fertilise a given set of ova (Parker, 1970), and it is rare for a male to gain sole access to a batch of eggs. A key determinant of male reproductive success under future ocean conditions is not the average success of a group of males, but the proportion of offspring contributed by each individual male under the changed environmental conditions (Schlegel *et al.*, 2012). I investigated the relationships between ejaculate traits and success in paired competitive fertilisation trials under current ocean conditions and then established whether these relationships held under OA conditions. I addressed these questions in the sea urchin *Paracentrotus lividus* taking advantage of the microsatellite loci characterised for this species, which were used to assign paternity to one of two male competitors. *P. lividus* spawns synchronously and can be found inhabiting rock pools and shallow sub-tidal habitats where sperm competition may be intense. I tested the following hypotheses:

Hypothesis 4: The number and speed of motile sperm in a male's ejaculate significantly influences success in paired competitive fertilisation trials in current ocean conditions in *Paracentrotus lividus*.

Hypothesis 5: The relationships between male ejaculate traits and competitive fertilisation success will not change under simulated future OA conditions in *Paracentrotus lividus*. Whilst the number and speed of motile sperm in a male's ejaculate may change in the new conditions, the relationships between gamete traits and competitive fertilisation success will hold.

Overall, through this body of work I have addressed the potential for enhanced copper toxicity at pH reductions associated with OA for sperm DNA integrity and swimming performance, a physiological investigation of OA and sperm swimming behaviour in an extended exposure to OA conditions and finally the influence of OA-induced changes to sperm motility for competitive fertilisation success.

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Research paper I 2

Anthropogenic impacts on freely spawned sperm in marine invertebrates: a systematic map

In preparation for submission to: Frontiers in Ecology and the Environment

Anthropogenic impacts on freely spawned sperm in marine invertebrates: a systematic map

2.1 ABSTRACT

Marine ecosystems worldwide are increasingly subjected to a wide array of contaminants and debris resulting from human activities. Against this backdrop of existing and emerging marine pollution pressures, climate change is progressing at an alarming rate. Large-scale changes in oceanic physicochemical conditions are currently being recorded and projected to accelerate over the coming century. Despite much recent work there remains a pressing need to establish the reproductive consequences of human activities for marine species. Sperm play a central and critical role in reproduction, key to fitness in sexually reproducing species. Hence, understanding the impacts of environmental stressors for sperm function is of paramount importance to establishing population level effects. Sperm freely spawned into seawater may be particularly vulnerable, as they must function from directly within the altered conditions. We undertook a systematic search of the literature aiming to record and synthesise the results of studies that have investigated the impacts of anthropogenic stressors on freely spawned sperm. We adopted a sperm cellular life-time approach encompassing male reproductive investment and sperm phenotype. We found a narrow coverage of species within broad taxonomic groups with cnidarians and ascidians very poorly studied. Only a handful of studies have investigated interactions between anthropogenic drivers, despite multiple co-occurring stressors in natural systems. No study exposed adults and sperm to the same driver(s) leaving significant knowledge gaps on the potential accumulation of effects across successive life-stages or trans-generational plasticity. Hypoxia may be emerging as an anthropogenic stressor of environmental concern, as the handful of studies that investigated sperm responses to hypoxic conditions have revealed negative effects across the board. Percentage sperm motility appears to be more sensitive to simulated OA conditions than sperm swimming speed, but the paucity of sperm physiological investigations prevents a mechanistic understanding of species-specific responses. In

conclusion, this systematic map has revealed an overall paucity of knowledge on the potential for anthropogenic activities to impact upon this important cell type crucial to marine invertebrate life histories. Such information could be critical to an understanding of population success in the face of a rapidly changing climate.

2.2 INTRODUCTION

The world's estuaries and oceans are the ultimate recipients of a startling array of anthropogenic contaminants and debris. For centuries the oceans were regarded as having an infinite capacity to assimilate wastes, with coastal waters used as disposal mechanisms for the wastes and by-products of industrial, agricultural and domestic activities (Coe and Rogers, 2012). However, the limits of ocean systems are becoming evident. Marine pollution is a global and growing issue, with marine ecosystems worldwide increasingly subjected to a multitude of contaminants with the potential to negatively affect the health of aquatic life (Matthiessen and Law, 2002). The immediate and often most acute impacts occur in estuarine and coastal zones close to human settlements, activities and industries (Bryan and Langston, 1992). But the open ocean is not immune to pollution, where shipping operations beyond the continental shelf alongside the atmospheric transport and deposition of toxic chemicals can adversely affect open waters.

Heavy metals and persistent organic pollutants (POPs) are ubiquitous contaminants of a wide range of aquatic habitats across the globe. They are toxic, persist in the environment for long periods of time and may enter the food-chain and accumulate in biological tissue. Aquatic sediments are a major sink for a wide variety of organic and inorganic pollutants (Zoumis *et al.*, 2001). They act as repositories for hydrophobic, recalcitrant and hazardous compounds that may become available to benthic organisms and aquatic organisms in the water column through the sediment-water interface (Perelo, 2010). Contaminants may be mobilised from their sediment stores through changes in the geochemical conditions, bioturbation or resuspension, diffusion along a concentration gradient and through potential degradation to more mobile forms (Booij *et al.*, 1992). Environmental conditions such as the seawater pH/pCO_2 conditions can also alter the behaviour of contaminants bound to sediments, for example influencing metal fluxes from contaminated sediments (Roberts *et al.*, 2013). In addition to these existing historical pollution pressures, there are several emerging groups of pollutants of environmental concern. Pharmaceuticals have been present in marine ecosystems for

decades, but the technology allowing their quantification in the environment has only recently become available (e.g. Kolpin *et al.*, 2002). Biologically active compounds; they are designed to interact with a receptor in a target species (human or animal). Following their use, human pharmaceuticals and any metabolites are excreted into the sewerage system (Daughton and Ternes, 1999) and depending on their persistence, water solubility and polarity they may then be released into surface waters persisting for several months or years (Ternes *et al.*, 2004). Concern has been mounting about the consequences of pharmaceutical uptake into non-target aquatic species and their bioaccumulation in the environment (Hird *et al.*, 2016).

The commercial use and application of engineered nanomaterials (ENMs) is rapidly expanding fuelled by the unique properties these materials display at this size (Cross *et al.*, 2015). ENMs are defined as having one dimension of <100 nm (Klaine *et al.*, 2008) and they have wide-ranging applications spanning medicine, environmental remediation and commercial produce (Cross *et al.*, 2015). ENMs are at risk of release into the aquatic environment through their use and methods of disposal, where their high reactivity could represent an environmental risk. The final emerging group of pollutants are plastics: persistent synthetic polymers that accumulate as waste in marine ecosystems. They tend to break down into smaller fragments known as microplastics (<1 mm diameter) or may enter the environment at a microscopic size from a variety of sources such as personal care products, clothing and industrial processes (Cole *et al.*, 2011). Their ubiquitous distribution and small size indicates that microplastics may be readily ingested by aquatic filter feeders, with concern growing about the consequences of intake (Gall and Thompson, 2015). Plastic polymers are rarely used by themselves, and are typically mixed with substantial quantities of chemical additives to improve performance (Meeker *et al.*, 2009). Polyvinylchloride (PVC) for example may contain bisphenol-A (BPA), phthalates, flame retardants, organotins including tributyltin (TBT) and the heavy metals cadmium and lead (Thompson *et al.*, 2009). As plastics breakdown toxic additives are known to leach into both fresh and marine waters (Flint *et al.*, 2012) increasing their levels in the environment.

Against this backdrop of existing and emerging marine pollution pressures, seawater conditions are currently changing at an unprecedented rate (Hönisch *et al.*, 2012) as a

consequence of climate change (Stocker *et al.*, 2013). Since the industrial revolution over 350 billion tons of carbon stored in fossil fuels has been oxidised and released into the atmosphere through human activities (Boden *et al.*, 2010) and this is responsible for driving large-scale changes in the Earth's climate system. Long-lived, heat-trapping greenhouse gases (dominated by carbon dioxide: CO_2) are warming the Earth's surface. A large proportion of this excess energy in the climate system (~90 %) has been stored in the oceans (1971-2010; Stocker *et al.*, 2013), and this is elevating global sea surface temperatures (SSTs). SSTs have warmed by on average +0.7 °C over the last 100 years (Orr *et al.*, 2005), with further warming projected over the coming century unless emissions are dramatically cut (Stocker *et al.*, 2013). Predictions vary across models and regions, but average global SST is expected to rise by a further 1-3 °C by the year 2100 (Stocker *et al.*, 2013; Bopp *et al.*, 2013). The oceans play a key role in reducing atmospheric CO_2 concentrations: moderating the impacts of human activities. Since industrialisation approximately one third of anthropogenic CO_2 emissions have been absorbed by the oceans (Doney *et al.*, 2014), but this has serious implications for seawater chemistry. Termed ocean acidification (OA), oceanic uptake of rising atmospheric CO_2 levels drives reduced seawater pH and carbonate mineral saturation states. The pH of surface seawater has already decreased by 0.1 pH unit since industrialisation, with further reductions of 0.22-0.35 pH units projected by the end of this century (Bopp *et al.*, 2013). The rate at which these changes are taking place is faster than at any other time for the past 300 million years, raising the possibility that we are entering unknown territory of marine ecosystem change (Hönisch *et al.*, 2012).

Over the past decade there have been dramatic changes in the dissolved oxygen (DO) content of coastal waters, and an increasing occurrence of hypoxic events. The DO content of coastal waters normally varies between 5.0 and 8.0 mg L^{-1} and hypoxic events take place when this drops below 2.8 mg L^{-1} (less than 30 % oxygen saturated; Diaz and Rosenberg, 1995). These events mainly occur as a consequence of increased amounts of bioavailable nitrogen reaching coastal areas from human activities. Coastal hypoxia is likely to be exacerbated by climate change through the reduced solubility of oxygen in warmer waters and reduced ventilation as a result of changes to patterns of circulation and stratification, which will also reduce the DO content of open oceans (Keeling *et al.*, 2010). A warmer atmosphere is also expected to affect the hydrological cycle, driving

increased rates of evaporation and precipitation that are expected to freshen regions of low-salinity and increase the salinity of saltier regions (Doney *et al.*, 2012). Additionally, an increased frequency of extreme rainfall events is predicted under climate change, which is expected to enhance fresh-water runoff into coastal waters (Stocker *et al.*, 2013) leading to shock low-salinity events.

Although climate change variables and marine pollutants are often discussed in relation to a single environmental driver (e.g. temperature or copper), in reality marine species may be exposed to an array of simultaneously co-occurring stressors with the potential to interact (Przeslawski *et al.*, 2008). Multiple stressors may interact additively to affect physiological or ecological processes, whereby the combined effect represents the additive accumulation of effects resulting from the single stressors. Or in combination the effects of multiple stressors may be greater than the sum of effects of the individual stressors i.e. a synergistic interaction. Alternatively, effects may be antagonistic, whereby in combination exposure to one stressor dampens the effects of another. Given the rapid rate of change currently being recorded and projected to accelerate in the world's oceans, set against a backdrop of existing and emerging marine pollution pressures, there is a pressing need to establish the reproductive consequences of this environmental melee of potential interacting stressors for free spawning marine invertebrate species.

Most marine species freely spawn their sperm into the seawater column, where fertilisation can then either be external or the female can draw sperm into a brooding chamber. Species with this reproductive strategy may be vulnerable to environmental stressors such as climate change and marine pollutants as their reproduction relies upon the successful functioning of sperm from directly within the seawater conditions (Lewis and Ford, 2012). The gametic phase is often the most sensitive stage in an organism's life (Marshall, 2006), as gametes face all of the challenges of environmental stressors, but at a small size and with only limited protective mechanisms. Sperm may be particularly susceptible as they lack any actively transcribing nuclear genes or biochemistry, limiting their ability to respond to environmental conditions through regulation. Sperm are perceived as having very limited DNA repair capabilities and lacking antioxidant defences or cellular repair mechanisms (Aitken *et al.*, 2004). They also have an abundance of

polyunsaturated fatty acids that act as substrates for reactive oxygen species (ROS) making them susceptible to oxidative damage (Ivanina *et al.*, 2013). In the face of potential environmental challenge, sperm have a critical primary function to carry out that is central to fitness in sexually reproducing species: the transport and transfer of the male genetic contribution. The environment sperm experience can have far-reaching consequences influencing fertilisation and offspring developmental success (Lewis and Galloway, 2009; Ritchie and Marshall, 2013). Marine invertebrates generally lack the blood-gonad barrier found in mammalian species, hence their sperm may experience an extended exposure to any contaminants present in seawater during gametogenesis, subsequent storage and upon release into seawater (Lewis and Watson, 2012).

Reproductive studies investigating the impacts of anthropogenic stressors in free spawning species have tended to focus on fertilisation and early embryonic development rather than investigating impacts directly on sperm (e.g. Geffard *et al.*, 2001; Marshall, 2006; Byrne *et al.*, 2010; Lewis *et al.*, 2008). Sperm concentration is arguably the single greatest influence on external fertilisation success (Levitan, 1991; Levitan *et al.*, 1991). When multiple sperm concentrations have been tested, numerous studies report the influence of a contaminant or environmental variable on fertilisation to be sperm-concentration dependent i.e. the presence or magnitude of effects depended on the sperm: egg ratio (Gonzalez-Bernat *et al.*, 2013; Ericson *et al.*, 2010; Hollows *et al.*, 2007). Fertilisation assays often select a single or small number of sperm concentrations, making it challenging to interpret the results with the knowledge that effects may vary across concentrations that were not tested (Hollows *et al.*, 2007). There is also a paucity of field data on sperm concentrations during natural spawning events. Whilst, these are likely to vary across spatial and temporal scales and be highly population-specific, a better understanding of fertilisation environments in nature is essential in order to appropriately design and interpret the results of ecologically relevant fertilisation assays. If reductions in fertilisation success are observed in the presence of a contaminant at a small range of sperm concentrations, knowledge of the frequency at which these concentrations are likely to be found for the population in question is highly desirable to conclusions.

Consequently, we decided to focus on freely spawned sperm here, and analyse what is currently known on the potential for anthropogenic stressors to impact this important cell type. We conducted a systematic search of the biological literature for studies that have investigated the impacts of anthropogenic drivers of marine environmental change on a sperm endpoint(s) in free spawning marine invertebrate species. We adopted a sperm cellular lifetime approach, which enabled the inclusion of experimental endpoints of male reproductive development and paternal provisioning alongside any sperm-related endpoints (see Figure 1). Preliminary literature searches revealed there was insufficient data to conduct a full meta-analysis comparing sensitivities across free spawning taxa and stressors. Hence, a systematic map protocol was adopted, as this is a fully transparent, robust and repeatable method to identify and synthesise relevant literature and identify future research needs (Snilstveit *et al.*, 2013). The aim was for rigour, objectivity and transparency in assembling existing knowledge on the potential for anthropogenic impacts on freely spawned sperm across two broad categories: marine climate change variables and pollutants of marine environments. By synthesising the current scientific knowledge, we aimed to identify drivers of emerging concern and highlight future research priorities.

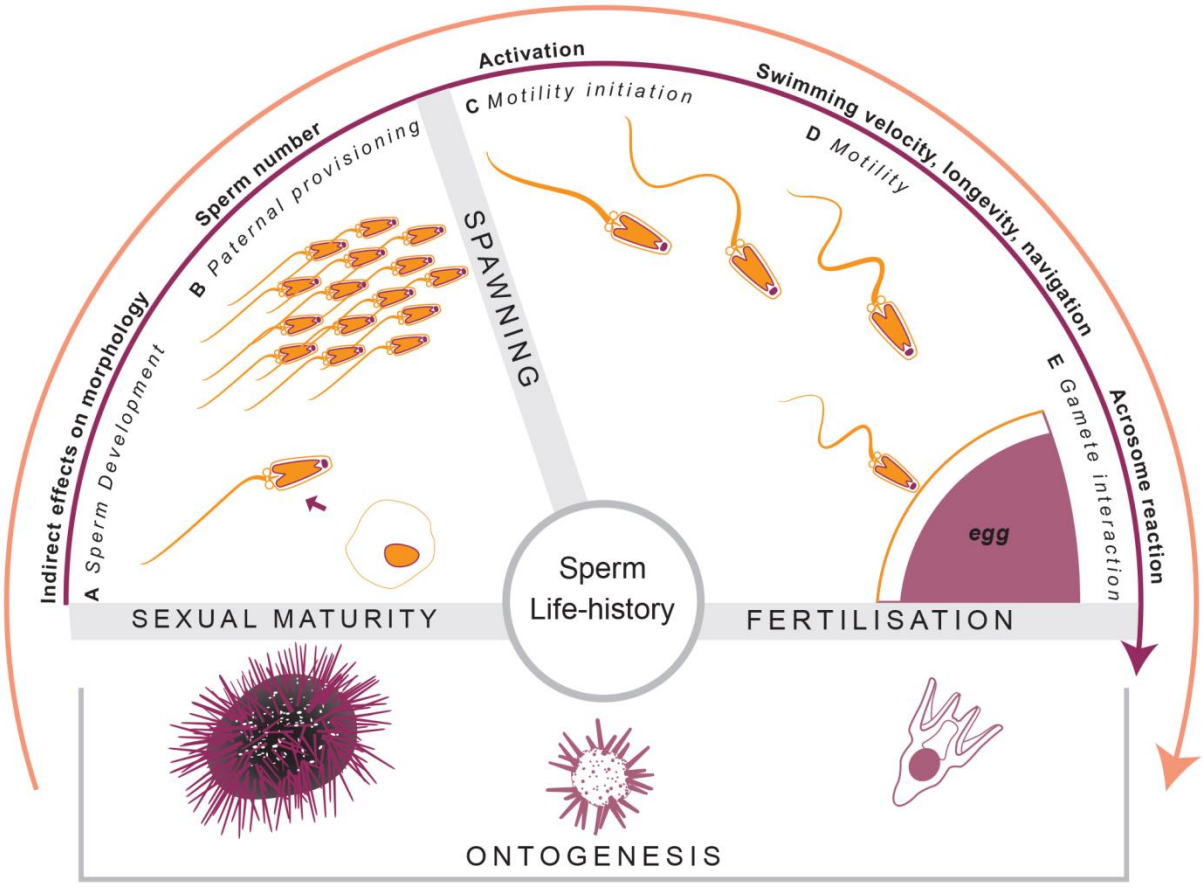

Figure 1. A conceptual figure illustrating sperm life-history for a typical free spawning species, the purple sea urchin *Paracentrotus lividus*. Examples of indirect and direct effects of anthropogenic drivers on sperm are provided.

2.3 METHODS

The literature search was conducted using the ISI Web of Science database to source published peer reviewed research articles. The final search terms and Boolean operators can be found in Table 1. Studies were assessed against our inclusion criteria firstly based upon the title, followed by the abstract and finally the full text article. Every effort was made to access a study's full text, which had to be available to access in English.

2.3.1 Primary research question

Taking a longitudinal cellular lifetime approach, what is the current extent of scientific knowledge on the potential for human activities to impact marine invertebrate sperm function in species that reproduce by freely spawning sperm into the seawater column?

2.3.2 Secondary research question(s)

Have studies considered the potential for interactions between anthropogenic drivers of marine environmental change to impact freely spawned marine invertebrate sperm and/or investigated impacts across multiple life history stages?

2.3.3 Time period used for literature search

No time restrictions were included in the literature search hence all publication dates were valid.

Table 1. Search criteria used in the systematic literature search.

Search terms		**Web of science hits**
Subject	"external fertili*" OR "marine invertebrate*" OR broadcast OR cnidari* OR mollusc* OR bivalv* OR echinoderm* OR polychaet* OR oyster* OR mussel* OR coral* OR urchin* OR starf* OR cucumber*	229, 052
	AND	
Intervention	"climate change" OR "anthropogenic stress*" OR acidif* OR pH OR pCO2 OR salinit* OR hyposalinit* OR temperature* OR warm* OR pollut* OR toxic* OR metal* OR chemical* OR nano*OR nano-* OR plastic* OR microplastic* OR copper OR zinc OR cadmium OR lead OR pharmaceutical* OR hypox* OR oxygen OR hypercapn* OR oil* OR "oil spill*"OR "global change" OR future OR "future condition*" OR pesticide* OR estrogenic OR oestrogenic OR "endocrine disrupt*" OR genotox* OR steroid*	70,092
	AND	
Outcome	"paternal provision*" OR "reproductive investment*" OR "male investment*" OR "investment* in reproduction" OR "gonad index" OR "gonad condition" OR "gonad weight" OR "gonad wet weight" OR "gonad dry weight" OR "spermatogenesis" OR "sperm development*" OR "sperm maturation" OR "spawn* volume" OR "spawn* qualit*" OR "gonad size" OR "gonad* development" OR "gonad growth" OR "reproductive cycle" OR "reproductive outcome*" OR "reproductive toxic effect*" OR "paternal effect*" OR "sperm provision*" OR "sperm number*" OR "number* of sperm" OR "sperm velocit*" OR "sperm swimming" OR "sperm motilit*" OR "sperm speed*" OR "sperm longevit*" OR "sperm chemotaxi*" OR "sperm MMP" OR "sperm metabolism" or "sperm respiration" OR "sperm ATP" OR "sperm oxygen" OR "sperm qualit*" OR "sperm behavio*" OR "sperm performance" OR "sperm concentration" OR "motile sperm" OR "sperm navigation*" OR "acrosome" OR "sperm perturbation" OR "gamete trait*" OR "gamete longevit*"OR "flagellar motility" OR "sperm activity" OR "sperm function*"OR "gamete function*" OR "sperm cell function*" OR spermiotox* OR "sperm ultrastruct*")	1,562

* indicates wildcard search operator

2.3.4 Study inclusion criteria

All studies highlighted using the search terms (Table 1) were then assessed against the following set of criteria. Studies that passed these criteria at the full text level qualified for the synthesis.

Relevant subject/population: We included marine invertebrate species that reproduce by freely spawning sperm into the seawater column. This included sequential or simultaneous hermaphrodites, but in the case of the latter, there should be no evidence in the scientific literature that self-fertilisation is favoured over sexual reproduction to allow inclusion.

Relevant intervention: We searched for experimental manipulations of marine-relevant climate change variable(s) or pollutants of marine habitats in laboratory, mesocosm or field settings. Exposures could either be chronic or acute. We included studies which exposed adult animals (sex may either be unspecified or male) or sperm rather than studies which exposed isolated somatic cells of tissues. To satisfy our criteria, treatment level(s) of an environmental variable must be relevant to climate change, for example warming above current ambient levels to align with projected SST increases rather than a cooler treatment. We accepted studies that report the outcome of statistical analysis of at least one qualifying experimental endpoint. Studies that cryopreserved sperm prior to experimental manipulation were excluded. Experimental manipulations of OA needed to manipulate seawater pCO_2/pH levels via CO_2 addition rather than through acid addition or the use of buffered solutions to satisfy our inclusion criteria.

Relevant comparator: We compared sperm exposed to treatment conditions with sperm from either the same male or from other males from the same study population exposed to control conditions. Alternatively, adult animals exposed to treatment conditions were compared to adults from the same study population exposed to control conditions. We defined control conditions as a treatment level selected to represent present day conditions, with or without present day environmental variability, for climate change variables. Whilst for pollutants, we defined control conditions as either (1) the absence of a pollutant(s), (2) conditions selected to mimic a relatively clean i.e. non-polluted natural site or (3) the least polluted site along a suspected pollution gradient. We defined

experimental conditions as a treatment level(s) selected to mimic projected future climate change scenarios or extreme conditions for climate change drivers. These may or may not include present day environmental variability or a forecasted future signal. In contrast, for studies of marine pollutants, experimental conditions can be defined as either (1) the addition of a pollutant(s) at any concentration, (2) conditions selected to mimic a polluted natural site or (3) a more polluted site(s) along an expected pollution gradient.

Relevant outcomes: Experimental endpoints that satisfied our inclusion criteria were quantitative or semi-quantitative measures of sperm phenotype, ejaculate composition or adult reproductive investment.

2.3.5 Assessment of included studies

Studies that satisfied our inclusion criteria at the full text level were included in the synthesis and key details of their methodology and results were extracted by the reviewers and recorded. The information extracted included; the exposure and details, subject and details and the outcome measure and result. Reviewer consistency was checked for the two independent reviewers via the kappa statistic, which measures the level of agreement between reviewers. A high score in this quantitative measure of interrater reliability confirms the consistency of data collection (McHugh, 2012). The kappa score was found to be 0.64 (95 % CIs: 0.36, 0.92) indicating substantial agreement between the independent reviewers (Landis and Koch, 1977).

2.3.6 Grouping of studies by stressor

The climate change variables we investigated were warming (SST increase), OA, hypoxia and salinity change. We decided to split the marine pollutants into broad categories with similar modes of action. The broad categories of pollutants we included were the heavy metals, pesticides (including herbicides), endocrine disrupting chemicals (EDCs) which

were grouped with pharmaceuticals, organic compounds, simulated oil spills, other pollutants; a group of compounds whose modes of action did not fit with any of the other groups and finally field pollution studies. We also included a category which we called interactions, where studies investigated two or more anthropogenic drivers within any category.

We grouped the EDCs with the pharmaceuticals as the systematic search revealed that the vast majority of pharmaceuticals studied in relation to the research question were synthetic or natural hormones, which aligned well with EDCs which target the endocrine (hormone) system. The 'other' category of pollutants was made up of dibutyltin (DBT), microplastics and 2,3,7,8-TCDD. DBT is related to TBT, but unlike TBT is not included on lists of known EDC compounds. In the absence of chemical additives, microplastics are thought to be chemically inert, with the main route of perturbation presumed to be through adverse effects of ingestion on energy homeostasis. Finally, 2,3,7,8-TCDD is a dioxin-like compound and an unintentional by-product of incomplete combustion, which did not align well with any other group.

2.4 RESULTS AND DISCUSSION

The systematic progression of the literature search is summarised in Figure 2. A total of 1562 papers were identified in this systematic literature search. Seventy nine studies qualified against our inclusion criteria from scientific papers spanning 27 years (1990 - 2016). The earliest qualifying study investigated the reproductive consequences of diets contaminated with polychlorinated biphenyls (PCBs): a confirmed EDC, for the starfish *Asterias rubens* (Den Besten *et al*., 1990).

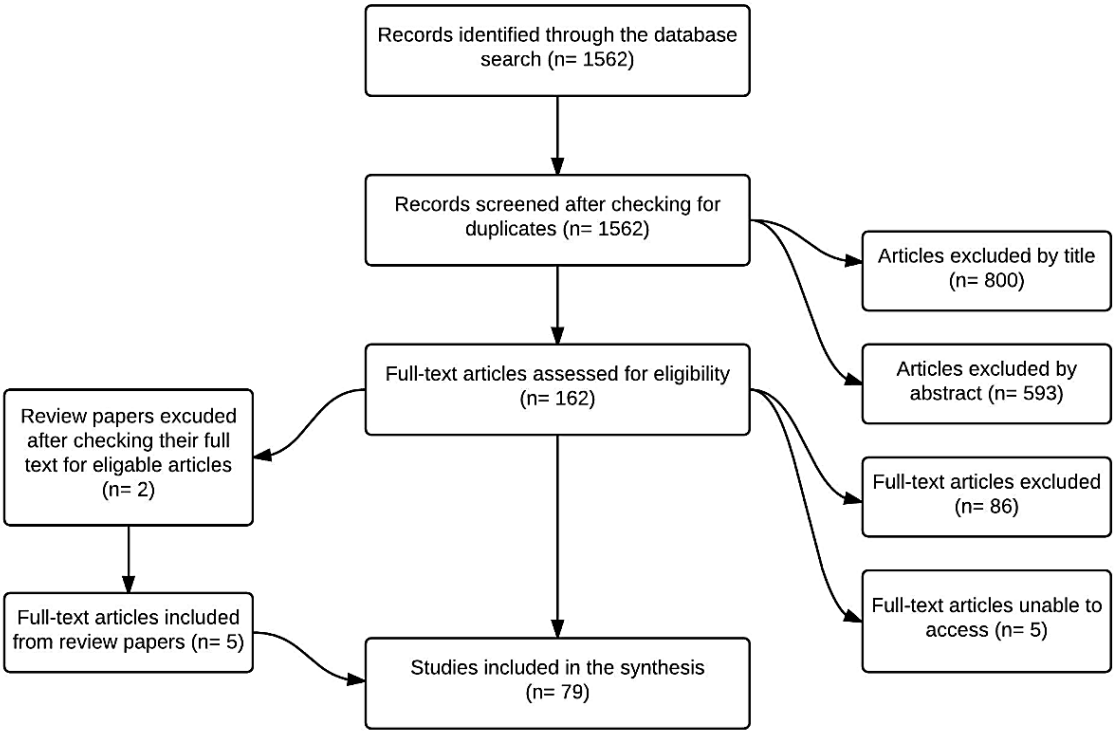

Figure 2. Flow diagram of literature search. Review papers were screened for references to qualifying studies and then excluded from the synthesis.

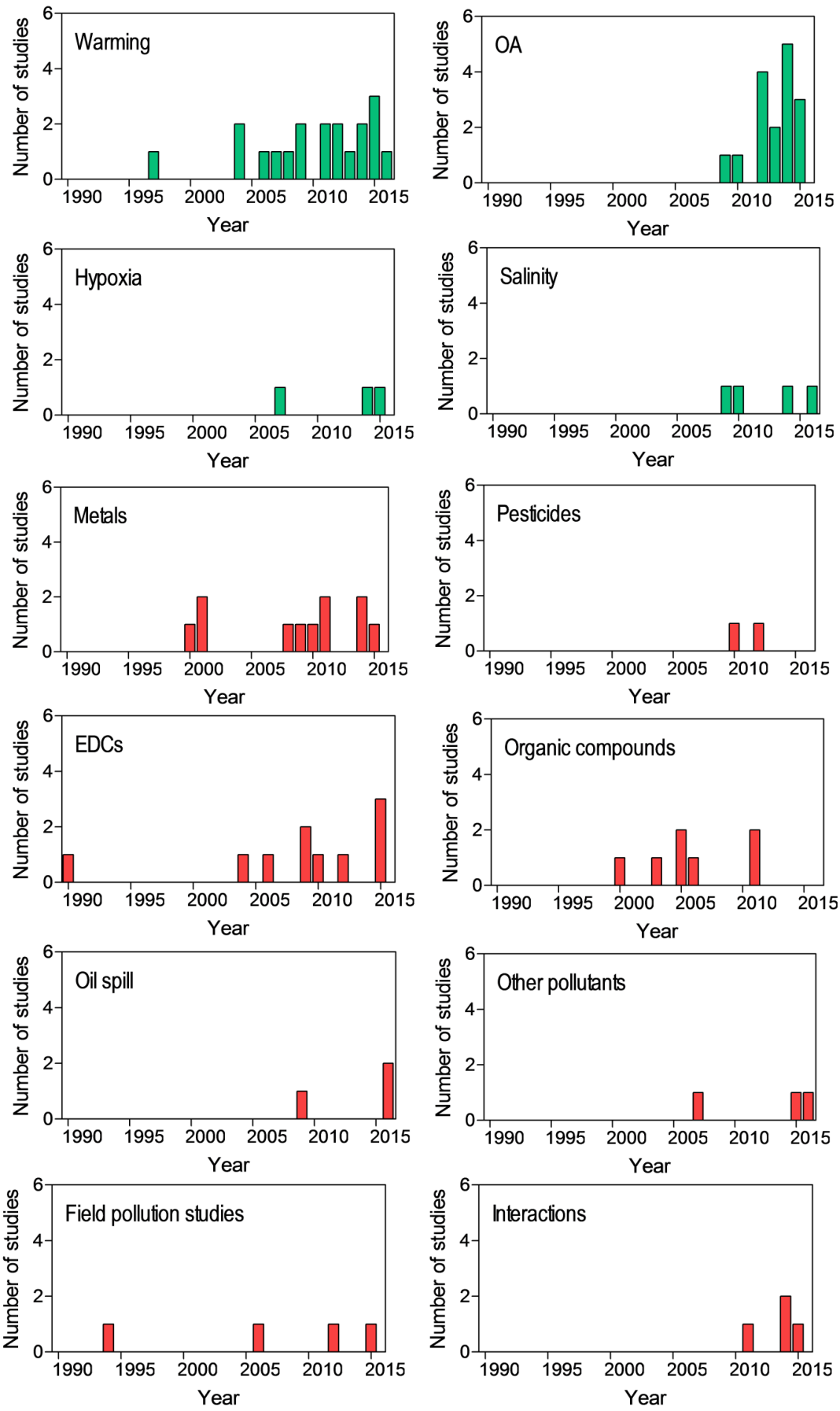
Warming
OA
Hypoxia
Salinity
Metals
Pesticides
EDCs
Organic compounds
Oil spill
Other pollutants
Field pollution studies
Interactions
Number of studies
Year
0
2
4
6
1990
1995
2000
2005
2010
2015

Figure 3. The number of studies published by year identified through the systematic literature search for each anthropogenic driver. Marine pollution studies (red bars) and investigations of climate change variables (green bars).

Recent work has been dominated by attempts to understand the impacts of oceanic uptake of CO_2 (OA and warming) on freely spawned sperm (Figure 3). Warming has been one of the most widely studied anthropogenic drivers in this area, although a lot of the early work was conducted within an aquaculture context. The other two climate change variables included in this synthesis, hypoxia and reduced salinity, are relatively new but emerging fields of research in relation to freely spawned sperm with the first study identified through this search published in 2007 and 2009 for each driver respectively. Interest in these stressors may accelerate in popularity based on the trends found in Figure 3. Scientific interest in the influence of organic compounds on freely spawned sperm has tailed off in recent years with the most recent study published in 2011, whereas metal investigations have remained popular, with three studies published in the past three years in addition to the more historical work from 2000-2001. There has been significant recent interest in EDCs (including pharmaceuticals) and freely spawned sperm (see peak at 2015 in Figure 3), and this may be related to technological advances allowing their quantification at very low concentrations in the environment (Kolpin *et al.*, 2002). It is surprising that there has not been more work on the historic anthropogenic stressors such as heavy metals and oil spills, as these have been chronic contaminants for decades with the potential to negatively influence male reproductive health.

Most of the early work on the response of gametes to toxicants have used fertilisation success and/or embryonic development as the experimental endpoint(s), often following rigorous Organisation for Economic Co-operation and Development (OECD) guidelines on chemical testing (for example Dinnel *et al.*, 1989; Ghirardini *et al.*, 2001; Arslan *et al.*, 2007). These guidelines instruct researchers to conduct spermiotoxicity and embryotoxicity tests which assess fertilisation rates and embryonic development respectively, and they remain popular methodologies employed by studies assessing perturbation of external fertilisation. The majority of spermiotoxicity tests do not meet

our inclusion criteria as effects were not explored directly for freely spawned sperm. Numerous processes determine external fertilisation success including various cellular characteristics of spermatozoa (Lu and Wu, 2005; Kazama *et al.*, 2014; Espinoza *et al.*, 2009), the number and speed of motile sperm (Vogel *et al.*, 1982), the number of viable sperm and the efficiency of the polyspermy block (Reuter *et al.*, 2011). Indirectly assessing sperm effects via fertilisation rates precludes a mechanistic understanding of the routes of perturbation and hence, the mode of toxicity for a compound. The effects of a toxicant on external fertilisation are likely to be sperm concentration dependent (Hollows *et al.*, 2007; Marshall, 2006). Hence, studies should have extensive knowledge on sperm concentrations during population spawning events in order to appropriately design and interpret the results of spermiotoxicity tests, although this is rarely the case.

There is certainly the potential for biases in study publication and outcome reporting to influence the number of studies assessing sperm endpoints identified through the systematic search. There is empirical evidence that studies with statistically significant results are more likely to be published than studies showing no effect i.e. a publication bias exists (Dickersin and Min, 1993). Studies without statistically significant results also take longer to achieve publication (Stern and Simes, 1997), adding potential further bias to the evidence available over time. Within studies, authors may choose to include a subset of the original variables recorded for publication, leading to a selective reporting bias (Hutton and Williamson, 2000). There are varied reasons why authors might reduce the number of variables for publication such as to conform to strict journal word count restrictions, a desire to tell a catchy 'story' that may generate interest from higher impact journals or presumed anomalous results which may be challenging to interpret.

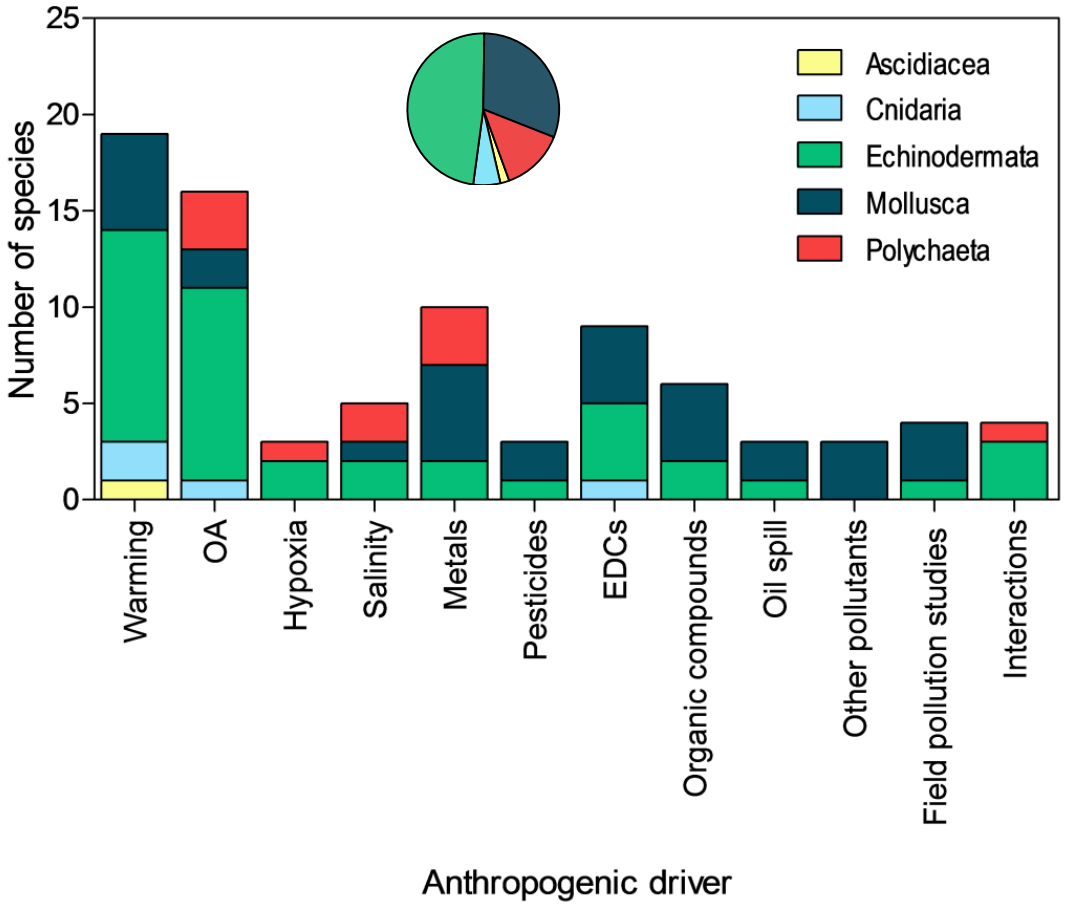

Figure 4. Free spawning marine invertebrate species, split into broad taxonomic groups, whose male reproductive and/or sperm responses to anthropogenic drivers have been studied to date (bar chart), and the percentage of total species studied in each taxonomic group across anthropogenic drivers (pie chart).

Across marine free spawning taxonomic groups, the echinoderms have been best-studied, with 25 species investigated across anthropogenic drivers (see pie chart Figure 4). They have been especially popular study species in climate change investigations of warming, OA, hypoxia or salinity change (see bar chart Figure 4). Mollusc species are the second most-studied group with 16 species investigated across anthropogenic drivers and they have dominated investigations into the impacts of marine contaminants. As a group polychaetes have not been widely studied, with a small number of species

included in investigations across only a few anthropogenic drivers. In total seven polychaete species have been studied across drivers. Polychaetes are common foundation species with ecologically important roles that modify habitats and enhance biodiversity (Smith *et al.*, 2005). The best-studied polychaete identified here, is *Galeolaria caespitose* with sperm responses examined in two independent investigations (Schlegel *et al.*, 2014; Falkenberg *et al.*, 2016). G. *caespitose* dominates the intertidal region on exposed rocky shores in temperate Australian climates (Bulleri *et al.*, 2005). Whilst a common species in Southern Australia, *G. caespitose* is not a widely distributed cosmopolitan species and their sperm responses may not be representative of polychaetes found in a variety of habitats across the globe, highlighting the need for additional research effort.

By far the least studied groups are the cnidarians and ascidians, with only three and one species respectively investigated to date (Figure 4 pie chart). Cnidaria includes the corals, with communities supported by reef building corals some of the most important ecosystems on the planet (Sorokin, 2013). Ascidians are also ecologically important components of marine ecosystems, but with numerous invasive species and notorious biofoulers, as a whole group their reputation may have been somewhat unfairly tarnished. Given the huge ecological importance of both of these groups, the lack of investigations into how sperm functioning may be impacted by marine pollutants and the accelerating rate of climate change is worrying, and represents significant gaps in the scientific knowledge. Some of this deficit may have been driven by the diverse range of reproductive modes exhibited by coral species. Sexual reproduction is important for evolutionary processes and at least 444 of more than 1500 recognised coral species have been recorded utilising aspects of sexual reproduction often through broadcast spawning (Harrison, 2011). Hence, determining broadcast spawned coral sperm responses to anthropogenic stress is important to understanding the response of external fertilisers. In fact, there are thousands of extant species of free spawning marine invertebrates and with only tens of species investigated to date across a small number of drivers and sperm endpoints, research into this area has only scratched the surface. The species whose sperm responses have been explored, are often easy to collect (often intertidal or shallow subtidal species; Przeslawski *et al.*, 2015) and maintain under laboratory

conditions. Hence, their responses are unlikely to provide a representative sample of marine invertebrates from a wide variety of habitats.

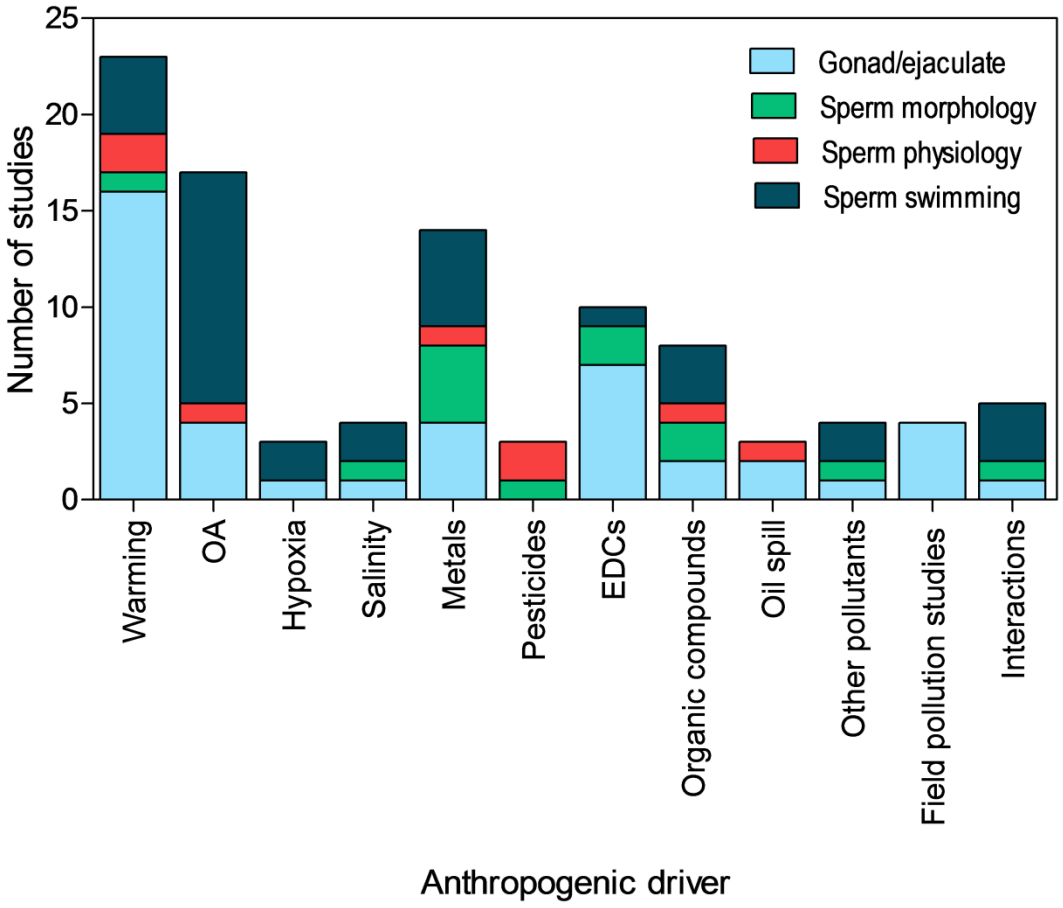

Figure 5. The number of peer-reviewed studies to investigate male reproductive and/or sperm responses to anthropogenic drivers in free spawning marine invertebrate species split by endpoint 'type'. Endpoints have been grouped into broad categories ('types'); gonad or ejaculate endpoints, sperm morphology (includes sperm DNA damage), sperm physiology (includes sperm viability) and sperm swimming performance (includes longevity). Studies that investigated more than one endpoint type are included once in each category.

Studies can also be grouped according to the sperm functional endpoints measured, illustrated in Figure 5. There has not been an even distribution of research effort across endpoint types for each driver. These decisions may be informed by predicted or known modes of action, or they may simply highlight knowledge gaps for understudied endpoints. Investigations into the impacts of warming have mainly focussed on gonad or ejaculate endpoints i.e. reproductive investment, whereas the majority of OA-based investigations have studied sperm swimming behaviour. Field pollution studies have solely focussed upon reproductive investment (Krause, 1994; Zorita *et al.*, 2015; Zorita *et al.*, 2006), whilst pesticides have only been investigated in relation to sperm physiology or DNA damage (Favret and Lynn, 2010; Akcha *et al.*, 2012)

Across anthropogenic drivers, gonad and ejaculate endpoints have been most widely utilised, closely followed by investigations into sperm swimming performance, sperm morphology and finally the least studied endpoint group are the sperm physiological measures. Intriguingly none of the studies identified in this literature search reported the influence of warming as a single variable on sperm swimming speed to confirm a widely held assumption that speeds will be enhanced in climate change relevant treatments. Caldwell *et al.* (2011a) identified a significant interaction between seawater temperature and pCO_2/pH conditions that enhanced the sperm swimming performance of the sea urchin, *Psammechinus miliaris*. This interaction precludes the interpretation of either driver individually, but given that seas are projected to concurrently warm and acidify may be a more accurate representation of future ocean conditions than investigations of either variable individually.

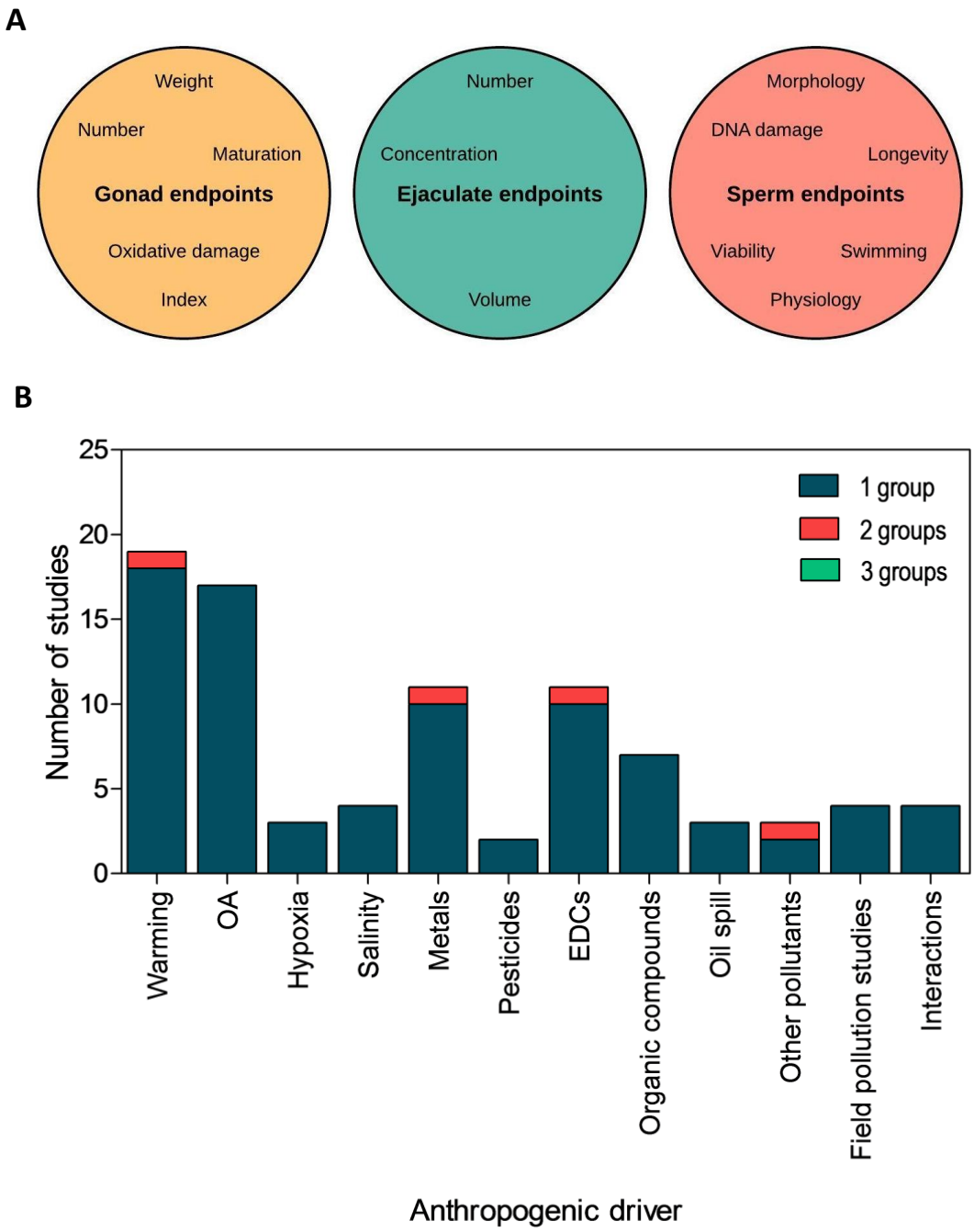

Figure 6. (A) Conceptual figure to illustrate the breakdown of study endpoints into three broad sperm life-history stages ('groups') and (B) the number of studies identified through the systematic literature search that investigated endpoints categorised by one, two or all three groups.

Experimental endpoints were split into three broad categories; gonad, ejaculate and sperm, to span three distinct sperm life history stages (Figure 6A). We then assessed how

many categories of endpoints had been investigated by studies to establish the frequency at which impacts on multiple sperm life history stages have been considered by researchers (Figure 6B). Investigations into anthropogenic drivers have rarely been undertaken for multiple sperm life history stages. Out of the total of 79 studies, only four investigated more than one endpoint category, and in all cases this was only two categories (Wintermyer and Cooper, 2007; Tian *et al.*, 2015; Boni *et al.*, 2015; Au *et al.*, 2001b). All four studies exposed adult animals to the anthropogenic driver before measuring gonad, ejaculate and/or sperm endpoints, so in all cases sperm were not exposed to the same conditions as the adult animals.

During natural spawning events, sperm are released in the close vicinity of the adult male and hence, are very likely to experience similar environmental conditions (temperature, salinity, pH) depending on the local temporal heterogeneity in environmental conditions. Of course, adults may be sediment dwelling, whereas their sperm are usually realised into the overlying seawater. In these cases, the environmental conditions experienced by the adult male and sperm are likely to vary. Firstly, as environmental conditions in the sediment may be different to the seawater conditions, and may be modified by the organism itself (e.g. through respiration). And secondly, as aquatic sediments are a major sink for a wide variety of organic and inorganic pollutants (Zoumis *et al.*, 2001), and interactions between the sediment, pore water and contaminants can be complex influencing their availability, toxicity and exposure routes for an organism (Cross *et al.*, 2015). A more thorough investigation of local conditions characterising natural habitats is required to better understand adult and sperm environments in externally fertilising sediment dwelling fauna.

Experiments conducted over successive life stages are required to assess carry-over effects i.e. an accumulation of effects across successive stages, that may result in later vulnerability to further stress as a consequence of exposure earlier in development (Pechenik, 2006). Alternatively, performance may be enhanced by the shared environmental conditions through selection on specific phenotypes or via phenotypic modification. Ritchie and Marshall (2013) found that performance was generally higher when the salinity conditions experienced by sperm and larvae of the polychaete, *Galeolaria gemineoa* matched. Their findings provide empirical evidence that

performance may be enhanced when successive stages are exposed to the same conditions and this may play some role in ameliorating some of the negative effects of exposure to environmental stressors. This systematic map has revealed a paucity of investigations assessing anthropogenic impacts on sperm across multiple sperm life history stages, which are required in order to assess carry-over effects, trans-generational plasticity and the influence of parental environmental history on the performance of gametes and offspring.

Only a small number of investigations have considered interactions between multiple anthropogenic drivers and their influence on freely spawned sperm (Figures 4, 5 and 6). Four studies identified through the systematic search investigated more than one environmental driver. All four of these studies empirically tested the influence of OA in conjunction with an additional driver; temperature (Uthicke *et al.*, 2014; Caldwell *et al.*, 2011a), hypoxia (Graham et al., 2015) or the heavy metal copper (presented in Chapter 3 of this thesis; Campbell *et al.*, 2014). These investigations were conducted in three species of echinoderm and one polychaete species (Table S1-12). This handful of studies constitutes the entirety of scientific knowledge on the potential for multiple stressors to impact freely spawned sperm, which is clearly a significant knowledge gap.

Environmental stressors rarely act in isolation, instead they co-occur with other natural or anthropogenic stressors, with a high likelihood of interactions amongst them. Global environmental change is multi-faceted; the oceans are concurrently warming, acidifying and deoxygenating alongside changes to ocean circulation patterns, stratification and salinity (Stocker *et al.*, 2013). Of course there are a myriad of possible stressors combinations that could be tested in complex and hence unfeasible fully crossed multi-factorial experimental designs. One solution to this challenge, was adopted by Boyd *et al.* (2015) in their study of physiological responses of a subantarctic diatom to complex future ocean scenarios. They utilised a collapsed factorial experimental design to investigate the combined influence of multifaceted ocean change scenarios on diatom growth rates. A similar approach could be adopted by studies investigating the performance of freely spawned sperm under future ocean conditions.

The four studies that investigated potential synergisms between stressors confirm the potential for anthropogenic drivers to interact and affect freely spawned sperm. Uthicke *et al.* (2014) observed slower gonad maturation in the sea urchin, *Echinometra sp. A* under combined warming and OA scenarios. OA conditions were also found to increase the toxicity of copper to sperm from the coastal polychaete, *Arenicola marina,*and this resulted in enhanced spermatozal DNA damage in combined treatments (Campbell et al., 2014). Not all of the interactions identified to date appear to have negative effects. Caldwell *et al.* (2011a) observed enhanced sperm swimming performance in combined warming and OA scenarios in the sea urchin, *P. miiliaris*. There clearly is a potential for interactions between anthropogenic drivers to influence freely spawned sperm, with significant research effort required to address the current knowledge deficit.

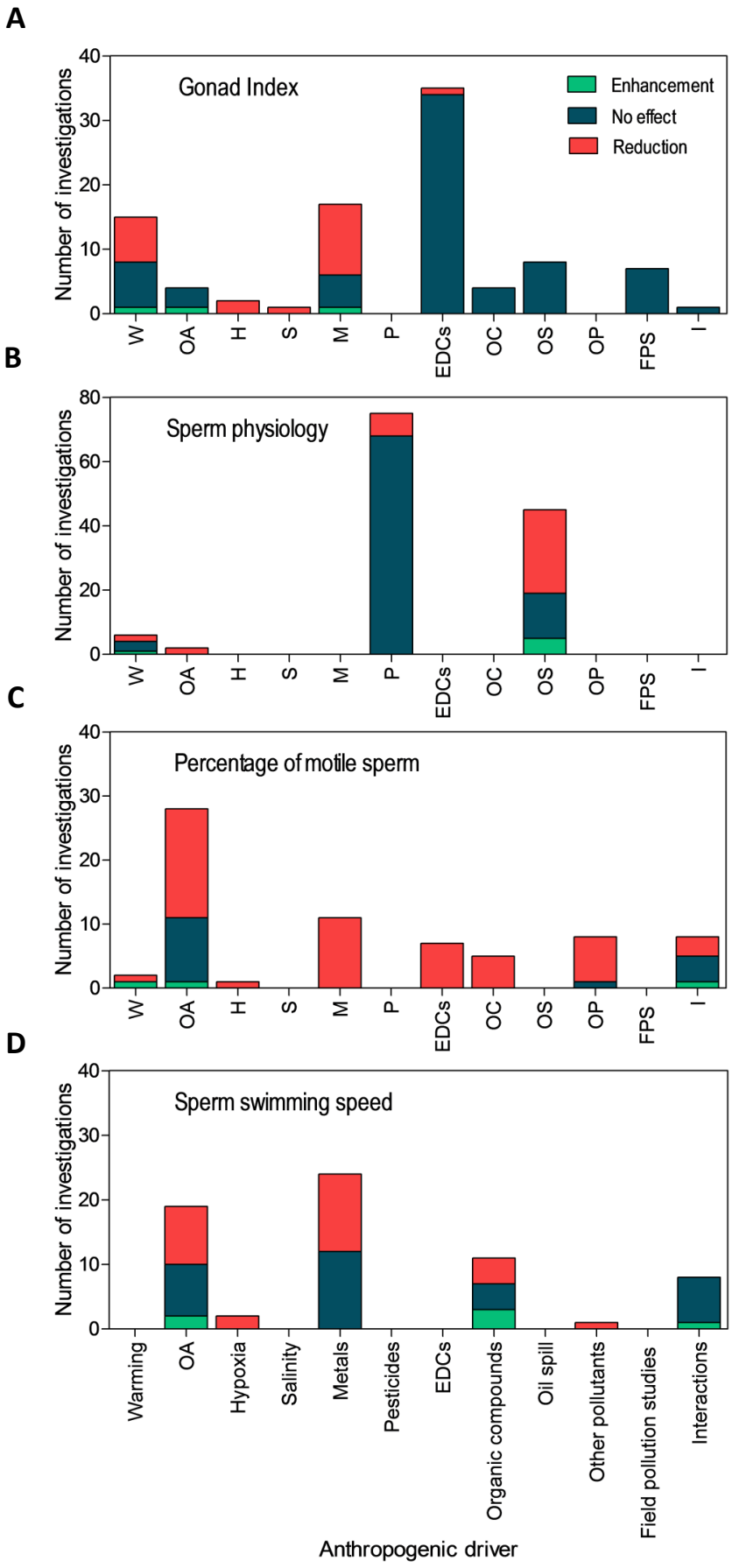

Figure 7. The effects direction of studies that have investigated male reproductive and/or sperm responses to anthropogenic drivers in free spawning marine invertebrate species

for (A) gonad index, (B) any sperm physiological endpoint (including viability), (C) the percentage of motile sperm and (D) average sperm swimming speed. Findings are the reported outcomes of statistical analyses conducted by qualifying studies. Investigations are individual combinations of study, species and treatment levels and in the case of sperm physiology, physiological endpoints. When post-hoc tests were omitted by a study with more than one treatment level, the study can only be included as a single investigation per study species with the overall statistical finding presented. The findings of interactions relate solely to the interaction term and when non-significant, and the significance of main effects can be individually assessed, these investigations have been included in the columns relevant to the individual drivers.

We selected the four best-studied endpoints to analyse in greater depth. A summary of the results of these investigations can be found in Figure 7. Gonad index (GI) has been widely studied across anthropogenic drivers. GI can be defined as the proportion of an organism‘s total weight occupied by the gonad(s). The majority of investigations of GI have found that exposure to climate change variables or marine pollutants had no significant effect (Figure 7A). This is particularly true for marine pollution studies, where the overwhelming majority of investigations failed to elicit a change in GI from control animals. The most-studied driver in relation to GI has been the endocrine disrupting chemicals (EDCs) which are also grouped with pharmaceuticals. However, it appears that reproduction in invertebrates is not affected by exposure to the compounds tested, often at relatively high exposure levels (Table S1-7). One study identified reduced GI values in the bivalve, *Gomphina veneriformis* in a 36 week exposure to tributyltin (TBT; Park et al., 2015). The authors reported significantly lower GI values, but only at the highest concentration of TBT they tested (0.8 μgL^{-1}). In another study of the same species, *G. veneriformis*, animals were exposed to a range of TBT concentrations including higher concentrations (0.1-20 μgL^{-1}) for 28 weeks (Park *et al*., 2012). However, in this study there were no differences in GI between animals in any of the TBT treatments in comparison to the control.

Reproductive investment appears more sensitive to heavy metals than other groups of pollutants at the levels studied (Figure 7A). The majority of investigations have identified reduced GI values in free spawning species in metal exposures, although responses appear species-specific (Table S1-5). For example GI was unaffected in the sea urchin, *Anthiocidaris crassipina* exposed to cadmium for 4 weeks at high concentrations (0.01-0.1 mgL^{-1}; Au *et al.,* 2001b). However, a lower cadmium concentration (0.055 mgL^{-1}) reduced the GI of the mollusc, *Tegillarca granosa* following a similar exposure duration (Liu *et al*., 2014). *T. granosa* GI values were also reduced in exposures to copper, lead and zinc for 30 days in the same study (Liu *et al*., 2014), and their reproduction may be particularly vulnerable to a wide spectrum of metals. Reproduction in another mollusc species, *G. veneriformis* was insensitive to much higher concentrations of zinc than *T. granosa* (3-4 orders of magnitude higher) and their GI was even enhanced at one treatment level (Ju *et al.*, 2009) highlighting the species-specificity of perturbation.

As an entire group, reproductive investment has been perturbed by exposures to climate change variables rather than marine pollutants (Figure 7A). Most investigations have identified reduced GI values in animals exposed to future climate change scenarios. The majority of these investigations have been conducted in relation to warming, and these studies have produced mixed results (Table S1-1). These results are discussed in more detail in relation to Figure 8, where the influence of exposure length, temperature elevation above the control and taxa are investigated. The systematic search revealed that only a handful of studies have considered OA influences on GI in free spawning marine invertebrates. GI appears to be insensitive to seawater pCO_2/pH conditions as a single stressor as all investigations to date found no effect or even enhanced GI values in animals under simulated OA conditions (Table S1-2). However, these studies have solely focussed on echinoderms and there may be sensitive species from other free spawning taxa, whose responses have not yet been tested. The tendency for studies to provide food in excess or *ad libitum* may also influence susceptibility (Pansch *et al.*, 2014), and there needs to be a greater understanding of the interplay between food availability and OA conditions for male reproductive investment. There has only been one investigation apiece of the influence of hypoxic conditions or salinity reductions on GI (Tables S1-3 and S1-4). Both revealed negative responses to the experimental conditions, highlighting the need for further research into these emerging groups of stressors.

It is surprising how few investigations of sperm physiology have taken place across anthropogenic drivers (Figure 7B). There is a paucity of data on sperm physiological responses to climate change variables and for most groups of marine pollutants. Understanding mechanisms of perturbation aids an understanding of modes of disruption, and could help to characterise sensitive and tolerant sperm phenotypes in order to identify vulnerable populations beyond the small numbers tested to date for each driver. The two best-studied drivers in sperm physiological investigations have been the pesticides and simulated oil spills. Overall sperm physiology appears to be sensitive to the extremely high concentrations of polycyclic aromatic hydrocarbons (PAHs) selected to simulate catastrophic oil spills (Table S1-9). Although unlikely, these spills can take place during marine invertebrate reproductive seasons. For example the Deepwater Horizon (DWH) oil spill took place off the coast of Louisiana in April 2010; at the beginning of the *Crassostra virginica* spawning season (Volety *et al.*, 2016). Exposure *of C. virginica* sperm to a field collected DWH oil slick and oil dispersant resulted in alterations to several sperm physiological measures and these may have influenced population fertilisation success during the 2010 spill (Volety *et al.*, 2016).The vast majority of investigations into pesticides have found no effects on sperm physiology (Table S1-6). Several commercially available herbicides (glyphosate, Roundup (a formulation of glyphosate), diuron) and one pesticide (bayluscide) have been tested. These investigations have been focussed on two oyster species, *Crassostrea gigas* and *C.viginica* along with one echinoderm species, *Lytechinus variegatus*. Out of these investigations sperm physiology was only affected in *C. virginica* exposed to the pesticide bayluscide (0.06-1 mgL^{-1}). The pesticide reduced sperm mitochondrial activity and sperm membrane integrity at these concentrations (Favret and Lynn, 2010).

Investigations of sperm responses to OA have mainly focussed on swimming performance and impacts on the number and/or speed of motile sperm (Figure 7C, D). Investigations into both of these parameters have produced mixed results, which are discussed in greater detail in reference to Figure 9. Despite variation in sperm sensitivity to OA-induced motility perturbation, there have been few physiological investigations to aid a mechanistic understanding of effects when observed to help appreciate why some populations are affected but others are more robust to changes in seawater chemistry associated with OA. The one study that has investigated sperm physiology in response to

OA conditions, identified significant alterations to sperm mitochondrial membrane potential (MMP) in the sea urchin, *Centrostephanus rodgersii* (Schlegel *et al.*, 2015). MMP is generated through the activity of mitochondrial energy metabolism, hence the reductions in sperm MMP observed by the authors suggests a mechanism for perturbation in this species via aerobic energy metabolism.

Across anthropogenic drivers the majority of investigations into percentage sperm motility have identified reductions upon exposure to experimental conditions (Figure 7C), suggesting this characteristic ejaculate trait is generally vulnerable to anthropogenic perturbation. The number of sperm actively swimming influences effective sperm concentrations in the vicinity of a given ovum, and there is a well-established relationship between sperm concentration and external fertilisation success (Lillie, 1915; Benzie and Dixon, 1994). There has been limited research effort into the effects of pesticides, simulated oil spills, field pollution studies, warming, hypoxia and salinity reductions. Percentage sperm motility may also be sensitive to these groups of drivers, and research should be focussed on characterising these relationships and investigating potential interactions amongst them. All of the work to date on hypoxic conditions has revealed negative effects on freely spawned sperm (Table S1-4). This includes reduced sperm swimming speeds in the polychaete, *Hydroides elegans* (Shin *et al*., 2014), reductions in the speed and number of motile sperm in the urchin, *Paracentrotus lividus* (Graham *et al.*, 2015) and reductions in GI in another sea urchin species, *Strongylocentrotus droebachiensis* (Siikavuopio et al., 2007). Whilst there have only been a small number of investigations, there is concern that the projected increase in hypoxic events in coastal waters, exacerbated through climate change (Keeling *et al.*, 2010), could represent a real threat to reproduction in coastal species. There is a strong possibility that hypoxic events could coincide with gametogenesis and reproductive investment or with population spawning events with an increased frequency in future coastal waters. Our synthesis suggests that the consequences for population fertilisation success in coastal external fertilisers could be severe. There is much further experimental research required to assess species' vulnerabilities to hypoxia and ascertain the broad applicability of these findings across free spawning taxa.

Sperm swimming speed responses to anthropogenic drivers have been less widely studied, and the results appear to be more variable than for investigations of percentage sperm motility (Figure 7C, D). Whilst, the percentage of motile sperm was reduced in all exposures to metals and organic compounds conducted to date, there have been more varied responses observed for sperm swimming speed (Tables S1-5 and S1-8). Identifying the mechanisms underlying perturbation of each sperm swimming parameter and the modes of action for each compound would enable a clearer understanding of these results, highlighting the need for more investigations of sperm physiology. Sperm swimming responses are discussed in greater depth in relation to Figures 9 and 10.

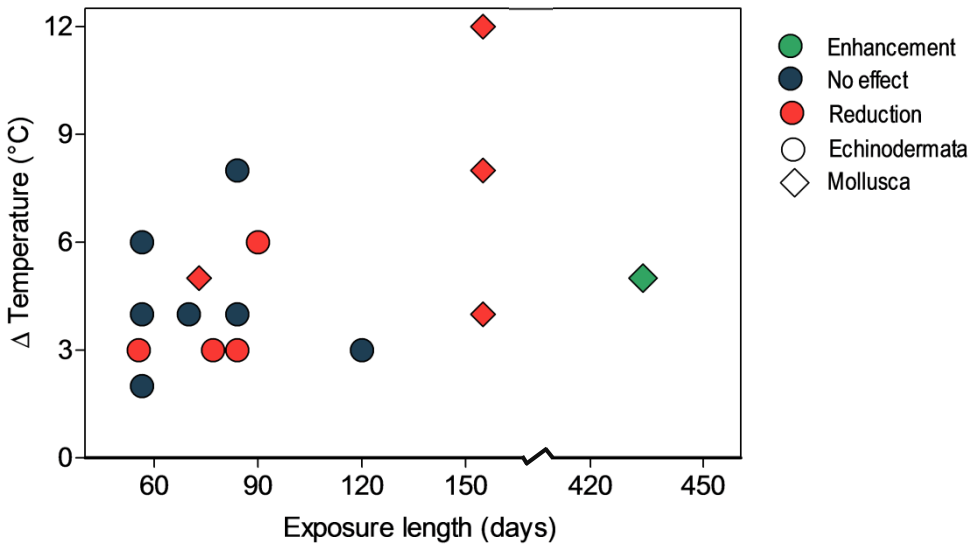

Figure 8. The findings of studies that investigated gonad index in response to elevated sea surface temperature in free spawning marine invertebrate species split into broad taxonomic groups. Points are coloured by the reported outcome of statistical analysis conducted by qualifying studies. The exposure length was taken from the exposure in days reported by a study or calculated from exposures reported in other units using assumptions of 7 days in a week and 31 days in a month. ΔTemperature has been calculated as the treatment level – the control level, and when controls have not been clearly identified by studies they have been selected from the available treatments (Table S1-1 for further details on selection criteria). Each point represents an individual combination of study, species and treatment level.

Studies of GI in elevated temperature treatments have focussed on echinoderm and mollusc species (Figure 8). There does not seem to be a clear relationship between the temperature elevation above control conditions and the sensitivity of GI values to perturbation by warming across echinoderms. Reproduction in mollusc species appears much more sensitive to elevated temperature treatments, with most studies observing reductions in GI at elevated temperatures ranging from +4 to +12 °C and exposures lasting for between 73 and 155 days (Table S1-1). Only one investigation of GI in a mollusc species failed to find a reduction at an elevated temperature. Guerra *et al.*

(2012) kept *Argopecten ventricosus* at 5 °C above the control for 14 months and found that this enhanced GI values. Their findings highlight the potential for adaptive responses in reproductive investment to climate change.

In marine invertebrates the gonads are often the main nutritive storage organ, and there are usually seasonal changes in their composition and the percentage of total body weight they constitute i.e. the GI (Garrido and Barber, 2001). Kurihara *et al.* (2013) identified a significant interaction between time and seawater pCO_2/pH conditions that influenced GI in their nine month study of reproductive investment in the sea urchin, *Hemicentrotus pulcherrimus* (Kurihara *et al*., 2013). This interaction resulted in lower GI values in urchins kept under control conditions as the experiment progressed, which might be interpreted as a reduced investment in reproduction in control animals. However, the authors also carried out histological analyses, which revealed that control urchin gonads matured more quickly and these animals spawned earlier than urchins kept at elevated temperature. Their findings highlight the need for dual investigations of GI with histological analyses in order to fully understand reproductive responses to experimental conditions. Additionally there may be sex-specific differences in GI values (Hazan *et al.*, 2014) or in the response of GI to environmental variables or stressors (Uthicke *et al.*, 2014). Yet, the response of males and females are rarely assessed independently, and the influence of sex is infrequently included in statistical analyses of reproductive investment in experimental conditions. If the sexes respond equally to an anthropogenic driver but in opposite directions, their responses may cancel each other out given a sex ratio of approximately 50: 50. This needs to be addressed in future experimental work.

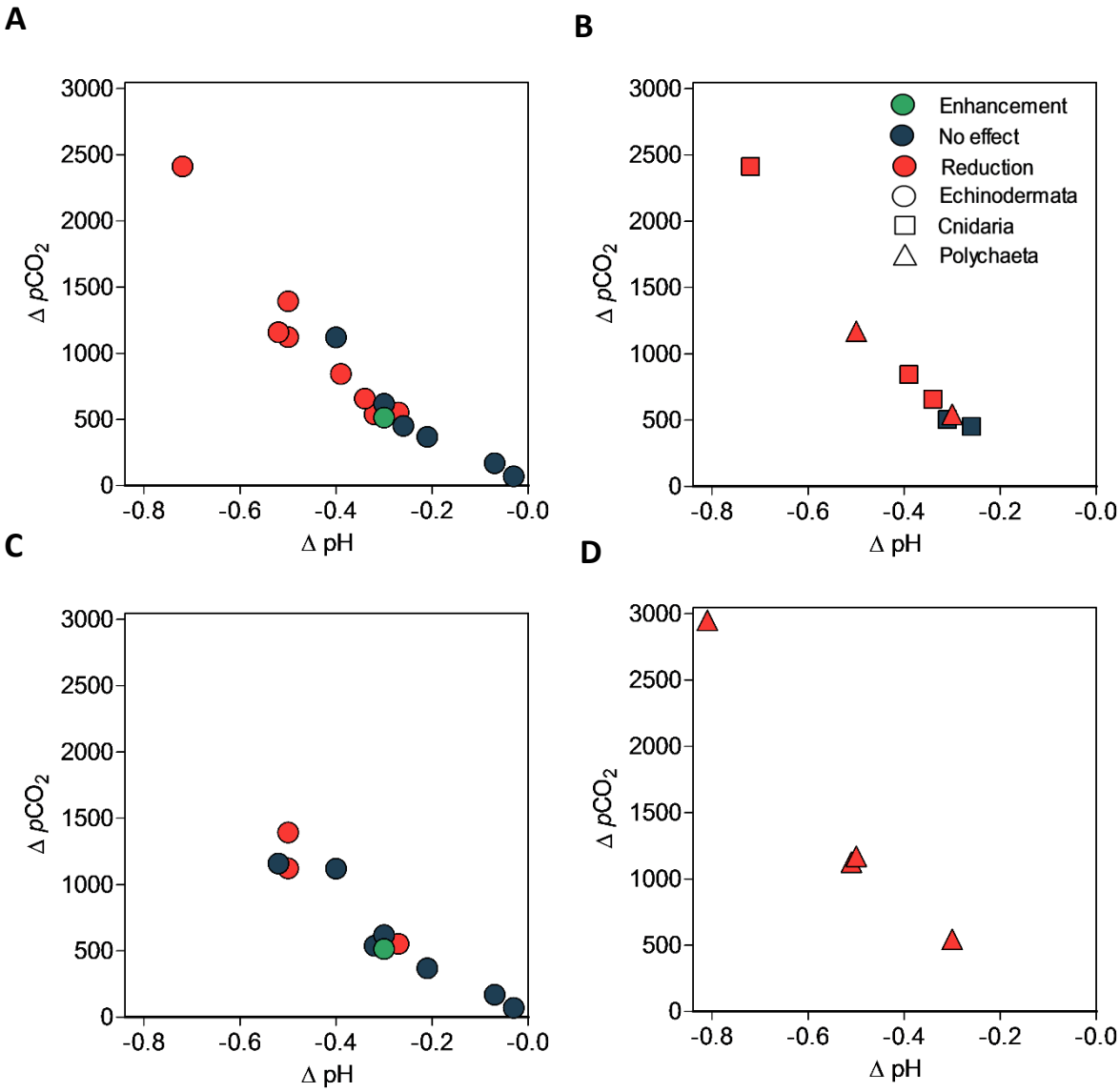

Figure 9. The findings of studies that investigated sperm swimming performance in response to sperm exposure to OA conditions in free spawning marine invertebrate species. Firstly, the percentage of motile sperm (top row; A and B) and secondly average sperm swimming speed (bottom row; C and D). The figures have been split by broad taxonomic groups into echinoderms (A and C) and other taxa (B and D). Points are coloured by the reported outcome of statistical analyses conducted by qualifying studies. ΔpH and ΔpCO_2 have been calculated as the treatment level minus the control level. The values have been preferentially taken from reported seawater pCO_2 values following chemical analysis and pH measurements of experimental seawaters. If either has been omitted by a study, targeted values reported by the study have been substituted when available. The pH values are on the pH scale reported for measurements (Table S1-2 for more details). Studies that failed to undertake post-hoc tests on multiple treatment

levels, where significant results were identified in an ANOVA, have been excluded from the figure alongside any results where the influence of OA was found to significantly interact with another anthropogenic driver. Each point represents an individual combination of study, species and treatment level.

Investigations into sperm swimming behaviour under OA conditions have yielded mixed results (Table S1-2). In an attempt to disentangle some of this variability we have plotted the results of investigations by the coordinates of experimental treatments (Figure 9); ΔpH (treatment –control), ΔpCO_2 (treatment –control). Sperm swimming in polychaetes appears sensitive to perturbation under OA conditions. Whilst there have only been a small number of investigations, OA treatments have all elicited reductions in the number and speed of motile sperm in the polychaete species tested to date. Cnidaria have also not been widely studied and the influence of OA on sperm swimming performance has only been investigated in one species to date, *Acropora digitifera*. The percentage motility of *A. digitifera* sperm appears to be sensitive to OA conditions above a threshold treatment level. There was no effect of OA conditions on sperm motility at only small changes to seawater pCO_2/ pH conditions from control conditions (-0.31 pH units, +505 µatm pCO_2 and below). Whereas stronger OA treatments (-0.34 pH units, +658 µatm pCO_2 and above) acted to reduce the percentage of motile sperm compared to *A. digitifera* sperm incubated under the control (Morita *et al.*, 2010).

There has been a great deal of work on the sperm swimming performance of echinoderm species in response to OA conditions. Percentage sperm motility in echinoderms appears to be generally sensitive to OA above an approximate threshold (~-0.32 pH units, ~+620 µatm pCO_2). However, responses vary between species; with some reductions in percentage motility observed below this threshold, and some robust percent motility responses above the threshold. Percentage sperm motility may be particularly robust to changes in seawater pCO_2/pH conditions in *Strongylocentrotus nudus* as there was no effect of more extreme OA conditions (-0.4 pH units, +1120 µatm pCO_2; Sung *et al.*, 2014) in a study of this species. In *C. rodgersii* percentage sperm motility was enhanced by

slight OA conditions (-0.3 pH units, +515 µatm pCO_2), but reduced at a greater OA treatment level (-0.5 pH units, +1123 µatm pCO_2; Schlegel *et al*., 2015). It seems intuitive that there may be a range of seawater conditions at which sperm swimming performance is maximal. Small deviations in conditions are unlikely to result in any observable changes to swimming performance, which may even be slightly enhanced (Calabrese, 2008). However, a change in conditions that brings a sperm outside of the optimal range is likely to negatively impact on swimming performance. Factors such as a male's developmental, parental and evolutionary history may influence the position and limits to these optimal conditions.

There have been fewer investigations of echinoderm sperm swimming speed responses to OA than for percentage sperm motility (Figure 9). The results also appear more variable, without a clear threshold of sensitivity to perturbation of sperm swimming speeds under OA conditions. A similar pattern was found in sperm swimming speeds in *C. rodgersii* as for percentage sperm motility under the same OA treatments; enhancement under slight OA conditions but reduced speeds in the greater OA treatment (Schlegel *et al.*, 2015). Sperm swimming speeds in *S. nudus* were also robust to a relatively strong OA treatment level (-0.4 pH units, +1120 µatm pCO_2) agreeing with the observed sperm motility response (Sung *et al.*, 2014). Despite sensitive sperm motility responses to the same OA treatment levels, sperm swimming speeds were not affected by OA treatments of up to -0.52 pH units and a +1159 µatm pCO_2 elevation in *Heliocidaris erythrogramma* (Schlegel *et al*., 2012). This finding highlights the different mechanisms that may underlie changes in these two ejaculate traits and the species-specificity with which they might be affected by OA conditions.

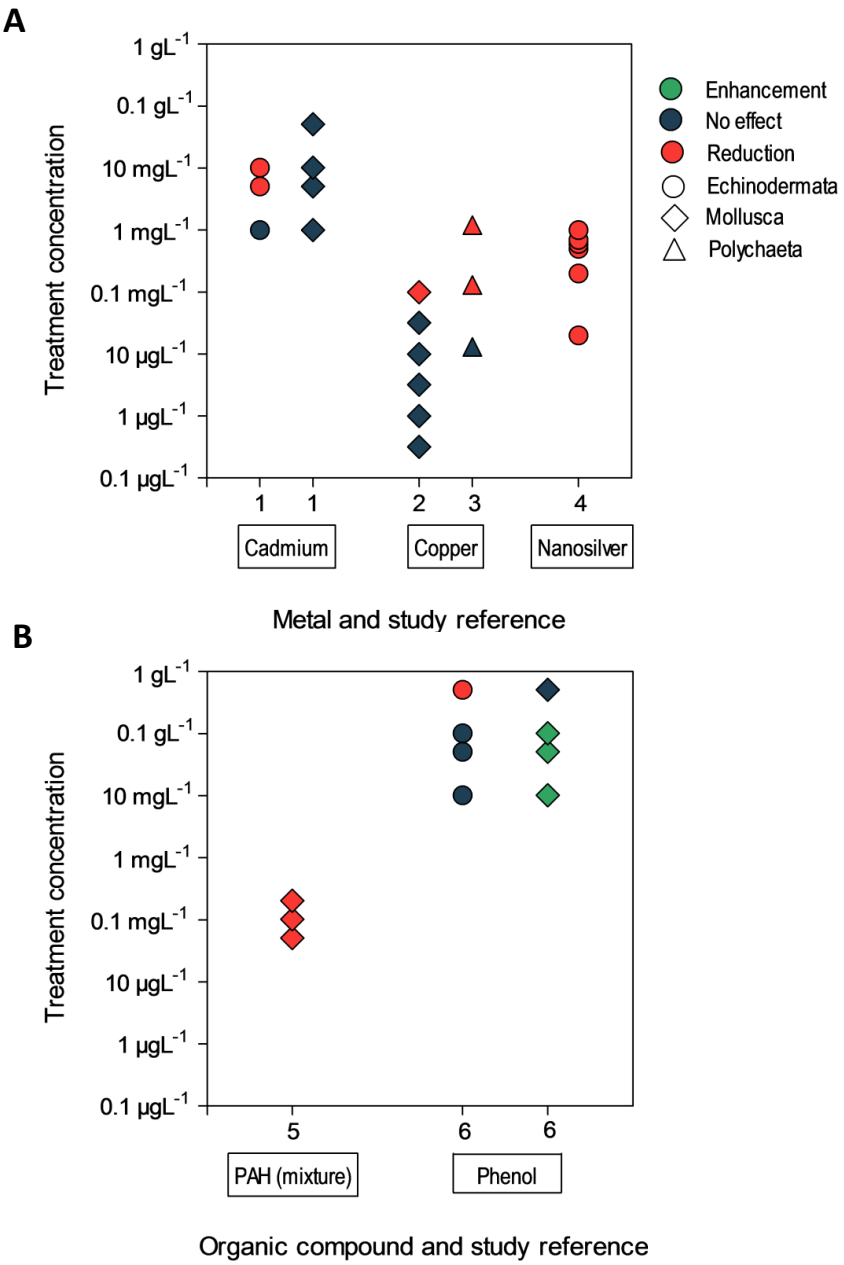

Figure 10. The findings of studies that investigated sperm swimming speed in response to sperm exposure to (A) metals and (B) organic compounds in free spawning marine invertebrate species split into broad taxonomic groups. Points are coloured by the reported outcome of statistical analyses conducted by qualifying studies. The y axis is a log scale. When studies reported concentrations in alternative units, conversions were applied (Table S1-5 and S1-8 for further details). 1: *Anthiocidaris crassipina* and *Perna viridis* (Au *et al.*, 2000), 2: *Mytilus trossulus* (Fitzpatrick *et al*., 2008), 3: *Arenicola marina* (Campbell *et al.*, 2014), 4: *Paracentrotus lividus* (Gambardella *et al.*, 2015), 5: *Crassostrea gigas* (Jeong and Cho, 2005), 6: *Anthiocidaris crassipina* and *Perna viridis* (Au *et al*., 2000).

There have been surprisingly few investigations into sperm swimming responses to heavy metals and organic compounds (Figure 10 and Tables S1-5 and S1-8). This paucity of data makes comparisons across compounds and taxa tricky, as the same compound has rarely been tested in different species within the same taxa or in different species from other free spawning taxa. Sperm swimming speeds appear less sensitive to perturbation by metals or organic compounds in species of mollusc than in echinoderms. For example swimming speeds were more sensitive to perturbation by cadmium in the sea urchin *A. crassipina* than in the mollusc *Perna viridis* (Au *et al.*, 2000). Speeds were reduced in exposures to 5 mgL^{-1} cadmium in *A. crassipina,* but were robust to concentrations of up to 51 mgL^{-1} in *P. viridis*. Similar findings were found in these species exposed to the organic compound phenol; swimming in *P. viridis* was robust to extremely high phenol concentrations of up to 510 mgL^{-1} and sperm swimming speeds were even enhanced by concentrations of up to 102 mgL^{-1} (Au *et al.*, 2000). Whereas sperm swimming speeds in *A. crassipina,* were reduced by exposure to 510 mgL^{-1} phenol. The concentrations used by these studies are extremely high and environmentally unrealistic. It is highly unlikely that sperm swimming speeds would be affected by field relevant concentrations in either species.

Sperm swimming speeds appear more sensitive to perturbation by copper in free spawning species. Lower concentrations of copper reduced sperm speeds in the mollusc *Mytilus trossolus* (0.1 mgL^{-1}; Fitzpatrick *et al.*, 2008) and the polychaete *A. marina* (0.13 mgL^{-1}; Campbell *et al.*, 2014) than for phenol or cadmium in their respective species. The concentrations at which effects were found in these two studies are at the very upper limit of environmentally realistic concentrations (Bryan and Gibbs, 1983) and represent a heavily impacted site. Hence, the reductions in sperm swimming speeds observed by these studies are unlikely to be found under field conditions for either species. Of the compounds investigated to date, sperm swimming speeds were most sensitive to PAHs and nanosilver. *C. gigas* sperm swimming speeds were reduced in all treatments, where they were exposed to a mixture of PAHs at concentrations as low as 51 μgL^{-1} (Jeong and Cho, 2005). Sperm from the sea urchin *P. lividus* were sensitive to all concentrations of nanosilver tested, with speeds reduced at concentrations as low as 20 μgL^{-1} (Gambardella *et al*., 2015). Their results may provide evidence that nanoparticles are more toxic than bulk forms, but with no direct comparison of sperm swimming in exposures to the bulk

and nano form of the same material there is further experimental work required to support this hypothesis.

2.5 CONCLUSIONS

There is overall a paucity of data on the response of free spawned sperm to anthropogenic drivers of marine environmental change. Sperm are critical to marine invertebrate life histories, and any alteration to their function has the potential to have severe consequences for marine invertebrate populations. Given the rapid rate at which climate change is accelerating against a backdrop of existing and emerging marine pollution pressures, this knowledge deficit is concerning and limits our understanding of marine invertebrate reproductive health in future oceans. Future work should broaden investigations to cover a greater range of species from a diverse mix of habitat types and free spawning taxa. Only a handful of studies have investigated interactions between anthropogenic drivers, despite multiple co-occurring stressors in natural systems. Studies should consider potential interactions between multiple drivers, which may be more feasible using a condensed factorial experimental design.

This systematic literature search has highlighted salinity change and hypoxia as emerging drivers of concern and research effort should be focussed on understanding their impacts on the sperm of coastal free spawning species. The systematic map has also highlighted a paucity of sperm physiological investigations, which are required in order to uncover the mechanisms underlying changes to sperm performance and function. There is a real impetus to conduct investigations of sperm physiology under simulated OA conditions to clarify the physiological basis for the species-specific reductions in sperm swimming speed and percentage motility observed in studies to date. With this information, researchers may gain predictive power to identify vulnerable populations and species beyond the small number whose swimming responses have been studied to date. No study identified in this synthesis exposed adults and sperm to the same driver(s) leaving significant knowledge gaps on the potential accumulation of effects across successive

life-stages and trans-generational plasticity, which are both needed to fully understand reproduction in future seas.

2.6 SUPPORTING INFORMATION

2.6.1 Supporting tables

Key to supporting tables:

- Statistical analysis conducted by the study identified a significant increase
- Statistical analysis conducted by the study identified no significant effect
- Statistical analysis conducted by the study identified a significant reduction

Table S1-1. The methodology and results of studies that have investigated the influence of warming for freely spawned sperm.

Taxa	**Species**	Treatment (°C)	Temperature follows natural cycles (Y/N)	**Duration (units)**	Life stage exposed	**Endpoint**																**Scope**	
						Gonad						Ejaculate		Sperm									
						Weight	Index	Diameter	Number	Occupation index	Maturation	Ejaculate volume	Sperm number/conc.	DNA damage	Lipid peroxidation	Ca^{2+} conc.	MMP	Mito. activity	pHi	Motile (%)	Longevity	Climate change	Aquaculture
Ascidiacea	*Botryllus schlosseri* (Johnson and Yund, 2004)	+7		3 days	S																■		
Cnidaria	*Acropora digitifera* (Paxton et al., 2015)	+2	Y	1 month	A								■									X	
	Paramuricea clavata (Arizmendi-Mejía *et al.*, 2015)	+9	N	20 days	A			■	■													X	
Echinodermata	*Echinometra mathaei* (Rahman *et al.*, 2009)	+5[A]		85 mins	S																■		
	Echinometra sp. A (Uthicke *et al.*, 2014)	+3	N	77 days	A		■															X	
	Evechinus chloroticus (Delorme and Sewell, 2016)	+6	N	90 days	A		■															x	
	Evechinus chloroticus (James and Heath, 2008)	+4[D]	N	10 weeks (spring (Lamare *et al.*, 2002))	A		■																X
	Heliocidaris tuberculate (Binet and Doyle, 2013)	+4		3 hours	S													■			■	x	
		+6		1 hour	S													■			■	x	
	Lytechinus variegatus (Watts *et al.*, 2011)	+6[B]	N	8 weeks	A	■																	x
	Paracentrotus lividus (Shpigel *et al.*, 2004)	+2-4	N	12 weeks	A		■																X
	Strongylocentrotus droebachiensis (Siikavuopio *et al.*, 2006)	+2[C]	N	8 weeks (winter (Norderhaug *et al.*, 2016))	A		■																X

Echinodermata (cont.)	*Strongylocentrotus droebachiensis* (cont)	+4[C]	N	8 weeks (winter (Norderhaug *et al.*, 2016))	A																			X
		+6 [C]	N	8 weeks (winter (Norderhaug *et al.*, 2016))	A																			x
	Strongylocentrotus franciscanus (McBride *et al.*, 1997)	+3	N	120 days	A																			X
	Strongylocentrotus intermedius (Lawrence *et al.*, 2009)	+5[C]	N	4 months	A																			X
		+10[C]	N	4 months	A																			X
	Strongylocentrotus purpuratus (Azad et al., 2011)	+4[A, C]	N	12 weeks	A																			x
		+8[A, C]	N	12 weeks	A																			x
	Tripneustes gratilla (Rahman *et al.*, 2009)	+5[A]		85 mins	S																			
		+10[A]		85 mins	S																			
Mollusca	*Argopecten ventricosus* (Guerra *et al.*, 2012)	+5	Y	14 months	A																			x
	Mytilus galloprovincialis (Boni *et al.*, 2015)	+13	N	14 days	A																		X	
		+13	N	30 days	A																		X	
	Panopea generosa (Marshall *et al.*, 2012)	+4[A, C]	N	155 days	A																			x
		+8 [A, C]	N	155 days	A																			x
		+12[A, C]	N	155 days	A																			x
	Panopea zelandica (Viet Le *et al.*, 2014)	+5[C]	N	73 days	A																			X
	Ruditapes philippinarum (Delgado and Camacho, 2007)	+4[C]	N	70 days	A																			x

Notes: A= adult; S= sperm; conc. = concentration; mins= minutes.Treatment is calculated as the control level subtracted from the treatment level.For studies which did not clearly identify a control temperature treatment, a control has been selected from the available treatment levels for one of the following four possible reasons; the temperature closest to the reported temperature during collection of the study population[A], the regional annual mean sea surface temperature (SST) reported by the study[B], the annual mean SST at the reported collection site (or closest location for which data is available on the internet)[C] or finally the average SST recorded by the study at the study population collection site during the experiment[D]. When experiments were repeated in multiple seasons, the season that best represents the reproductive season for the study population in the broad region it was collected from was selected based upon the available scientific literature. In such cases the season and literature used for decision making has been included in the 'duration' column.

Table S1-2. The methodology and results of studies that have investigated the influence of ocean acidification for freely spawned sperm.

Taxa	Species	Treatment (pH: pH units)	Treatment (pCO_2: µatm)	pH/pCO_2 follows natural cycles (Y/N)	Duration (units)	Life stage exposed	Endpoint							
							Gonad			Sperm				
							Index	RNA/DNA	Maturation	MMP	Speed (unknown)	Speed (VCL)	Speed (VSL)	Motile (%)
Cnidaria	*Acropora digitifera* (Nakamura and Morita, 2012)	-0.31[x]	+505		(-)	S								
	Acropora digitifera (*Morita et al.*, 2010)	-0.26[x]	+453		(-)	S								
		-0.34[x]	+658		(-)	S								
		-0.39[x]	+845		(-)	S								
		-0.72[x]	+2413		(-)	S								
		-1.48[x]	+16413		(-)	S								
Echinodermata	*Acanthaster plancii* (Uthicke *et al.*, 2013)	-0.27	+554		(-)	S								
		-0.50	+1393		(-)	S								
	Centrostephanus rodgersii (Schlegel *et al*., 2015)	-0.3	+515		15 mins	S								
		-0.5	+1123		15 mins	S								
	Echinometra sp. A (Uthicke et al., 2014)	-0.19	+342	N	77 days	A								
	Echinometra sp. EE (Hazan et al., 2014)	-0.45	+998	Y	343 days	A								
	Heliocidaris erythrogramma (Schlegel *et al.*, 2012)	-0.32	+539		(-)	S								
		-0.52	+1159		(-)	S								
	Hemicentrotus pulcherrimus (Kurihara *et al*., 2013)	-0.27	+490	N	9 months	A								
	Holothuria spp (Morita *et al*., 2010)	-0.26[x]	+453		(-)	S								
		-0.34[x]	+658		(-)	S								
		-0.39[x]	+845		(-)	S								
		-0.72[x]	+2413		(-)	S								
		-1.48[x]	+16413		(-)	S								
	Paracentrotus lividus (Graham *et al.*, 2015)	-0.15	+239	N	6 months	A								
	Paracentrotus lividus (Catarino *et al*., 2012)	-0.33	+666	N	19 days	A								
		-0.61	+1825	N	19 days	A								
	Sterechinus neumayeri (Suckling *et al*., 2015)	-0.29	+491	N	2 years	A								

Echinodermata (cont.)		-0.46	+968	N	2 years	A								
	Strongylocentrotus nudus (Sung *et al.*, 2014)	-0.03	+70		(-)	S								
		-0.07	+170		(-)	S								
		-0.21	+370		(-)	S								
		-0.30	+620		(-)	S								
		-0.40	+1120		(-)	S								
Mollusca	*Crassostrea gigas* (Havenhand and Schlegel, 2009)	-0.33			(-)	S								
	Mytilus galloprovincialis (Vihtakari *et al*., 2013)		+620*		(-)	S								
Polychaeta	*Arenicola marina* (Campbell *et al.*, 2014)	-0.51	+1127		10 mins	S								
		-0.81	+2950		10 mins	S								
	Galeolaria caespitose (Schlegel *et al*., 2014)	-0.30	+544		(-)	S								
		-0.50	+1170		(-)	S								
	Pomatoceros lamarckii (Lewis *et al.*, 2012)	-0.18, -0.38, -0.43, -0.58, -0.73, -0.78, -0.98	+139, +463, +590, +966, +1649, +3479		10 mins	S								

Notes:

A= adult; S= sperm; mins= minutes; (-) indicates immediate analysis following sperm dilution, mins= minutes.

Treatment is calculated from the treatment level minus the control level. Reported averages were used in these calculations unless a range of values were reported by a study, and then the median value of that range was calculated and used to work out the treatment level. pH values are reported on the pH scale used by each study, and presumed to be NBS scale unless otherwise stated. If an alternative scale was utilised by a study the treatment pH is followed by '^{x}'.

If a study has omitted a post-hoc test to confirm which treatment levels are significantly different from the control (when significant differences are found) they are presented in the same row along with the outcome of the statistical analysis.

When calculated pCO_2 values and/or pH measurements have been omitted by a study, they have been included in the table with the reported targeted value(s) followed by a '*'. When a study has omitted calculated values and/or measurements with no reference to a targeted value the cell has been left blank.

Table S1-3. The methodology and results of studies that have investigated the influence of salinity reductions for freely spawned sperm.

Taxa	**Species**	**Treatment (salinity units)**	**Duration (units)**	Life stage exposed	**Endpoint**				
					Gonad		Sperm		
					Index	Weight	Morphology	SAAS	Longevity
Echinodermata	*Anthocidaris crassipina* (Lau *et al.*, 2009)	-6.5 to 8.5	24 weeks	A					
	Echinarachnius parma (Allen and Pechenik, 2010)	-14	30 mins	S					
Mollusca	*Crassostrea gigas* (Falkenberg *et al*., 2016)	-5, -10, -15, -20, -25	10 mins	S					
Polychaeta	*Galeolaria caespitose* (Falkenberg *et al*., 2016)	-5, -10, -15, -20, -25, -30	10 mins	S					
	Hydroides diramphus (Jensen *et al.*, 2014)	-9	2 weeks	A					

Notes:

A= adult; S= sperm; mins= minutes; SAAS= sperm accumulated at the surface.

Treatment is calculated from the treatment level minus the control level. If a significant linear relationship has been modelled between salinity and an endpoint, the treatment levels and overall relationship are presented in the same row.

Table S1-4. The methodology and results of studies that have investigated the influence of hypoxia for freely spawned sperm.

Taxa	**Species**	Control oxygen level (units)	Treatment oxygen level (units)	Percentage change (%)	Duration (units)	Life stage exposed	**Endpoint**				
							Gonad	Sperm			
							Index	Speed (VCL)	Speed (VSL)	Speed (VAP)	Motile (%)
Echinodermata	*Paracentrotus lividus* (Graham *et al.*, 2015)	190 µatm	54 µatm	-72	60 mins	S		■			■
	Strongylocentrotus droebachiensis (Siikavuopio *et al.*, 2007)	9.5 mgL^{-1}	6.0 mgL^{-1}	-37	54 days	A	■				
			4.0 mgL^{-1}	-58	54 days	A	■				
Polychaeta	*Hydroides elegans* (Shin *et al.*, 2014)	6.0 mgL^{-1}	1.0 mgL^{-1}	-83	60 mins	S		■	■	■	

Notes:

A= adult; S= sperm; mins= minutes.

Table S1-5. The methodology and results of studies that have investigated the influence of heavy metals for freely spawned sperm.

Metal	Taxa	Species	Conc. (units)	Conc. (order of magnitude)							Duration (units)	Life stage exposed	Endpoint									
				gL^{-1}		mgL^{-1}			μgL^{-1}				Gonad			Sperm						
				0.1	0.01	1	0.1	0.01	1	0.1			Index	Maturation	DNA damage	Viability	Morphology	Speed (VCL)	Speed (VSL)	Speed (VAP)	Motile (%)	Trajectory
Cadmium	Echinodermata	*Anthiocidaris crassipina* (Au *et al.*, 2001a)	0.01 mgL^{-1}					■			4 weeks	A						■	■	■	■	■
			0.1 mgL^{-1}				■				4 weeks	A						■	■	■	■	■
		Anthiocidaris crassipina (Au *et al.*, 2001b)	0.01 mgL^{-1}					■			4 weeks	A	■				■					
			0.1 mgL^{-1}				■				4 weeks	A	■				■					
		Anthiocidaris crassipina (Au *et al.*, 2000)	1 mgL^{-1A}			■					1 hour	S					■	■	■	■		
			5.1 mgL^{-1A}			■					1 hour	S					■	■	■	■		
			10.2 $mgL^{-1\,A}$		■						1 hour	S					■	■	■	■		
	Mollusca	*Perna viridis* (Au *et al.*, 2000)	1 mgL^{-1A}			■					I hour	S					■	■	■	■		
			5.1 mgL^{-1A}			■					I hour	S					■	■	■	■		
			10.2 mgL^{-1A}		■						I hour	S					■	■	■	■		
			51 mgL^{-1A}		■						I hour	S					■	■	■	■		
		Tegillarca granosa (Liu *et al.*, 2014)	55 μgL^{-1}					■			30 days	A	■									
			110 μgL^{-1}				■				30 days	A	■									
			220 μgL^{-1}				■				30 days	A	■									
	Polychaeta	*Perinereis nuntia* (Zheng *et al.*, 2010)	1.12 μgL^{-1}						■		50 days	A		■								
			23.1 μgL^{-1}					■			50 days	A		■								
			0.6 mgL^{-1}				■				50 days	A		■								
Copper	Mollusca	*Mytilus trossulus* (Fitzpatrick *et al.*, 2008)	0.32 μgL^{-1}							■	100 mins	S								■		
			1 μgL^{-1}						■		100 mins	S								■		
			3.2 μgL^{-1}						■		100 mins	S								■		
			10 μgL^{-1}					■			100 mins	S								■		
			32 μgL^{-1}					■			100 mins	S								■		
			100 μgL^{-1}				■				100 mins	S								■		
		Tegillarca granosa	7.1 μgL^{-1}						■		30 days	A	■									

Copper (cont.)		(Liu *et al.*, 2014)	14.2 μgL^{-1}								30 days	A											
			28.4 μgL^{-1}								30 days	A											
	Polychaeta	*Arenicola marina* (Campbell *et al*., 2014)	13 μgL^{-1B}								10 mins	S											
			130 μgL^{-1B}								10 mins	S											
			1.2 mgL^{-1B}								10 mins	S											
		Nereis virens (Caldwell *et al.*, 2011b)	13 μgL^{-1B}								30 mins	S											
			130 μgL^{-1B}								30 mins	S											
			1.2 mgL^{-1B}								30 mins	S											
			12 mgL^{-1B}								30 mins	S											
Lead	Mollusca	*Tegillarca granosa* (Liu *et al*., 2014)	43 μgL^{-1}								30 days	A											
			86 μgL^{-1}								30 days	A											
			172 μgL^{-1}								30 days	A											
Nanosilver	Echinodermata	*Paracentrotus lividus* (Gambardella *et al*., 2015)	0.02 mgL^{-1}								1 hour	S											
			0.2 mgL^{-1}								1 hour	S											
			0.5 mgL^{-1}								1 hour	S											
			0.6 mgL^{-1}								1 hour	S											
			0.7 mgL^{-1}								1 hour	S											
			1 mgL^{-1}								1 hour	S											
Zero-valent nanoiron (stabilised)	Mollusca	*Mytilus galloprovincialis* (Kadar *et al.*, 2011)	0.1 mgL^{-1}								2 hours	S											
			1 mgL^{-1}								2 hours	S											
			10 mgL^{-1}								2 hours	S											
Zero-valent nanoiron (unstabilised)	Mollusca	*Mytilus galloprovincialis* (Kadar *et al*., 2011)	0.1 mgL^{-1}								2 hours	S											
			1 mgL^{-1}								2 hours	S											
			10 mgL^{-1}								2 hours	S											
Zinc	*Mollusca*	*Gomphina veneriformis* (Ju *et al.*, 2009)	0.64 mgL^{-1}								24 weeks	A											
			1.07 mgL^{-1}								24 weeks	A											
			1.79 mgL^{-1}								24 weeks	A											
		Tegillarca granosa (Liu *et al.*, 2014)	0.84 μgL^{-1}								30 days	A											
			1.68 μgL^{-1}								30 days	A											
			3.36 μgL^{-1}								30 days	A											

Notes: A= adult; S= sperm; conc.= concentration; mins= minutes. When sperm swimming performance has been assessed at multiple time-points the significance of treatments effects at the final time-point in comparison to the control has been included in the table. [A] Concentrations (mgL^{-1}) were calculated from the seawater salinity, seawater temperature and metal concentration (ppm) reported by the study, if omitted temperature was assumed to be 20 °C and salinity to be 30 salinity units. [B] Concentrations (mgL^{-1}) were calculated from the reported molar concentrations and the molecular weight of the metal salt (copper sulphate pentahydrate).

Table S1-6. The methodology and results of studies that have investigated the influence of pesticides for freely spawned sperm.

Pesticide	Taxa	Species	Conc. (units)	Conc. (order of magnitude)									Duration (units)	Life stage exposed	Endpoint						
				gL^{-1}		mgL^{-1}			μgL^{-1}						Sperm						
				0.1	0.01	1	0.1	0.01	1	0.1	0.01	0.001			Viability	DNA damage	Membrane integrity	ATP content/activity	Mitochondrial activity	Acrosome reacted (%)
Bayluscide	Echinodermata	*Lytechinus variegatus* (Favret and Lynn, 2010)	0.2 μgL^{-1}							■			20 mins	S						
			1 μgL^{-1}						■				20 mins	S						
			3.9 μgL^{-1}						■				20 mins	S						
			15.6 μgL^{-1}					■					20 mins	S						
			62.5 μgL^{-1}					■					20 mins	S						
			250 μgL^{-1}				■						20 mins	S						
			1 mgL^{-1}			■							20 mins	S						
	Mollusca	*Crassostrea virginica* (Favret and Lynn, 2010)	0.2 μgL^{-1}							■			20 mins	S						
			1 μgL^{-1}						■				20 mins	S						
			3.9 μgL^{-1}						■				20 mins	S						
			15.6 μgL^{-1}					■					20 mins	S						
			62.5 μgL^{-1}					■					20 mins	S						
			0.3 mgL^{-1}				■						20 mins	S						
			1 mgL^{-1}			■							20 mins	S						
Diuron	Mollusca	*Crassostrea gigas* (Akcha *et al.*, 2012)	50 ngL^{-1}								■		1 hour	S						
			0.1 μgL^{-1}							■			1 hour	S						
			0.25 μgL^{-1}							■			1 hour	S						
			0.5 μgL^{-1}							■			1 hour	S						
Glyphosate	Mollusca	*Crassostrea gigas* (Akcha *et al.*, 2012)	0.5 μgL^{-1}							■			1 hour	S						
			1 μgL^{-1}						■				1 hour	S						
			1.5 μgL^{-1}						■				1 hour	S						
			2.5 μgL^{-1}						■				1 hour	S						
			5 μgL^{-1}						■				1 hour	S						

Round-up	Echinodermata	*Lytechinus variegatus* (Favret and Lynn, 2010)	0.3 mgL^{-1}										20 mins	S						
			1 mgL^{-1}										20 mins	S						
			4 mgL^{-1}										20 mins	S						
			16 mgL^{-1}										20 mins	S						
	Mollusca	*Crassostrea gigas* (Akcha *et al*., 2012)	0.5 μgL^{-1}										1 hour	S						
			1 μgL^{-1}										1 hour	S						
			1.5 μgL^{-1}										1 hour	S						
			2.5 μgL^{-1}										1 hour	S						
			5 μgL^{-1}										1 hour	S						
		Crassostrea virginica (Favret and Lynn, 2010)	0.3 mgL^{-1}										20 mins	S						
			1 mgL^{-1}										20 mins	S						
			4 mgL^{-1}										20 mins	S						
			16 mgL^{-1}										20 mins	S						

Notes: A= adult; S= sperm; conc.= concentration; mins= minutes

Table S1-7. The methodology and results of studies that have investigated the influence of endocrine disrupting chemicals (EDCs) and pharmaceuticals for freely spawned sperm.

EDC	Taxa	Species	Conc. (units)	Conc. (order of magnitude)									Duration (units)	Life stage exposed	Endpoint							
				gL^{-1}		mgL^{-1}			μgL^{-1}						Gonad			Ejaculate	Sperm			
				0.1	0.01	1	0.1	0.01	1	0.1	0.01	0.001			Index	Condition	Oxidative damage	Number	DNA damage	Motility (%)	Longevity
Benzo(a) pyrene (B[a]P)	Mollusca	*Chlamys farreri* (Tian *et al.*, 2015)	25 ngL^{-1}								■		10 days	A			■	■			
			0.5 μgL^{-1}							■			10 days	A			■	■			
			10 μgL^{-1}					■					10 days	A			■	■			
		Mytilus edulis (Lewis and Galloway, 2009)	10 μgL^{-1}					■					72 hours	A					■		
			0.1 mgL^{-1}				■						72 hours	A					■		
			1 mgL^{-1}			■							72 hours	A					■		
Bisphenol-A	Mollusca	*Mytilus edulis* (Ortiz-Zarragoitia and Cajaraville, 2006)	51.3 μgL^{-1A}					■					3 weeks	A	■						
Cyproterone acetate (CPA)	Echinodermata	*Antedon mediterranea* (Sugni *et al.*, 2010)	30 ngL^{-1}								■		2 weeks	A	■						
			300 ngL^{-1}							■			2 weeks	A	■						
			3 μgL^{-1}						■				2 weeks	A	■						
		Paracentrotus lividus (Sugni *et al.*, 2010)	30 ngL^{-1}								■		2 weeks	A	■						
			300 ngL^{-1}							■			2 weeks	A	■						
			3 μgL^{-1}						■				2 weeks	A	■						
p,p'-DDE (DDE)	Echinodermata	*Antedon mediterranea* (Sugni *et al.*, 2010)	100 ngL^{-1}							■			2 weeks	A	■						
			500 ngL^{-1}							■			2 weeks	A	■						
			2.5 μgL^{-1}						■				2 weeks	A	■						
p,p'-DDE (DDE) (cont.)	Echinodermata (cont.)	*See below Paracentrotus lividus* (Sugni *et al.*, 2010)	100 ngL^{-1}							■			2 weeks	A	■						
			500 ngL^{-1}							■			2 weeks	A	■						
			2.5 μgL^{-1}						■				2 weeks	A	■						
17β-estradiol	Cnidaria	*Montipora capitate* (Tarrant *et al.*, 2004)	18 to 100 ngL^{-1}							■	■		3 weeks	A				■			

	Echinodermata	*Dendraster excentricus* (Rempel *et al.*, 2009)	0.1 mgL^{-1}										40 mins	S							
			1 mgL^{-1}										40 mins	S							
			10 mgL^{-1}										40 mins	S							
Fenarimol (FEN)	Echinodermata	*Antedon mediterranea* (Sugni *et al.*, 2010)	24 ngL^{-1}										2 weeks	A							
			240 ngL^{-1}										2 weeks	A							
			2.4 μgL^{-1}										2 weeks	A							
		Paracentrotus lividus (Sugni *et al.*, 2010)	24 ngL^{-1}										2 weeks	A							
			240 ngL^{-1}										2 weeks	A							
			2.4 μgL^{-1}										2 weeks	A							
4-hydroxyestradiol (4OHE2)	Echinodermata	*Dendraster excentricus* (Rempel *et al.*, 2009)	1 μgL^{-1}										40 mins	S							
			0.1 mgL^{-1}										40 mins	S							
			10 mgL^{-1}										40 mins	S							
Methyltestoster-one (MET)	Echinodermata	*Antedon mediterranea* (Sugni *et al.*, 2010)	10 ngL^{-1}										2 weeks	A							
			100 ngL^{-1}										2 weeks	A							
			1 μgL^{-1}										2 weeks	A							
		Paracentrotus lividus (Sugni *et al.*, 2010)	10 ngL^{-1}										2 weeks	A							
			100 ngL^{-1}										2 weeks	A							
			1 μgL^{-1}										2 weeks	A							
PCB (mixture)	Echinodermata	*Asterias rubens* (Den Besten *et al.*, 1990)	19.89 $\mu g\ g^{-1B}$										7 months	A							
Tributylin (TBT)	Mollusca	*Gomphina veneriformis* (Park *et al.*, 2012)	0.1 μgL^{-1}										28 weeks	A							
			1 μgL^{-1}										28 weeks	A							
			20 μgL^{-1}										28 weeks	A							
		Gomphina veneriformis (Park *et al.*, 2015)	0.4 μgL^{-1}										36 weeks	A							
			0.6 μgL^{-1}										36 weeks	A							
			0.8 μgL^{-1}										36 weeks	A							
		Mytilus galloprovincialis (Mazzei *et al.*, 2015)	0.1 μgL^{-1A}										1 hour (longevity) 10 mins (motility)	S							
			1 μgL^{-1A}											S							
			10.2 μgL^{-1A}											S							
			0.1 mgL^{-1A}											S							
			1 mgL^{-1A}											S							
Tributylin (cont.)	Mollusca (cont.)	*Mytilus galloprovincialis* (cont.)	10.2 mgL^{-1A}											S							
			102.1 mgL^{-1A}											S							
Triphenyltin (TPT)	Echinodermata	*Antedon mediterranea* (Sugni *et al.*, 2010)	100 ngL^{-1}										2 weeks	A							
			225 ngL^{-1}										2 weeks	A							

			500 ngL^{-1}									2 weeks	A							
		Paracentrotus lividus (Sugni *et al*., 2010)	100 ngL^{-1}									2 weeks	A							
			225 ngL^{-1}									2 weeks	A							
			500 ngL^{-1}									2 weeks	A							

Notes:

A= adult; S= sperm; conc.= concentration; mins= minutes

[A] Concentrations (mgL^{-1}) were calculated from the seawater salinity, seawater temperature and concentration (ppm) reported by the study, if omitted temperature was assumed to be 20 °C and salinity to be 30 salinity units. [B] Concentration measured in mussel tissues (mussels exposed to the PCB mixture for 1 month and fed to starfish during the experimental exposure).

Table S1-8. The methodology and results of studies that have investigated the influence of organic compounds for freely spawned sperm.

Organic compound	Taxa	Species	Conc. (units)	Conc. (order of magnitude)									Duration (units)	Life stage exposed	Endpoint								
				gL^{-1}		mgL^{-1}			μgL^{-1}						Gonad		Sperm						
				0.1	0.01	1	0.1	0.01	1	0.1	0.01	0.001			Index	Weight	Morphology	ATP content/activity	Speed (VCL)	Speed (VSL)	Speed (VAP)	Trajectory (linearity)	Motile (%)
DAP	Mollusca	*Mytilus edulis* (Ortiz-Zarragoitia and Cajaraville, 2006)	51.3 μgL^{-1A}					■					3 weeks	A									
DMP	Mollusca	*Haliotis diversicolor supertexta* (Zhou *et al*., 2011)	1 μgL^{-1}						■				1 hour	S									
			10.2 μgL^{-1}					■					1 hour	S				■					
			102 μgL^{-1}				■						1 hour	S				■					
Nonylphenol	Mollusca	*Crassostrea gigas* (Nice, 2005)	1 μgL^{-1}						■				72 hours (A) grown to sexual maturity in control conditions	A									■
			100 μgL^{-1}				■							A									■
PAH (mixture)	Mollusca	*Crassostrea gigas* (Jeong and Cho, 2005)	204.2 μgL^{-1A}				■						30 days	A								■	
			51.1 μgL^{-1A}					■					150 mins	S					■	■			■
			102.1 μgL^{-1A}				■						150 mins	S					■	■			■
			204.2 μgL^{-1A}				■						150 mins	S					■	■			■
Phenanthrene	Echinodermata	*Psammechinus miliaris* (Schäfer *et al.*, 2011)	5 μgL^{-1}						■				10 days	A									
			150 μgL^{-1}				■						10 days	A									
Phenol	Echinodermata	*Anthiocidaris crassipina* (Au *et al*., 2000)	10.2 mgL^{-1A}		■								1 hour	S									
			51 mgL^{-1A}		■								1 hour	S									
			102 mgL^{-1A}	■									1 hour	S									
			510 mgL^{-1}	■									1 hour	S									
		Anthiocidaris	0.1 mgL^{-1}				■						4 weeks	A									

Phenol (cont.)		*crassipina* (Au *et al*., 2003)	10 mgL^{-1}										4 weeks	A								
	Mollusca	*Perna viridis* (Au *et al*., 2000)	10.2 mgL^{-1A}										1 hour	S								
			51 mgL^{-1A}										1 hour	S								
			102 mgL^{-1A}										1 hour	S								
			510 mgL^{-1A}										1 hour	S								
Tetrabromod-iphenylether	Mollusca	*Mytilus edulis* (Ortiz-Zarragoitia and Cajaraville, 2006)	5.1 μgL^{-1A}										3 weeks	A								

Notes:

A= adult; S= sperm; conc.= concentration; mins= minutes

[A] Concentrations (mgL^{-1}) were calculated from the seawater salinity, seawater temperature and organic compound concentration (ppm) reported by the study, if omitted temperature was assumed to be 20 °C and salinity to be 30 salinity units.

Table S1-9. The methodology and results of studies that have investigated the influence of oil spill relevant exposures for freely spawned sperm.

Taxa	Species	Oil spill component	Conc. (units)	Conc. (order of magnitude)									Duration (units)	Life stage exposed	Endpoint				
				gL^{-1}		mgL^{-1}			μgL^{-1}						Gonad	Sperm			
				0.1	0.01	1	0.1	0.01	1	0.1	0.01	0.001			Index	Viability	Acrosomal activity	ROS production	MMP
Echinodermata	*Asterias rubens* (Joly-Turquin *et al.*, 2009)	Erika oil spill samples	3.4 $\mu g\ g^{-1}$ [A]										3 months	A					
Mollusca	*Chlamys islandica* (Frantzen *et al.*, 2016)	Mechanically dispersed North sea crude oil	3.3 mgL^{-1}			■							48 hours, followed by 71 days in control conditions	A					
			66.7 mgL^{-1}		■									A					
			0.33 gL^{-1}	■										A					
		North sea crude oil + dispersant (Dasic NS)	3.3 mgL^{-1} (+13.3 mgL^{-1} dispersant)			■								A					
			66.7 mgL^{-1} (+13.3 mgL^{-1} dispersant)		■									A					
			0.33 gL^{-1} (+13.3 mgL^{-1} dispersant)	■										A					
		Oil dispersant (Dasic NS)	13.3 mgL^{-1}		■									A					
	Crassostrea virginica (Volety *et al.*, 2016)	Mechanically dispersed Deepwater Horizon surface oil	31.25 mgL^{-1}		■								30 mins	S					
			62.5 mgL^{-1}		■								30 mins	S					
			125 mgL^{-1}	■									30 mins	S					
			250 mgL^{-1}	■									30 mins	S					
			500 mgL^{-1}	■									30 mins	S					
		Deepwater Horizon surface oil + dispersant (Corexit 9500A)	6.25 mgL^{-1} (+0.63 mgL^{-1} dispersant)			■							30 mins	S					
			12.5 mgL^{-1} (+1.25		■								30 mins	S					

Mollusca (cont.)	*Crassostrea virginica* (cont.)	Deepwater Horizon surface oil + dispersant (cont.)	mgL^{-1} dispersant)																
			25 mgL^{-1} (+2.5 mgL^{-1} dispersant)										30 mins	S					
			50 mgL^{-1} (+5 mgL^{-1} dispersant)										30 mins	S					
			100 mgL^{-1} (+10 mgL^{-1} dispersant)										30 mins	S					
		Oil dispersant (Corexit 9500A)	0.63 mgL^{-1}										30 mins	S					
			1.25 mgL^{-1}										30 mins	S					
			2.5 mgL^{-1}										30 mins	S					
			5 mgL^{-1}										30 mins	S					
			10 mgL^{-1}										30 mins	S					

Notes:

A= adult; S= sperm; conc.= concentration; mins= minutes, MMP= mitochondrial membrane potential.

[A] Concentration measured in mussel tissues (mussels exposed to an Erika oil spill sample for 3 days) frozen and fed to experimental animals for the duration of the experiment.

Table S1-10. The methodology and results of studies that have investigated the influence of other pollutants for freely spawned sperm.

Pollutant	Taxa	Species	Conc. (units)	Conc. (order of magnitude)									Duration (units)	Life stage exposed	Endpoint				
				gL^{-1}		mgL^{-1}			μgL^{-1}						Gonad	Sperm			
				0.1	0.01	1	0.1	0.01	1	0.1	0.01	0.001			Condition	Morphology	Speed (VAP)	Motile (%)	Longevity
DBT	Mollusca	*Mytilus galloprovincialis* (Mazzei *et al*., 2015)	0.1 μgL^{-1A}							■			1 hour (longevity) 10 mins (motility)	S				■	■
			1 μgL^{-1A}						■					S				■	■
			10.2 μgL^{-1A}					■						S				■	■
			0.1 mgL^{-1A}				■							S				■	■
			1 mgL^{-1A}			■								S				■	■
			10.2 mgL^{-1A}		■									S				■	■
			102.1 mgL^{-1A}	■										S				■	■
Polystyrene microplastics (2 and 6 µm)		*Crassostrea gigas* (Sussarellu *et al*., 2016)	23 μgL^{-1}					■					2 months	A			■	■	
2,3,7,8-TCDD		*Crassostrea virginica* (Wintermyer and Cooper, 2007)	2 $ngKg^{-1}$ (injected)										28 days	A	■	■			
			10 $ngKg^{-1}$ (injected)										28 days	A	■	■			

Notes:

A= adult; S= sperm; conc.= concentration; mins= minutes.

[A] Concentrations (mgL^{-1}) were calculated from the seawater salinity, seawater temperature and pollutant concentration (ppm) reported by the study, if omitted temperature was assumed to be 20 °C and salinity to be 30 salinity units.

Table S1-11. The methodology and results of studies that have conducted field pollution studies to investigate freely spawned sperm endpoints.

Taxa	Species	Location	Field sites	Control	Suspected contaminant(s)	Difference in concentration from control site (if measured)	Duration (units)	Life stage exposed	Endpoint		
									Gonad		
									Maturation	Index	Weight
Echinodermata	*Strongylocentrotus purpuratus* (Krause, 1994)	Carpinteria, California, USA	5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100 m from effluent discharge	1000 m from effluent discharge	Oil production effluent (a variable mixture of metal and organic pollutants)	Not measured	8 weeks	A			
Mollusca	*Mytilus edulis* (Zorita *et al*., 2006)	Visnes, Norway	Site 3 (Farther from the mine)	Reference site (no further details)	Copper mine (Copper)	Not measured for seawater. But chemical analysis of mussel tissue (+0.65 µg Cu g^{-1} dry weight)	3 weeks	A			
			Site 2 (close to the mine)	Reference site (no further details)	Copper mine (Copper)	Not measured in seawater. But chemical analysis of mussel tissue (+10.87 µg Cu g^{-1} dry weight)	3 weeks	A			
			Site 1 (inside the mine)	Reference site (no further details)	Copper mine (Copper)	Not measured in seawater. But chemical analysis of mussel tissue (+46.04 µg Cu g^{-1} dry weight)	3 weeks	A			
	Mytilus galloprovincialis (Zorita *et al.*, 2015)	Oiartzun estuary, Bay of Biscay,	Intermediate part of estuary	Outer estuary	Shipyard and coal fired power station (a mixture of metal and organic pollutants)	Not measured in seawater. But chemical analysis of mussel tissue (+3.38 Cu, +0.15 Ni, +0.06 As, +21 Zn, +8.3 ΣDDTs, +263.4 ΣPAHs: all values provided in µg g^{-1}	1 month	A			

Mollusca (cont.)	*Mytilus galloprovincialis* (cont.)	Spain Oiartzun estuary (cont.)				dry tissue)[A]					
			Inner estuary close to a shipyard and coal fired power station	Outer estuary	Shipyard and coal fired power station (a mixture of metal and organic pollutants)	Not measured in seawater. But chemical analysis of mussel tissue (+0.23 Cd, +3.25 Cu, +0.3 Cr, +0.32 Ni, +0.20 Pb, +0.26 As, +108 Zn, +17.1 ΣDDTs, +185.4 ΣPAHs: all measured in µg g^{-1} dry tissue)[A]	1 month	A			
	Saccostrea glomerata (Andrew-Priestley *et al.*, 2012)	Newcastle, Australia	100-150 m from effluent outfall	Two reference locations	Sewage effluent outfall (Estrogenic compounds)	Not measured in seawater or oyster tissue but analysis of the effluent confirmed the presence of 8 estrogenic compounds including E2, estrone and bisphenol-A.	6 weeks				
			<50 m from effluent outfall	Two reference locations	Sewage effluent outfall (Estrogenic compounds)		6 weeks				

Notes:

A= adult; S= sperm; Cd= cadmium; Cu= copper; Cr= chromium; Ni= Nickel; Pb= lead; As= arsenic; Zn= zinc; ΣDDTs= total concentration of dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyldichloroethane (DDD); ΣPAHs = total concentration of tested polycyclic aromatic hydrocarbons including naphthalene, acenaphthylene, acenaphthene, fluorene, phenenathrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(e)pyrene, benzo(a)pyrene, perilene, indenol(1,2,3-cd)pyrene, dibenzo(a,h)anthracene and benzo(g,h,i)perylene.

[A] The metals presented here are elevated by ≥ 0.05 mg Kg^{-1} dry tissue between animals exposed at the treatment and control sites. Other metals were analysed but there was found to be > 0.05 mg Kg^{-1} dry tissue difference.

Table S1-12. The methodology and results of studies that have investigated the influence of interactions between anthropogenic drivers for freely spawned sperm.

Taxa	**Species**	Variable 1	**Treatment relative to the control (units)**	Life stage exposed	Variable 2	**Treatment relative to the control (units)**	Life stage exposed	**Duration (units)**	**Endpoint**				
									Gonad		Sperm		
									Index	Maturation	DNA damage	Speed (VCL)	Motile (%)
Echinodermata	*Echinometra sp. A* (Uthicke *et al.*, 2014)	OA	-0.19 pH units and +342 µatm pCO_2	A	Temperature	+3 °C	A	77 days					
	Paracentrotus lividus (Graham *et al.*, 2015)	OA	-0.15 pH units and +239 µatm pCO_2	A	Hypoxia	-136 µatm pO_2 (-72%)	S	6 months (A); 1 hour (S)					
	Psammechinus miliaris (Caldwell *et al*., 2011a)	OA	-0.11, -0.24 and -0.39 pH units	S	Temperature	+3 and +6 °C	S	30 mins					
Polychaeta	*Arenicola marina* (Campbell *et al*., 2014)	OA	-0.51 pH units and +1127 µatm pCO_2	S	Heavy metal (copper)	13 μgL^{-1A}	S	10 mins (swimming) 1 hour (DNA damage)					
		OA	-0.51 pH units and +1127 µatm pCO_2	S	Heavy metal (copper)	130 μgL^{-1A}	S	10 mins (swimming) 1 hour (DNA damage)					
		OA	-0.51 pH units and +1127 µatm pCO_2	S	Heavy metal (copper)	1.2 mgL^{-1A}	S	10 mins (swimming) 1 hour (DNA damage)					
		OA	-0.81 pH units and +2950 µatm pCO_2	S	Heavy metal (copper)	13 μgL^{-1A}	S	10 mins (swimming) 1 hour (DNA damage)					
		OA	-0.81 pH units and +2950 µatm pCO_2	S	Heavy metal (copper)	130 μgL^{-1A}	S	10 mins (swimming) 1 hour (DNA damage)					
		OA	-0.81 pH units and +2950 µatm pCO_2	S	Heavy metal (copper)	1.2 mgL^{-1A}	S	10 mins (swimming) 1 hour (DNA damage)					

Notes: A= adult; S= sperm; mins= minutes. Treatment is calculated from the treatment level minus the control level. The significance provided is in relation to the interaction only; when interactions are not significant (allowing main effects to be interpreted independently) the findings can be found in the tables relevant to the individual variables. If a study has omitted a post-hoc test to confirm which treatment levels are significantly different from the control (when significant differences are found) they are presented in the same row along with the outcome of the statistical analysis. [A] Concentrations (mgL^{-1}) were calculated from the reported molar concentrations and the molecular weight of the metal salt (copper sulphate pentahydrate).

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Research paper II 3

Ocean acidification increases copper toxicity to the early life history stages of the polychaete *Arenicola marina* in artificial seawater

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Ocean acidification increases copper toxicity to the early life history stages of the polychaete *Arenicola marina* in artificial seawater

3.1 ABSTRACT

The speciation and therefore bioavailability of the common pollutant copper is predicted to increase within the pH range anticipated under near-future ocean acidification (OA), hence the potential exists for copper toxicity to marine organisms to also increase. We investigated the impact of OA (seawater pH values of 7.77 (pCO_2 1400 µatm) and 7.47 (pCO_2 3000 µatm)) upon copper toxicity responses in early life history stages of the polychaete *Arenicola marina* and found both synergistic and additive toxicity effects of combined exposures depending on life history stage. The toxicity of copper on sperm DNA damage and early larval survivorship was synergistically increased under OA conditions. Larval survival was reduced by 24 % when exposed to both OA and copper combined compared to single OA or copper exposures. Sperm motility was negatively affected by both OA and copper singularly with additive toxicity effects of the two stressors when combined. Fertilization success was also negatively affected by both OA and copper individually, but no additive effects when exposed as combined stressors were present for this stage. These findings add to the growing body of evidence that OA will act to increase the toxicity of copper to marine organisms, which has clear implications for coastal benthic ecosystems suffering chronic metal pollution as pCO_2 levels rise and drive a reduction in seawater pH.

3.2 INTRODUCTION

Ocean acidification (OA), the change in pH and carbonate chemistry of the world's oceans as a result of increasing atmospheric concentrations of carbon dioxide (CO_2; Feely *et al.*, 2009), is now broadly considered to represent a major threat to global marine biodiversity (Halpern *et al.*, 2008; Doney *et al.*, 2009; Dupont and Pörtner, 2013). Recent projections forming part of the Representative Concentration Pathways (RCP) see atmospheric pCO_2 surpassing 1000 µatm shortly into the next century (RCP 8.5; Riahi *et al.*, 2011). Average ocean surface pH has already dropped by 0.1 units since the industrial revolution, indicating a rise of 30 % in seawater H^+ concentration (Ericson *et al.*, 2012), with climate models projecting a further decrease by as much as 0.43 units by the end of this century to a global average pH of around 7.73 (Bao *et al.*, 2012). A wide body of evidence now suggests that this change in ocean chemistry has the potential to negatively influence the health and fitness of a wide range of marine invertebrate species and life history stages (Dupont *et al.*, 2010b; Kroeker *et al.*, 2010; Doney *et al.*, 2009; Ross *et al.*, 2011).

OA is not happening in isolation and marine habitats face a wide array of current and evolving threats from multiple anthropogenic stressors. Pollution of the marine environment is a continued and growing global issue with metals in particular known to be persistent ubiquitous contaminants of a wide range of habitats (Luoma *et al.*, 2008; Rogers and Laffoley, 2011). Copper remains a common contaminant in coastal waters (Rogers and Laffoley, 2011), with concentrations ranging from 0.004 µM (Jones and Bolam, 2007) to in excess of 1.6 µM in highly contaminated areas (USEPA, 2007) with the 76/464/EEC directive environmental quality standard (EQS) set at 0.08 µM. Whilst copper is a naturally occurring trace element essential for some biological functions, elevated levels can be toxic to a range of marine organisms (Viarengo, 1989; Fitzpatrick *et al.*, 2008). Metals, such as copper, exert toxicity via the production of reactive oxygen species (ROS). At elevated levels these can overwhelm an organism's antioxidant defences and induce oxidative damage of cellular components such as proteins, lipids

and DNA (Krumschnabel *et al.*, 2005; Lushchak, 2011) or directly through the covalent binding of free ionic copper to macromolecules.

The behaviour, speciation and therefore bioavailability of many heavy metals in seawater is strongly dependant on seawater chemistry, with a number of metals known to be sensitive to speciation changes within the pH range projected for near-future OA (Richards *et al.*, 2011; Byrne *et al.*, 1988). OA may also alter the behaviour of metals bound to sediments, influencing metal fluxes from contaminated sediments (Roberts *et al.*, 2013). OA is predicted to increase the toxic free ion concentration of copper (Cu^{2+}) in coastal waters by as much as 115 % in the next 100 years (Richards *et al.*, 2011). In addition, the inorganic speciation of copper is dominated by complexation to the carbonate ion (CO_3^{2-}) which will reduce under OA conditions (Byrne *et al.*, 1988). Hence it might be predicted that the toxicity of copper to marine organisms will increase under near-future OA (Millero *et al.*, 2009). Only a few studies to date have investigated this potential for altered bioavailability and/or toxicity of metals under near-future projections for seawater pH. Bioaccumulation of a number of trace metals was found to be altered in the eggs of the squid *Loligo vulgaris* and the cuttlefish *Sepia officinalis* under elevated pCO_2, such that accumulation of some metals increased whilst others decreased (Lacoue-Labarthe *et al.*, 2011; Lacoue-Labarthe *et al.*, 2009). Roberts *et al.* (2013) identified a 2.7 fold increase in toxicity, measured as DNA damage, induced by exposures to field collected contaminated sediment at elevated pCO_2 (750 µatm, pH 7.90) in the amphipod *Corophium volutator*. Elevated pCO_2 has also been shown to increase the sensitivity of larvae to copper in the polychaete *Pomatoceros lamarckii* (Lewis *et al.*, 2012) and decrease larval production rates in the copepod *Tisbe battagliai* (Fitzer *et al.*, 2013).

The early life history stages of marine invertebrates are generally much more sensitive to environmental stressors than their adult forms (Xie *et al.*, 2005), hence are likely to form the bottlenecks in any population experiencing environmental stress. As such they are regularly used for regulatory toxicity testing, with the US EPA's standardised water toxicity tests including a sea urchin and sand dollar fertilisation assay (Anderson, 1990). Fertilisation and sperm motility are known to be sensitive to copper, with responses ranging from a slight increase in sperm swimming speeds under very low doses (Young

and Nelson, 1974) to reduced sperm swimming speeds and fertilisation success at elevated copper concentrations (Caldwell *et al.*, 2011b). For example sea urchin fertilisation appears to be generally quite sensitive to copper exposure with reported EC_{50}s of between 1.9 - 59 μgL^{-1} (Dinnel *et al.*, 1989), whilst other species such as the polychaete *Nereis virens* appear less sensitive, with no observable reduction in fertilisation success below 500 μgL^{-1} (Watson *et al.*, 2008).

The polychaete *Arenicola marina* is an ecologically important benthic species inhabiting intertidal sediments across Northern Europe with important roles as a sediment engineer and prey species for wading birds and fish. Its responses to environmental stressors are therefore of key importance to coastal ecosystems, yet they remain understudied with respect to OA. *A. marina* reproduce by the female spawning eggs into her burrow whilst sperm is shed onto the surface of the sand on an incoming tide, with fertilisation and early larval development thought to occur within the female burrow (Farke and Berghuis, 1979). As such these stages are likely to be exposed to any coastal metal contamination present which often accumulates in sediment and pore waters. Here we investigate the response of the early life history stages of *A. marina* to combined exposure to OA conditions and copper in order to test the hypothesis that copper toxicity will be enhanced at reduced seawater pH.

3.3 METHODS

Animal collection and maintenance

Animals were collected from Mothecombe beach, Devon, UK (50°31''23 N, -3°94''58 W) during November 2013 and assessed for sex and maturity. This site is considered to be relatively 'clean' and free of any significant contamination (Environment Agency, 2007). Males and females were maintained in separate static 16 litre tanks in a temperature controlled room (12 ± 0.5°C) for a minimum of seven days prior to experimental procedures. Tanks were filled with natural sediment collected from Mothecombe and well aerated artificial seawater (ASW, Tropic Marin) made up to a salinity of 35 psu. Salinity was monitored using a Mettler Toledo SG7 SevenGo pro conductivity meter to an accuracy of ± 0.1 psu.

Gamete collection

Spawning was induced in males by injection of 1 ml 8,11,14-eicosatrienoic acid (Sigma Co.) into the coelomic cavity (Pacey and Bentley, 1992). Spawning followed approximately 1 hour after injection with sperm collected 'dry' as it was extruded from the nephromixia to prevent premature activation and stored in micro-centrifuge tubes on ice until use within 4 hours. Females were injected with 1 ml prostomial homogenate (equivalent to one per individual) on the day prior to an experiment and kept overnight in individual crystallising dishes containing 180 ml of ASW filtered to 1 µm. Eggs were collected the following morning and stored in micro-centrifuge tubes in fresh ASW on ice until use within 6 hours of collection. Microlitres of dry sperm were diluted with known volumes of ASW and replicate sperm counts were performed using a Neubauer Haemocytometer. Egg counts were performed on three 1 µl micro-volumes and an average concentration of settled oocytes calculated for each female under a compound microscope.

Water chemistry

ASW was aerated for 2-4 hours prior to an experiment and constituted the ambient seawater pH treatment (pH 8.28, ~400 µatm pCO_2, described in Table 1). Seawater pH values of 7.77 and 7.47 were used to represent near and medium-term ocean acidification treatments (as projected according to scenario RCP 8.5 and the 2013 IPCC WGI AR5 (Stocker *et al.*, 2013), full seawater chemistry is provided in Table 1).

Table 1. Measured and calculated (CO_2SYS Pierrot *et al.*, 2006) seawater parameters for experimental treatments.

pH treatment	Measured parameters				Calculated parameters			
	T (°C)	**S***	**pH_{NBS}**	**DIC (µmol kg^{-1})**	**TA (µmol kg^{-1})**	**pCO_2 (µatm)**	**HCO_3^- (µmol kg^{-1})**	**CO_3^{2-} (µmol kg^{-1})**
8.28[A,B]	12 ± 0.1	35	8.29	2657.2	2976.0	359.5	2399.6	242.9
8.28[C]	12 ± 0.1	35	8.28	2847.9	3175.6	394.9	2576.8	254.9
8.28[D]	12 ± 0.1	35	8.26	2862.8	3176.9	417.5	2600.1	245.5
7.77 [A]	12 ± 0.1	35	7.76	3071.5	3129.0	1486.7	2925.7	74.2
7.77 [B]	12 ± 0.1	35	7.77	2918.3	2978.5	1394.5	2808.1	85.7
7.77 [C]	12 ± 0.1	35	7.76	3060.1	3117.5	1481.2	2912.6	86.8
7.77 [D]	12 ± 0.1	35	7.77	3089.2	3151.1	1459.7	2939.6	89.8
7.47 [A]	12 ± 0.1	35	7.48	3147.5	3092.7	3309.0	2971.5	40.5
7.47 [B]	12 ± 0.1	35	7.46	2986.6	2927.2	2873.1	2826.7	42.2

Notes: * Salinity was adjusted to 35 by blending commercial salt mixture with distilled water.

Used in A: sperm motility analysis, B: sperm comet assay, C: day 1 fertilisations, D: day 2 fertilisations and larval survival assays.

For the short term sperm exposures, ASW was acidified to the desired pH using a computerised control system (NBS scale, AquaMedic, Germany) which regulated pH via a solenoid valve and triggered the injection of pure gaseous CO_2 once seawater pH exceeded pre-programmed levels (+ 0.05) in conjunction with constant vigorous aeration. Seawater pH was monitored with an additional Metrohm (827 pH lab) pH_{NBS} electrode and NBS buffers to ensure water was collected at ± 0.02 the desired pH. The appropriate volume of copper sulphate stock solution (20 mM) was added to seawater pH treatments to give nominal copper concentrations representing natural background levels in coastal systems (0.2 µM; Jones and Bolam, 2007), a polluted site (2 µM; Bryan and Gibbs, 1983) and also a higher concentration (20 µM) found in acute preliminary exposures to induce similar levels of DNA damage as measured in the sperm and somatic cells of polychaetes, including *A. marina*, from chronically polluted field populations (Lewis and Galloway, 2008). This higher concentration was used for mechanistic insight and does not relate to a field-relevant concentration. Seawater samples were collected from experimental treatments for dissolved inorganic carbon (DIC) analysis which was carried out using a custom built system described by Friederich *et al.* (2002) and following the methodology detailed in Lewis *et al.* (2012). This analytical system allowed the measurement of seawater DIC with a precision of ± 3 µM. Total alkalinity (TA) and pCO_2 were calculated using CO2SYS (Pierrot *et al.*, 2006), the parameters selected by Findlay *et al.* (2013), the DIC, pH and salinity measurements using the NBS scale, the K_1 and K_2 values determined by Mehrbach (1973) (values refit by Dickson and Millero (1987)) and K_{SO4} as determined by Dickson (1990).

Sperm motility analysis

Sperm motility was assessed at the full range of seawater pH treatments and copper concentrations by computer assisted sperm analysis (CASA). Two ml of experimental seawater followed by 13 µl of sperm was added to 5 ml vials and repeated for each male (n=5). Vials were gently agitated to mix the sperm-seawater solution and incubated at 12 ± 0.1°C for 10 minutes. Three microlitre volumes of sperm-seawater solutions were immediately transferred to Leja 20 mm standard counting chambers. Motility analysis was carried out using a Microptic Sperm Class Analyser (Microm, UK) fitted with a Nikon Eclipse 50i negative phase contrast microscope (100x magnification and a Peltier cooled

stage) which was operated at 12 ± 0.1°C. Images were captured at a rate of 100 frames per second and individual sperm were tracked for 0.5 seconds. A minimum of 500 sperm were tracked in each sample with data on percent motility and a number of CASA derived motility parameters calculated. Threshold values of 10 μms^{-1} curvilinear velocity (VCL) and 3.2 μms^{-1} average path velocity (VAP) were utilised to remove non-motile sperm from the subsequent analysis and determine percentage sperm motility (i.e. the percentage of sperm with motility parameters recorded above threshold values of the total number analysed). The recommendations of Mortimer *et al.* (1995) on the use of CASA systems in research were followed where applicable to marine invertebrate sperm, e.g. sperm loading times and the avoidance of wall effects, in order to standardise the readings taken.

Comet assay

The comet assay was utilised to measure DNA strand breaks in sperm exposed to seawater pH and copper treatments using methodology previously described by Lewis and Galloway (2009) with minor modifications. A 10 µl aliquot of undiluted sperm from each male (n=8) was added to individual vials containing 1.85 ml of treatment seawater. Sperm-seawater solutions were incubated at 12 ± 0.1°C for 1 hour before being centrifuged for 4 minutes at 7826 g. The excess fluid was removed and the cell concentrate gently mixed with 1 % low melting point agarose heated to 37°C and dropped onto slides previously coated in 1 % high melting point agarose. Briefly, cells were subjected to 2 hours of lysis in alkaline conditions at 5°C, followed by 45 minutes of denaturation in electrophoresis buffer (0.3 M NaOH and 1 mM EDTA), 30 minutes of electrophoresis at 25 V and a final neutralisation step. Slides were stained with SYBR® Safe DNA Gel Stain and examined using fluorescence microscopy (excitation: 502 nm; emission: 530 nm). One hundred cells per slide were analysed using Comet Assay IV (Perceptive Instruments Ltd) to quantify the percentage of DNA in the comet tail (resulting from DNA strand breaks) to approximate the percentage of DNA damage induced in each treatment.

Fertilisations

The fertilisation experiments took place over two days with freshly spawned gametes collected on each day. The gametes from each male and female were completely crossed under each experimental treatment (day 1, n=3 females and n=3 males; day 2, n=3 females and n=2 males). We selected our near-future pH scenario (pH 7.77) and highest copper concentration (20 µM) as the experimental treatments. The volume equivalent to 1000 oocytes from each female was added to individual wells of a 12 well plastic tissue culturing plate containing 5 ml of treatment seawater. Sperm was diluted with seawater of the corresponding treatment to a final concentration of 1 x 10^4 sperm ml^{-1} (measured in the field as the sperm concentration for a spawning population by Williams *et al.* (1997)) and added to the corresponding wells; this was repeated individually for each cross. Each well was gently agitated to maximise sperm-oocyte encounters before being covered and incubated at 12 ± 0.1°C. After 10 minutes the eggs were washed three times in treatment seawater to remove excess sperm and prevent any further fertilisation before incubation for a further 10 hours. Wells were then fixed in 5 % paraformaldehyde in seawater and fertilisation success and developmental stage recorded from samples of fifty oocytes per well. Fertilisation success was calculated as the percentage of viable post-fertilisation stages (determined as having intact fertilisation membranes and no discolouration) recorded from the total number of oocytes scored.

Larval survival

To assess the combined effects of OA and copper on larval survivorship in *A. marina*, sperm from two males was pooled and diluted to a concentration of 1 x 10^4 sperm ml^{-1} before being added to individual beakers (n=3) each containing ambient ASW and 20,000 eggs from one female (3 females used in total). Beakers were covered and incubated at 12 ± 0.1°C for 1 hour to allow fertilisation under ambient conditions and checked for successful fertilisation. A volume equivalent to 7000 fertilised eggs was added to cylindrical 6 cm diameter plastic pots containing 180 ml of treatment seawater. We selected our near-future pH scenario (pH 7.77) and highest copper concentration (20 µM) as the experimental treatments. Pots (n=3) were incubated at 12 ± 0.1°C and either aerated to maintain ambient conditions or received a mixed gas input through a 21 g

hypodermic needle and a GFC mass flow controller to maintain a seawater pH of approximately 7.77 units. Daily pH measurements were taken of randomly selected pots from each treatment using a Metrohm (827 pH lab) pH_{NBS} electrode and NBS buffers and flow rate adjusted accordingly. Five days post-fertilisation 300 embryos or larvae from each pot were scored as to developmental stage and viability. Larval survival was calculated as the percentage of viable larvae from the post-fertilisation stages scored.

Statistical analysis

Linear mixed-effects modelling explored the influence of seawater pH and copper on average sperm VCL including male identity as a random term. The residuals of linear mixed-effects models on arcsine transformed percent motility data were not normally distributed so a generalized linear mixed-effects modelling approach with a binomial error family and logit link was adopted. Proportion sperm motility data was weighted by the number of sperm analysed in each sample and male identity was included as a random term. The percentage DNA damage, fertilisation success and larval survival data were arcsine transformed prior to analyses and linear mixed-effects modelling explored the influence of seawater pH and copper on each response variable. Where appropriate male identity, female identity, pair identity and/or day were included as random effects when considered biologically important. Missing data was omitted from analyses. Any non-significant terms were dropped from models to give the appropriate minimum adequate model (MAM). All parametric models were checked to ensure that residuals were normally distributed. All statistical analyses were conducted using R version 3.02 and the nlme, lme4 and ggplot2 packages (R Core Team, 2013).

3.4 RESULTS

Sperm motility

The swimming speeds of motile sperm ranged from 10.1 to 332.7 μms^{-1} across experimental treatments. Individually copper and reduced seawater pH were found to reduce sperm swimming speeds, measured as average VCL, compared to those under ambient conditions (Figure 1A, see Table SI-1 for the full output from the linear mixed-effects model). This was true for the two highest nominal copper concentrations; 2 µM (t=-3.839, df=49, p<0.001) and 20 µM (t=-3.817, df=49, p<0.001), and at a reduced pH of either 7.77 (t=-5.213, df=49, p<0.001) or 7.47 (t=-4.832, df=49, p<0.001). In combination, motility was reduced by up to 46 % from average speeds of 141.2 μms^{-1} in ambient conditions to as little as 78.3 μms^{-1} as a result of negative additive effects of the two stressors combined. Statistical analysis did not identify an interaction term (Likelihood ratio test, p=0.579) suggesting additive toxicity. Percent motility response ranged from a 1 % enhancement to a 10 % reduction across treatments from an average of 98 % motile sperm under ambient conditions (Figure 1B). We found a slight but significant motility enhancement under near-future OA but there were negative interactions between this pH and each concentration of copper (Table 2). Nominal copper concentrations of 2 and 20 µM reduced percentage motility by up to 4 %. Medium-term OA reduced the percentage of motile sperm by on average 3 % from ambient present-day conditions.

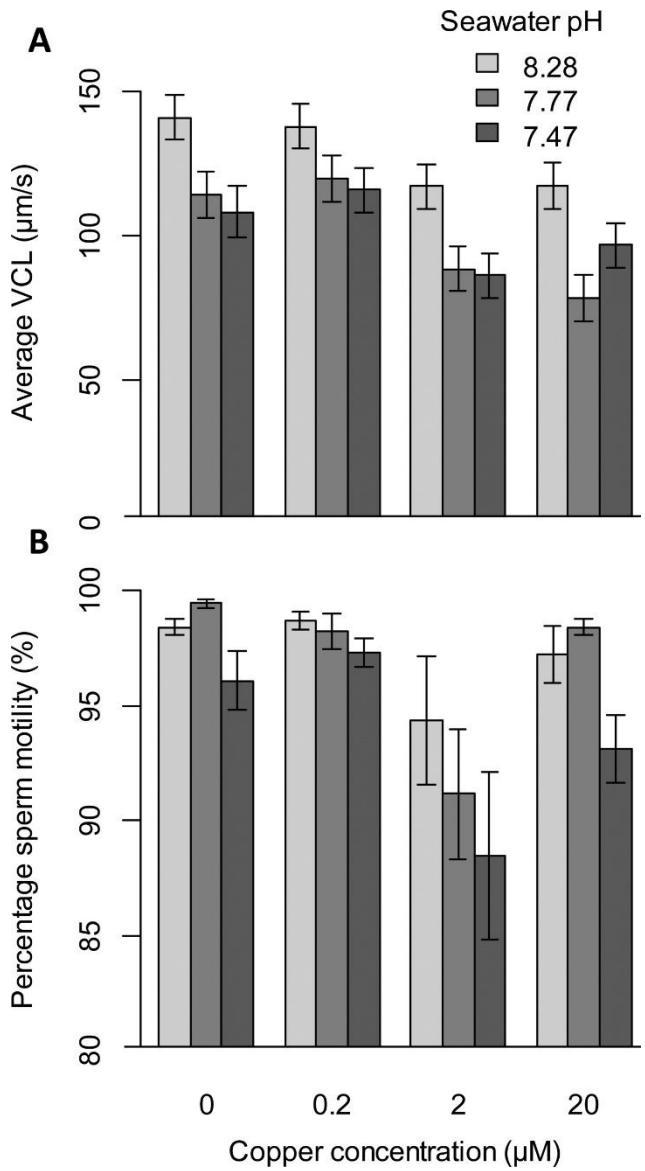

Figure 1. The effects of seawater pCO_2 and copper on (A) spermatozoal swimming velocity and (B) percentage sperm motility in *A. marina* (n=5). Data displayed as fitted group means (A) and group means (B) ± 1 se. Significance levels not included.

Table 2. The results of generalized linear mixed-effects modelling of the influence of seawater pH and copper on the percentage of motile sperm. Male identity was included as a random term and the analysis was weighted by the number of sperm analysed from each sample (significant *p* values ($p \leq 0.05$) are highlighted in bold and all results are in comparison to reference 'control' conditions (pH 8.28, 0 µM copper)).

	Estimate	Std. Error	Z	*p*
Intercept	4.157	0.195	21.303	**<0.001**
(A) pH				
7.77	1.050	0.290	3.636	**<0.001**
7.47	-0.820	0.185	-4.429	**<0.001**
(B) copper (µM)				
0.2	0.243	0.218	1.115	0.265
2	-1.280	0.169	-7.590	**<0.001**
20	-0.566	0.187	-3.019	**0.003**
(C) pH: copper interactions (pH units: µM)				
7.77: 0.2	-1.356	0.357	-3.795	**<0.001**
7.47: 0.2	0.120	0.268	0.447	0.655
7.77: 2	-1.554	0.307	-5.055	**<0.001**
7.47: 2	0.005	0.209	0.025	0.980
7.77: 20	-2.145	0.319	-6.730	**<0.001**
7.47: 20	-0.111	0.229	-0.484	0.629

Sperm DNA damage

There was an increase in spermatozoan DNA damage in the presence of copper across all pH treatments from an average of 14 % damage in our controls to 26 % in copper treatments (Figure 2, the full output from the linear mixed-effects model is presented in Table SI-2). Seawater pH and copper interactively induced sperm DNA damage and subsequent analysis identified that this interaction took place between copper and our lowest seawater pH ($t=4.380$, $df=32$, $p<0.001$). Medium-term OA significantly influenced sperm DNA damage ($t=2.418$, $df= 32$, $p=0.022$).

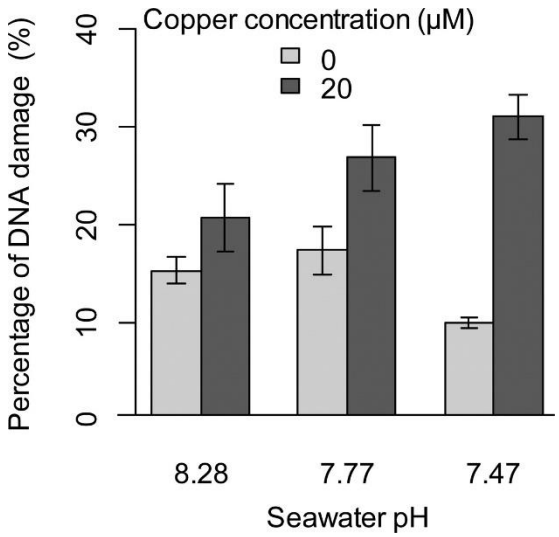

Figure 2. The effects of seawater pCO_2 and copper on the percentage of spermatozoal DNA damage in *A. marina* (n=8). Data displayed as group means ± 1 se. Significance levels not included.

Gamete fertilisation success

We observed an average 96 % fertilisation success in our ambient pH and no copper controls. Both copper exposure (t=-10.239, df=33, p<0.001) and seawater pH reduction (t=-6.146, df=33, p<0.001) significantly reduced fertilisation success (full linear mixed-effects model output is presented in Table SI-3). There was no further reduction in fertilisation success under combined copper and OA exposures, with a near identical percentage success in our two copper treatments irrespective of seawater pH (Figure 3A). Statistical analysis confirmed this to be a statistically significant positive interaction between the two stressors (t=5.037, df=33, p<0.001) rather than any additive or synergistic toxicity.

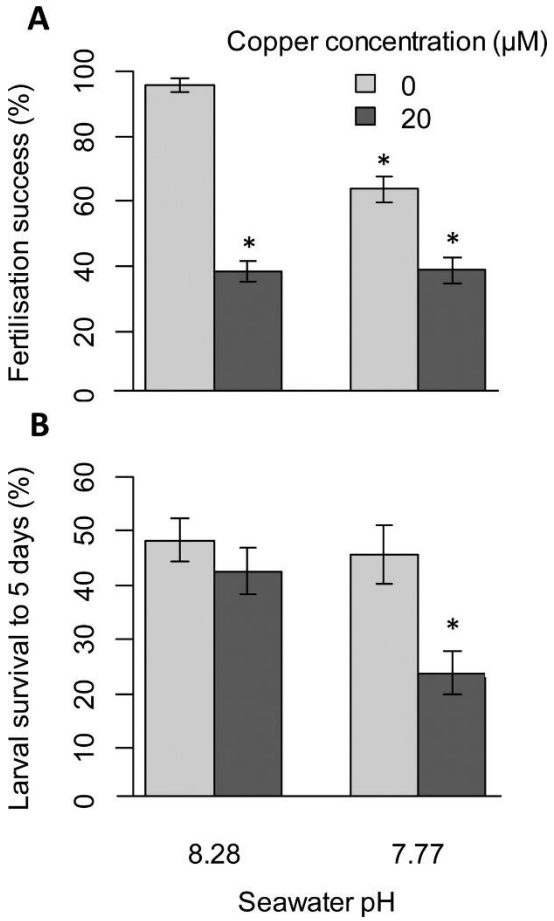

Figure 3. The effects of seawater pCO_2 elevation and copper on (A) fertilisation success and (B) larval survivorship 5 days post-fertilisation in *A. marina*. Data displayed as group means ± 1 se (* indicates a significant difference from success or survival under ambient seawater pCO_2 and no copper ($p \leq 0.05$).

Larval survival

Larval survivorship 5 days after fertilisation had dropped to 48 % in the control treatment of ambient pH and no added copper (Figure 3B). Individually, exposure to copper (t=-1.324, df=6, *p*=0.234) or reduced seawater pH (t=-0.610, df=6, *p*=0.564) had no significant influence on larval survival (full linear mixed-effects model output is presented in Table SI-4). However, a strong significant interaction was found between OA and

copper on larval survival, with a 24 % reduction in larval survival under combined exposures compared to them as individual stressors after 5 days (t=-2.871, df=6, *p*=0.028).

3.5 DISCUSSION

This series of combined OA relevant seawater pH–copper exposure experiments have clearly demonstrated the potential for OA to alter the toxicity of the common contaminant copper to early stages of the polychaete *Arenicola marina*. The influence of OA on copper toxicity varied according to life history stage. A strong negative synergism between OA and copper (i.e. greater effects in combination than the sum of effects of the individual stressors) was measured for sperm DNA damage and early larval survival, with significantly greater toxicity responses measured under reduced seawater pH (either 7.77 or 7.47) for these end points compared to ambient pH levels. Sperm swimming velocities were negatively influenced by both near-future OA and copper concentrations that mimic a heavily polluted coastal site, with additive toxicity effects of the two stressors when exposed in combination. These findings align with previous studies showing a similar influence of near-future ocean acidification on metal-induced DNA damage (Roberts *et al.*, 2013), larval mortalities (Lewis *et al.*, 2012) and larval production (Fitzer *et al.*, 2013) in a number of marine invertebrate species.

Sperm swimming speed and the number of motile sperm are important parameters determining fertilisation success for any broadcast spawning marine invertebrate, particularly when sperm concentrations are low (Levitan, 2000; Hollows *et al.*, 2007). We found that swimming speeds were strongly affected by both OA and copper exposures individually, and these stressors acted additively in combination leading to substantial speed reductions. The percentage of motile sperm (i.e. sperm which have been activated and initiated motility) appears more robust than swimming velocity to near-future OA in this species and was slightly but significantly enhanced in our near-future pH treatment. This disagrees with the majority of studies to date that have directly examined the impacts of reduced seawater pH/ elevated pCO_2 on percentage sperm motility and identified reductions under near-future OA (Nakamura and Morita, 2012; Morita *et al.*, 2010; Schlegel *et al.*, 2012; Havenhand *et al.*, 2008; Vihtakari *et al.*, 2013; Schlegel *et al.*, 2014). The 19 % OA-induced decrease in sperm swimming speeds we observed in *A. marina* was much greater than that measured by Lewis *et al.* (2012) in the tubeworm *Pomatoceros lamarckii* for similar pH levels, with *A. marina* also having much faster

sperm in general. They also exceed the swimming speed reductions reported in a sea urchin and a further polychaete species exposed to near-future OA (Havenhand *et al.*, 2008; Schlegel *et al.*, 2014) suggesting *A. marina* sperm are particularly sensitive to reduced pH/ elevated pCO_2. Our results add to increasing evidence that sperm responses to reduced seawater pH are highly species-specific and can range from decreased sperm velocities (Schlegel *et al.*, 2014) to robust swimming responses (Havenhand and Schlegel, 2009; Havenhand *et al.*, 2008) and even motility enhancement (Caldwell *et al.*, 2011a). Whilst other studies have also shown strong between-male differences within a species (Schlegel *et al.*, 2012; Schlegel *et al.*, 2014) our analysis took this inter-male variation into account in order to identify the influence of our seawater pH treatments across males.

Fertilisation success decreased by 32 % under near-future OA from our control treatment, most likely resulting from fewer gamete collisions as a consequence of reduced sperm swimming speeds. This is comparable to a 20 % reduction in successful fertilisation measured in the sea urchin *Helicidaris erythrogramma* under similar pH conditions (Havenhand *et al.*, 2008) and makes *A. marina* one of the more sensitive marine invertebrate species to OA-induced disruption of fertilisation success. Numerous studies have investigated the impacts of OA on fertilisation success as a single stressor across a wide range of invertebrate species, with conflicting results reported both within and between species (Byrne *et al.*, 2010; Pecorino *et al.*, 2014; Gonzalez-Bernat *et al.*, 2013; Foo *et al.*, 2012). Some studies have reported significant reductions in fertilisation success under near-future OA conditions (Havenhand *et al.*, 2008; Parker *et al.*, 2009; Parker *et al.*, 2010), whilst others have reported fertilisation to be robust to elevated seawater pCO_2 (Ericson *et al.*, 2010; Byrne *et al.*, 2009; Byrne *et al.*, 2010) although there may be differences in fertilisation response between individual mating pairs (Sewell *et al.*, 2013). These differences may be down to true intra-specific differences in OA response, driven by the strong inter-male and inter-species variation in sperm functional response to OA being observed (Schlegel *et al.*, 2014; Schlegel *et al.*, 2012). However, experimental design has also been suggested as a possible source of these differences in fertilisation response to OA (Byrne, 2011; Ross *et al.*, 2011) making interpretation difficult.

Our results are perhaps surprising for an intertidal polychaete, as an emerging paradigm within OA research suggests that any organism experiencing natural variability in pH conditions within their habitat will be more resilient to future OA (Lewis *et al.*, 2013; Moulin *et al.*, 2011). This hypothesis would predict that the gametes of intertidal species would be less sensitive to OA reflecting adaptation to the fluctuating pH conditions they may experience (Byrne *et al.*, 2010; Moulin *et al.*, 2011). In *A. marina* fertilisation takes place within intertidal female burrows on an incoming tide as sperm are washed into the burrows. Information on seawater pH and carbonate chemistry conditions for the intertidal zone in general is poor, however there is evidence that this environment is highly variable in terms of pH and pCO_2 (Artioli *et al.*, 2012; Saderne *et al.*, 2013). Conditions within an *A. marina* burrow at the time of fertilisation have not been measured but would be expected to show strong tidal and seasonal fluctuations, being influenced by the chemistry of the incoming tide and the respiration activity of the animals within the burrow. We cannot currently make any accurate predictions of the pH conditions fertilisation for this species would normally take place under.

Copper acted to strongly reduce fertilisation success irrespective of seawater pH. Copper toxicity to gametes during fertilisation has been reported at a range of environmentally relevant concentrations (as well as much higher concentrations) for a number of marine invertebrate species (Victor and Richmond, 2005; Ringwood, 1992; Reichelt-Brushett and Harrison, 1999). Echinoderms appear to be a more sensitive group of invertebrates, with EC_{50}s reported of between 1.9 - 59 μgL^{-1} (Dinnel *et al.*, 1989), whilst polychaetes appear to be more tolerant and are often found living in contaminated habitats (Bryan and Gibbs, 1983). Caldwell *et al.* (2011b) report an EC_{50} value of 2.2 µM in the polychaete *Nereis virens,* whilst Watson *et al.* (2008) report conflicting sensitivity for *N. virens* observing no reduction in fertilisation success below 500 μgL^{-1} copper (Watson *et al.*, 2008). Hollows *et al.* (2007) identified sperm concentration-specific reductions to fertilisation success at very low copper concentrations (≥0.16 µM) in another polychaete species, *Galeolaria caespitosa*. The reductions we observed are likely to be a result of direct copper toxicity upon processes such as sperm-egg binding or the sperm acrosome reaction which are crucial to fertilisation in a number of marine species (Vacquier and Moy, 1997), or via direct effects upon the oocyte membrane. Surprisingly, given our other results, there was no additive toxicity effect of combined OA and copper on

fertilisation success. We used a relatively high concentration of copper in these experiments to enhance any interaction between OA and copper that may exist from a mechanistic viewpoint; however this may actually have masked more subtle interactions between the two stressors such as those observed in the sperm swimming speed data where additive effects were observed at lower copper concentrations.

More interesting was the strong interaction between OA and copper upon sperm DNA damage observed in our short-term sperm exposures. A similar synergism was observed for somatic cells in the amphipod *Corophium volutator* exposed to metal contaminated sediment under a range of OA conditions (Roberts *et al*., 2013) providing tentative evidence that this pattern may be consistent across cell types and marine invertebrate species. For the same nominal copper exposure we observed a 10 % increase in sperm DNA damage under reduced seawater pH suggestive of increased oxidative stress under combined exposure scenarios. The most parsimonious explanation for this is the predicted increase in the toxic free ion concentration of copper under OA (Pascal *et al*., 2010) enhancing ROS production (Lushchak, 2011). Intriguingly this contrasts with a recent study using isolated mantle cells of the hard clam *Mercenaria mercenaria* which found that, whilst reduced seawater pH increased copper uptake into these cells, ROS generation was attenuated under OA (Ivanina *et al*., 2013). The authors suggested this may be due to an up-regulation of antioxidant proteins, which may explain the different response observed here since sperm are generally considered to be generally lacking in both antioxidant defences and DNA repair enzymes (Aitken *et al*., 2004). Sperm may also be more susceptible to oxidative damage due to the abundance of polyunsaturated fatty acids acting as substrates for ROS (Ivanina *et al.*, 2013). Our exposures were very short-term *in vitro* exposures of one hour with no prior paternal exposure. Sperm are perceived as having very limited DNA repair capability (Donnelly *et al.*, 2000) and thus oxidative DNA damage is likely to accumulate over time. Relatively high copper concentrations were used in our exposures to mimic the accumulated damage over longer chronic exposures measured previously in the sperm of polychaetes from polluted habitats (Lewis and Galloway, 2008). Sperm DNA damage has been shown to have consequences for population fitness, with embryos fathered by sperm with induced DNA damage suffering a higher incidence of severe developmental abnormalities (Lewis and Galloway, 2009).

This strong synergistic toxicity between OA and copper was also present in the early larval survivorship of A*. marina*. Data identifying the point the early larvae of *A. marina* are washed out of the female burrow and enter the water column is not available, but laboratory studies suggest they spend 3-4 days in the water column before returning to the benthic environment just prior to settlement. Larvae are often considered to be the most sensitive life history stage to environmental stressors, particularly in free spawning marine invertebrates with bi-phasic life histories. Whilst *A. marina* larvae were relatively robust to high concentrations of copper and near-future OA as single stressors, combined exposure caused a significant drop in survivorship to half that under ambient conditions after 5 days. This finding parallels earlier work in the intertidal polychaete *P. lamarckii* at an environmentally realistic copper concentration (0.002 µM; Lewis *et al.*, 2012). This synergistic toxicity may also simply be a result of enhanced copper bioavailability at reduced seawater pH, however the biological processes underpinning toxicity responses in larvae will be much more complex than for sperm due to the potential for repair and detoxification processes and energetic trade-offs. Oxidative damage to cellular components (lipids, DNA and proteins) may have overwhelmed larval anti-oxidant defences and the increased energetic costs of expensive DNA repair (Deerenberg *et al.*, 1998) and cellular detoxification may have exceeded larval energy budgets. Alternatively the energetic costs of coping with two stressors simultaneously may have overwhelmed larvae. According to the compensation hypothesis animals under stress will make energetic trade-offs between different physiological energy requiring processes in order to meet elevated energy demands under stress (Deerenberg *et al.*, 1998). Larval development under OA may be energetically expensive due to potential increases in ion regulatory processes (Stumpp *et al.*, 2011), OA compensatory mechanisms (Thomsen and Melzner, 2010), costs to maintaining cellular homeostasis (Lannig *et al.*, 2010) and/or oxidative stress (Tomanek *et al.*, 2011) and energy may have be diverted away from physiological maintenance processes. This in addition to the costs associated with copper detoxification and the repair of cellular oxidative damage may have exceeded an energetic 'tipping point' dramatically influencing larval survival when exposed to both stressors in combination. *A. marina* have lecithotrophic (i.e. non-feeding) larvae, hence have a limited energy reserve with which to reach the settlement stage where feeding commences. This means that as larvae they are not reliant on food supplies, possibly buffering them from single stressors such as OA in isolation (Dupont *et al.*, 2010a). This

may however, make them more sensitive to the additive energetic demands of multiple stressors compared to planktotrophic larvae in an environment with high food availability (or laboratory experiment where animals are fed *ad libitum*), which may be able to compensate the additional energetic costs with increased food intake (as shown by Thomsen *et al.* (2013)). Decreases in early larval survival are likely to negatively affect settlement and juvenile recruitment (Eckman, 1996) with potential consequences for population persistence and size maintenance.

Our results demonstrate the potential for the projected changes in ocean carbonate chemistry under OA to significantly alter the susceptibility of early life history stages of *A. marina* to the common coastal pollutant copper. Short-term 'shock' exposure experiments cannot simply be scaled-up for century-scale responses of organisms to OA, but they do provide us with an understanding of potential mechanisms of impact upon populations that warrant further investigation and that are relevant for looking at how OA might alter contaminant toxicities. Since coastal contamination is widespread this interaction has significant implications both for current predictions of OA impacts upon coastal marine invertebrates and for ecological risk management of marine habitats. There may be other existing and emerging contaminants whose toxicity are influenced by seawater pH and whose effects need to be considered within the context of OA if environmental risk assessments are to work effectively to protect our marine ecosystems over the coming century.

3.6 ACKNOWLEDGEMENTS

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3.7 SUPPORTING INFORMATION

3.7.1 Supporting tables

Table S1-1. The results of linear mixed-effects modelling of the influence of seawater pCO_2 and copper on average sperm curvilinear velocity (male identity was included as a random term, significant *p* values ($p \leq 0.05$) are highlighted in bold and all values relate to comparisons with reference 'control' conditions (pCO_2; 400 µatm, copper; 0 µM)).

	df	Value	Std. Error	t	*p*
Intercept	49	139.763	5.674	24.633	**<0.001**
(A) pCO_2					
1400	49	-28.102	5.390	-5.213	**<0.001**
3000	49	-26.416	5.467	-4.832	**<0.001**
(B) copper					
0.2	49	2.959	6.341	0.467	0.643
2	49	-24.345	6.341	-3.839	**<0.001**
20	49	-24.203	6.341	-3.817	**<0.001**

Table S1-2. The results of linear mixed-effects modelling of the influence of seawater pCO_2 and copper on the percentage of sperm DNA damage (male identity was included as a random term, significant *p* values ($p \leq 0.05$) are highlighted in bold and all values relate to comparisons with reference 'control' conditions (pCO_2; 400 µatm, copper; 0 µM)).

	df	Value	Std. Error	t	*p*
Intercept	32	0.399	0.029	13.674	**<0.001**
(A) pCO_2					
1400	32	0.0172	0.036	0.477	0.636
3000	32	-0.080	0.033	-2.419	**0.022**
(B) copper					
20	32	0.061	0.034	1.784	0.084
(C) pCO_2: copper					
1400:20	32	0.062	0.050	1.237	0.225
3000:20	32	0.209	0.048	4.380	**<0.001**

Table S1-3. The results of linear mixed-effects modelling of the influence of seawater pCO_2 and copper on fertilisation success (pair identity was accounted for as a random term nested within experimental day, significant *p* values ($p \leq 0.05$) are highlighted in bold and all values relate to comparisons with reference 'control' conditions (pCO_2; 400 µatm, copper; 0 µM)).

	df	Value	Std. Error	t	*p*
Intercept	33	1.347	0.061	21.993	**<0.001**
(A) pCO_2					
1400	33	-0.410	0.067	-6.146	**<0.001**
(B) copper					
20	33	-0.684	0.067	-10.239	**<0.001**
(C) pCO_2: copper					
1400:20	33	0.416	0.083	5.037	**<0.001**

Table S1-4. The results of linear mixed-effects modelling of the influence of seawater pCO_2 and copper on larval survival (female identity was accounted for as a random term, significant p values ($p \leq 0.05$) are highlighted in bold and all values relate to comparisons with reference 'control' conditions (pCO_2; 400 µatm, copper; 0 µM)).

	df	Value	Std. Error	t	p
Intercept	6	0.768	0.047	16.243	**<0.001**
(A) pCO_2					
1400	6	-0.027	0.044	-0.610	0.564
(B) copper					
20	6	-0.577	0.044	-1.324	0.234
(C) pCO_2: copper					
1400:20	6	-0.177	0.062	-2.871	**0.028**

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Research paper III 4

Impacts of ocean acidification on sperm accumulate over time

In preparation for submission to: Environmental Science & Technology

Impacts of ocean acidification on sperm accumulate over time

4.1 ABSTRACT

The majority of marine species release eggs and sperm into seawater for external fertilisation. Seawater conditions are currently changing at an unprecedented rate as a consequence of ocean acidification (OA), which will fundamentally alter marine fertilisation environments. Sperm are thought to be particularly vulnerable to these changes and may be exposed to external environmental conditions for variable periods of time between spawning and fertilisation. Yet we currently have a poor understanding of the potential for perturbation of sperm performance to develop or intensify during exposure to OA conditions. Here, we developed a novel exposure technique to control the seawater chemistry of sperm incubations that enabled us to undertake a mechanistic investigation of sperm swimming performance in the coastal polychaete *Arenicola marina* during an extended exposure to simulated OA (pH 7.77, 1000 µatm pCO_2). We found that key fitness-related aspects of sperm functioning declined after four hours under OA, whereas they remained consistently high in ambient conditions for longer. This decrease in the number and speed of motile sperm under OA could not be explained by alterations in sperm oxygen consumption, ATP content or viability but could act to reduce *A. marina* population fertilisation success due to their reproductive strategy. Our findings demonstrate that OA-perturbation of sperm function can develop over time, highlighting the importance of understanding the fertilisation dynamics of natural populations in order to accurately project impacts. As sperm play a central and critical role in reproduction, understanding the impacts of environmental change on sperm function is fundamental to projecting population-level effects.

4.2 INTRODUCTION

The vast majority of marine species reproduce via the ancestral mode; external fertilisation (Wray, 1995; Giese and Kanatani, 1987), whereby eggs and sperm are released into the seawater column to fertilise. Marine external fertilisers may be vulnerable to environmental stressors such as climate change and pollutants, as their reproduction relies upon the successful meeting of gametes in the seawater column (Lewis and Ford, 2012). The gametic phase is often the most sensitive stage in an organism's life (Marshall, 2006): gametes face all the challenges of environmental stressors at a small size and with only limited protective mechanisms. Sperm may be particularly susceptible as they are presumed to have no actively transcribing nuclear genes or biochemistry limiting their ability to respond to adverse environmental conditions through regulation. They also have little capacity to repair DNA damage and lack antioxidant defences or cellular repair mechanisms (Aitken *et al.*, 2004). The environment sperm experience can have far-reaching consequences and influence both fertilisation success and offspring developmental success (Ritchie and Marshall, 2013; Lewis and Galloway, 2009). Sperm have a critical primary function: the transport and transfer of the male genetic contribution to the egg, which is central to fitness in sexually reproducing species. Besides paternal DNA transfer, there is mounting evidence for additional sperm functions including egg activation (Gilbert *et al.*, 1996), mRNA delivery (Sendler *et al.*, 2013) and the origination of the zygote centrosome (Chemes, 2012). Motility is fundamental to a sperm's ability to function. Whilst sperm can be transported and dispersed rapidly in seawater by the action of currents, active swimming is likely to be required to bring sperm into direct contact with the surface of an egg (Kamp *et al.*, 1996). Hence, any environmental stressor that perturbs sperm motility could severely hamper sperm function.

Externally fertilising marine invertebrates tend to be sessile or sedentary and release sperm from their permanent positions. Their sperm remains fertilisation competent from just minutes (Pennington, 1985) after release into seawater to up to several days (Williams and Bentley, 2002). Hence, marine invertebrate sperm may experience an

extended exposure to environmental conditions before fusing with an egg. This differs from most species of marine fish, which tend to spawn in short bursts in close proximity to one another, favouring faster but shorter lived sperm lasting just seconds (Lahnsteiner and Patzner, 1998) to tens of minutes (Cosson *et al.*, 2008). The difficulties in predicting the timing and location of spawning events for most externally fertilising marine invertebrates means they have rarely been studied *in situ,* hence there is very little field data estimating the seawater contact time for successful sperm. Most of our knowledge on fertilisation dynamics comes from laboratory and theoretical studies. Early work by Pennington (1985) and Denny and Shibata (1989) highlighted the potential for rapid sperm dilution below fertilisable concentrations in seawater, hence it was widely accepted that most fertilisation takes place within seconds or minutes of sperm release (Levitan and Petersen, 1995). However, recent studies have challenged this view and proposed several factors that can extend the period before sperm are diluted below a threshold concentration for fertilisation (Yund, 2000). These include the release of sperm in very large numbers (Babcock and Mundy, 1992; Babcock *et al.*, 1994) or in viscous fluids (Thomas, 1994), which act to slow sperm dispersal. Spawning during calm periods (Serrao *et al.*, 1996), with reduced water motion or into semi-enclosed bodies of water such as rock pools or surge channels (Denny *et al.*, 1992) can also prevent rapid sperm dilution (Yund, 2000). In certain cases, such as when gamete collisions are rare, the period of sperm fertilising ability (termed longevity) influences fertilisation success (Levitan, 2006).

The seawater physico-chemical conditions into which these sperm are released are currently changing at a rate unprecedented in the geological record (Hönisch *et al.*, 2012), as increasing atmospheric carbon dioxide (CO_2) levels drive elevated sea surface temperatures and decreased oceanic pH and carbonate saturation states (termed ocean acidification: OA). The pH of surface seawater has already decreased by 0.1 pH unit since industrialisation (Stocker *et al.*, 2013), with further reductions of 0.22-0.35 pH units projected by the end of the century unless emissions are dramatically cut (Bopp *et al.*, 2013). A recent climate agreement aiming to reduce global CO_2 emissions in line with 2 °C of warming above pre-industrial levels was adopted at the 21st United Nations Conference (COP21) in December 2015. Without any further policy measures, the world is on a trajectory towards 4 °C of warming before 2100 (Clémençon, 2016; Stocker *et al.*,

2013) with serious implications for seawater chemistry. Globally we can emit an estimated 1000 billion tons of CO_2 until 2100 to stay below 2 °C of warming (Stocker *et al.*, 2013). Yet at current emissions growth rates, this allowance will be used up shortly after 2030 (Friedlingstein *et al.*, 2014). The extent to which countries implement measures to achieve COP21's aims will have large ramifications for the seawater conditions into which future populations of marine species spawn and fertilise within.

In most externally fertilising species, sperm are stored immotile in the gonad and are activated by the change in extrinsic conditions upon release into seawater, which triggers the onset of vigorous motility. Intracellular sperm pH plays a crucial role in the activation of marine invertebrate sperm swimming (Morisawa *et al.*, 1999) and determines the activity of dynein ATPase (Nishigaki *et al.*, 2014), the enzyme responsible for driving flagellar beating to propel a sperm forwards. Sperm are single cells with a greatly reduced cytoplasm, which presumably limits their ability to buffer their internal pH against changes in seawater pH (Melzner *et al.*, 2009). In combination with the numerous pH-dependent processes critical to fertilisation that take place inside a sperm cell (Nishigaki *et al.*, 2014), this implies that sperm functioning might be vulnerable to the predicted CO_2-driven changes in ocean chemistry for the coming century. Sperm have a central and critical role in reproduction, hence understanding the ways in which OA might affect sperm function is of paramount importance to understanding impacts at the population level.

A number of studies have shown that fertilisation success is negatively impacted by OA conditions for species across broad taxonomic groups including cnidaria (Albright *et al.*, 2010; Albright and Mason, 2013), echinodermata (Gonzalez-Bernat *et al.*, 2013b), mollusca *(Parker et al., 2009; Barros et al., 2013)* and polychaeta (Campbell *et al.*, 2014). However, many other studies have reported external fertilisation to be tolerant of experimental OA (Ho *et al.*, 2013; Byrne *et al.*, 2009; Chua *et al.*, 2013; Martin *et al.*, 2011; Havenhand and Schlegel, 2009) including a suite of marine invertebrates from South-East Australia (Byrne *et al.*, 2010). Contradictory responses, reported for the same species by different research groups, highlight the potential for population-specific gamete sensitivities that may be driven by adaptation to local environmental conditions. Or they may simply result from different methodologies employed by studies. When

sperm swimming has been directly examined, rather than using fertilisation success as the measured end point, most investigations identified significant reductions in sperm swimming speed and/or the percentage of motile sperm in an ejaculate for at least one OA treatment level (Havenhand *et al.*, 2008; Morita *et al.*, 2010; Schlegel *et al.*, 2012; Vihtakari *et al.*, 2013), implying a common sensitivity. Some studies however, found no effect for either swimming endpoint (Havenhand and Schlegel, 2009; Nakamura and Morita, 2012; Sung *et al.*, 2014) and one found that sperm swimming performance was enhanced under OA conditions adding to mounting evidence of species-specific sperm responses (Caldwell *et al.*, 2011).

The general consensus of fertilisation assays; that external fertilisation is robust to future OA conditions, contrasts with the consensus of sperm swimming analyses which implies a shared sensitivity. This disagreement is somewhat puzzling, given the well-established relationship between sperm swimming speed and external fertilisation (Styan, 1998; Vogel *et al.*, 1982; Levitan, 2000). Sperm concentration is arguably the single greatest influence on external fertilisation success (Levitan, 1991; Levitan *et al.*, 1991). When studies investigated a range of sperm concentrations, several found the influence of OA on fertilisation was sperm concentration-dependent (Gonzalez-Bernat *et al.*, 2013a; Ericson *et al.*, 2010) with stronger OA effects at lower, and potentially more environmentally relevant sperm concentrations (Levitan and Petersen, 1995), agreeing with the wider ecotoxicology literature (Hollows *et al.*, 2007). Fertilisation assays often test a single sperm concentration and biological effects could be missed by not investigating the full range of field-relevant sperm-to-egg ratios. There is also a tendency for assays to use relatively high sperm concentrations that can result in nearly 100 % fertilisation success across a range of experimental treatments (for example see; Martin *et al.*, 2011). This prohibits the identification of positive effects, and could mask the observation of more subtle, but biologically relevant, influences on fertilisation through sheer sperm numbers. Saturating sperm concentrations are unlikely to be ecologically relevant to most populations, as although field data is rare, when present it reveals that the percentage of a female's eggs fertilised is often much less than 100 % (Levitan, 1998; Williams *et al.*, 1997). Sperm motility is a key fitness-related aspect of sperm function and we propose there is a need for more ecologically relevant studies to look at OA impacts directly on sperm.

One of the logistical constraints to conducting OA-sperm exposures is that high sperm respiration rates rapidly alter the seawater pCO_2 levels modifying the exposure conditions. In order to overcome this, we developed a novel technique that allowed us to control the pH and oxygen content of sperm incubations, without mechanical disruption from direct air bubbling. We constructed self-contained incubation chambers from dialysis membranes designed to retain the sperm cells inside the chamber, but allow the rapid exchange of oxygen and carbon dioxide between the chamber and a large seawater reservoir where the carbonate chemistry was monitored and manipulated. Using this technique, we undertook a mechanistic exploration of sperm performance over 8 hours under simulated OA conditions in the coastal polychaete *Arenicola marina*. Found in intertidal sediments across Northern Europe, *A. marina* is a keystone species and ecosystem engineer (Volkenborn *et al.*, 2007). It plays important roles irrigating and bioturbating the sediment, and as a secondary producer and prey species for fish and wading birds. *A. marina* has an unusual reproductive strategy where interaction between sperm and eggs may take place several hours after spawning and dilution in seawater (Williams and Bentley, 2002). Under future ocean conditions this may result in a prolonged sperm exposure to OA in this species. Our aims were to (1) establish the influence of OA on sperm motility in *A. marina* over time and (2) to investigate potential mechanisms underlying any observed changes in sperm swimming behaviour by monitoring sperm oxygen consumption, ATP content and viability over time.

4.3 METHODS

Animals and experimental set-up

Ripe worms collected from Mothecombe beach, Devon, UK (50°31''23 N, -3°94''58 W) were acclimatised to laboratory conditions (14.5 ˚C, pH 8.10) for at least seven days prior to spawning. Animals were injected with 1 ml 8,11,14-eicosatrienoic acid (Sigma™) into the coelomic cavity (Pacey and Bentley, 1992), with sperm collected dry and kept on ice prior to use (≤ 2 hours). Temperature controlled water baths set to 14.5 ± 0.2˚C were filled with nine litres of artificial seawater (Tropic marin®) filtered to 0.2 µm, and either aerated to simulate current ocean conditions (ambient treatment; pH 8.10, 400 µatm pCO_2) or received a mixed gas input using a mass flow controlled system (Aalborg GFC 17) to simulate OA conditions (OA treatment; pH 7.77, 1000 µatm pCO_2). Salinity, temperature and pH in the water baths were monitored via a Mettler Toledo™ SG23 SevenGo pro™ pH and conductivity meter calibrated using NBS buffers.

An aliquot of 2 µl of sperm was activated in 1 ml of ambient seawater and immediately transferred into the lumen of a 5 cm length of dialysis tubing (Spectrum®, Spectra/Por™ MWCO: 6-8 kD, 6.4 mm diameter), and sealed at both ends by dialysis clips to form an incubation tube. This was repeated using OA seawater and with sperm from each male (n= 6). Dialysis tubes were then transferred into the water bath containing seawater of the corresponding treatment and incubated for 8 hours. At time-points (0, 1, 4 and 8 hours post-sperm activation) aliquots of sperm were sampled from each tube for the assessment of sperm concentration, swimming behaviour, viability, oxygen consumption and ATP content. The 8 hour exposure duration was selected to mimic a feasible period between tidal cycles for *A. marina* sperm functioning before presumed dilution below fertilisable concentrations.

Method validation

We conducted rigorous method development to validate our novel exposure technique and confirm no significant change in the pH or oxygen content of sperm-seawater incubations over the exposure period. Tube pH was monitored at various points during

the sperm incubations via a Mettler Toledo™ SG23 SevenGo pro™ pH meter fitted with a Mettler Toledo™ InLab® Ultra-Micro electrode (Table 1) and was found not to vary over the 8 hours. Tube pH remained within ± 0.02 pH units of target values during the exposures across seawater treatments (Table 1). We statistically analysed the oxygen concentration of sperm seawater samples taken at 0, 1, 4 and 8 hours to confirm there were no differences between treatments (see statistical analysis, Figure S1-1 and Table S1-1).

Table 1. Measured and calculated (CO2SYS; Pierrot *et al.*, 2006) seawater parameters for experimental treatments. Tube parameters were monitored during the exposures, and seawater samples were taken from water baths after 0, 4 and 8 hours of exposure. Data displayed as averages ± SE [T = temperature, S = salinity, DIC = dissolved inorganic carbon, TA = total alkalinity].

	Measured tube parameters			**Measured water bath parameters**				**Calculated water bath parameters**			
Treatment	**pH_{NBS}**	**T (°C)**	**S**	**pH_{NBS}**	**T (°C)**	**S**	**DIC (µmol kg^{-1})**	**pCO_2 (µatm)**	**TA (µmol kg^{-1})**	**HCO_3 (µmol kg^{-1})**	**CO_3^{2-} (µmol kg^{-1})**
Ambient	8.10 ± 0.01	14.3 ± 0.1	33.3 ± 0.1	8.10 ± 0.00	14.3 ± 0.1	33.3 ± 0.1	2003 ± 8	450 ± 1	2172 ± 8	1860 ± 7	125 ± 1
OA	7.75 ± 0.02	14.4 ± 0.1	33.1 ± 0.1	7.77 ± 0.01	14.4 ± 0.1	33.0 ± 0.1	2088 ± 6	1031 ± 12	2141 ± 6	1986 ± 5	62 ± 1

Sperm swimming analysis

Sperm swimming behaviour was analysed at each time-point by computer assisted sperm analysis (CASA, see Supporting Methods). We selected curvilinear velocity (VCL) as the swimming speed parameter. VCL is the calculated speed of the sperm head along the swimming path recorded in 100 frames taken over 0.5 seconds. As marine invertebrate sperm swimming paths are highly curved during the 'searching phase' before detecting

the presence of an egg, other derived speed parameters may bear little relevance to this study. For example, straight line velocity (VSL) is the speed travelled over a theoretical line drawn between the position of the sperm head in the first and last frame of analysis, and may not be a good indication of the average speed of sperm movement under these conditions. To determine the percentage of motile sperm and remove the influence of immotile sperm moving by capillary drift, a threshold value of 10 µm s^{-1} VCL was selected. Average sperm swimming speed was calculated as the average VCL of motile sperm (i.e. sperm swimming above the threshold value). Average sperm path linearity (LIN) was calculated for all motile sperm. This is calculated as the ratio of two CASA derived parameters (VSL/VCL). Sperm recorded with higher values of LIN have more linear swimming paths i.e. they are more progressively motile.

Sperm viability

Sperm viability at each time-point was assessed using a Molecular Probes™ LIVE/DEAD® sperm viability kit. To 200 µl aliquots of each sperm sample the fluorescent dyes SYBR® 14 (100 nM final concentration) and propidium iodide (12 µM final concentration) were added, and incubated at 14 ± 0.5 °C for 10 minutes. Using an Evos® FL Cell Imaging System the proportion of sperm fluorescing green (i.e. live) or red (i.e. membrane compromised/dead) was assessed for 720 ± 75 sperm (average ± 1 SE) per sample.

Sperm oxygen consumption

Aliquots of the sperm samples taken at each time-point were sealed in individual glass vials lacking an air space. Dissolved oxygen was measured using a fiber optic sensor (Firesting OXR 230) connected to a FSO2-4 optical oxygen meter. Oxygen consumption was calculated as the reduction in dissolved oxygen between an initial and final reading and corrected for any consumption in control vials containing seawater of the corresponding treatment only. Replicate sperm counts were conducted using an improved Neubauer haemocytometer and oxygen consumption adjusted accordingly to provide a final value per sperm cell.

Sperm ATP content

The sperm samples were prepared for Adenosine 5'-triphosphate (ATP) quantification using the methodology described by Perchec *et al.* (1995) [see Supporting methods]. ATP quantification took place using a Tecan Infinite® 200 PRO plate reader and an ENLITEN®ATP Assay System bioluminescence detection kit. Samples were quantified in triplicate and sample luminescence intensity compared to standards of known ATP concentration. Figures were adjusted by the sperm counts to provide the ATP content per sperm cell.

Seawater chemistry

Seawater samples were collected from each water bath at 0, 4 and 8 hours post sperm activation for dissolved inorganic carbon (DIC) analysis (see Supporting Methods).

Statistical analysis

Data was analysed using a two-way repeated measures ANOVA approach to investigate the influence of seawater conditions and exposure length on each aspect of sperm performance alongside the initial oxygen concentration of samples. Pairwise multiple comparisons were performed using the Holm-Sidak method. Data was checked for normality using the Shapiro-Wilk test and for equality of variance using Levene's median test. ANOVA's were performed in SigmaPlot (SigmaPlot), Levene's median tests in R version 3.2.3 (R Core Team, 2013) and all graphs were produced in GraphPad (GraphPad Prism 6).

4.4 RESULTS

All values of sperm performance provided in this section are the treatment averages ± 1 SE. Full seawater chemistry can be found in Table 1.

Sperm swimming performance

We identified significant interactions between the exposure time and seawater pH/pCO_2 conditions for all three investigated parameters of sperm swimming performance (Table 2); average sperm swimming speed (VCL), percentage sperm motility and average sperm path linearity. These interactions resulted in significant differences in swimming behaviour between sperm incubated in ambient and OA conditions that were observed 4 hours into the exposures.

Table 2. The results of two-way repeated measures ANOVAs to assess the influence of seawater conditions and exposure length on ejaculate traits in *A. marina.*

Ejaculate trait	Source of variation	df	SS	MS	F	*p*
Average sperm swimming speed	**Seawater conditions x exposure length**	**3**	**3336.975**	**1112.325**	**8.405**	**0.002**
Percentage of motile sperm	**Seawater conditions x exposure length**	**3**	**0.138**	**0.0460**	**4.822**	**0.015**
Average sperm path linearity	**Seawater conditions x exposure length**	**3**	**877.495**	**292.498**	**3.454**	**0.044**
Sperm viability	Seawater conditions	1	0.002	0.002	1.188	0.333
	Exposure length	3	0.028	0.009	2.934	0.074
	Seawater conditions x exposure length	3	0.009	0.003	0.704	0.569
Sperm oxygen consumption	**Seawater conditions x exposure length**	**3**	**17.381**	**5.794**	**7.368**	**0.003**
Sperm ATP content	Seawater conditions	1	0.052	0.052	0.428	0.542
	Exposure length	2	0.090	0.045	0.491	0.626
	Seawater conditions x exposure length	2	0.192	0.096	2.293	0.152

Notes: Significant terms are highlighted in bold ($p \leq 0.05$). The significance of individual terms cannot be inferred when they are included in a significant interaction.

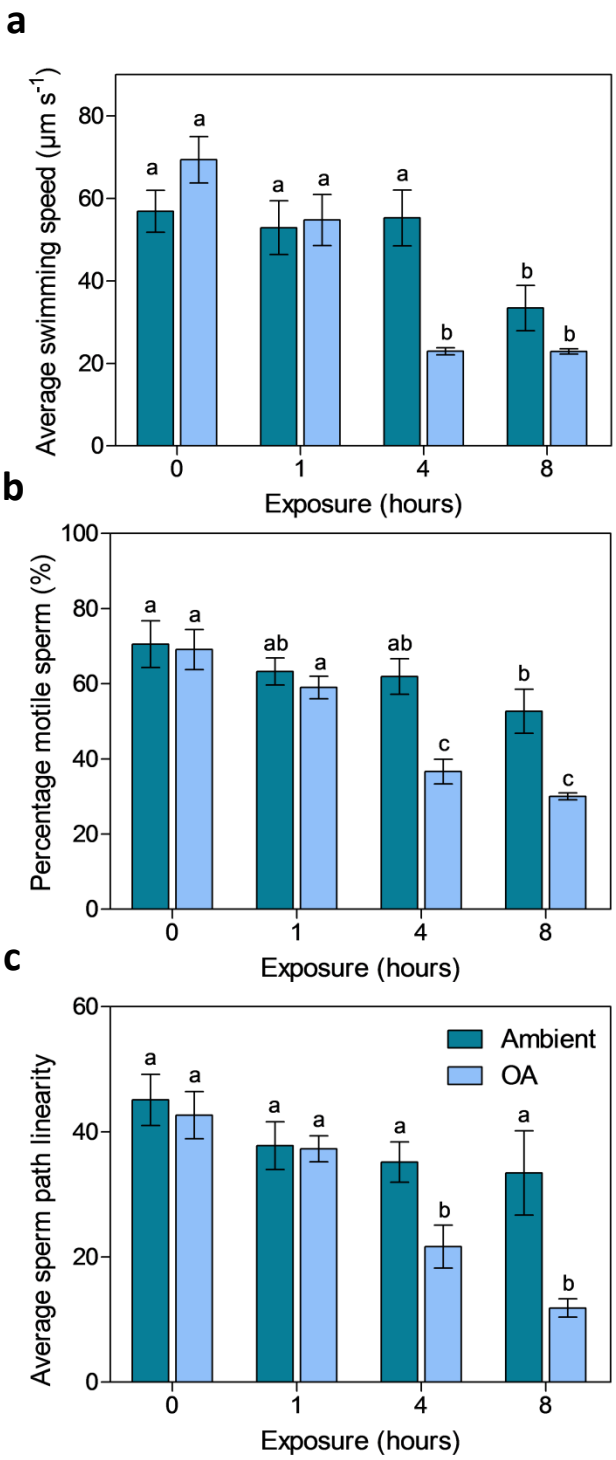

Figure 1. Impacts of ocean acidification on sperm swimming behaviour over an 8 hour exposure in *A. marina.* Average sperm swimming speed (a), the percentage of motile sperm (b) and the average linearity of sperm swimming paths for sperm incubated in either ambient of simulated OA seawater conditions. Data are means ± S.E. Lower case letters signify the results of the two-way repeated measures ANOVA (the absence of shared letters represents a significant difference ($p \leq 0.05$) between either seawater treatments or between time-points within the same seawater treatment). n=6 males.

Average sperm swimming speeds (VCL) were fastest immediately after activation in both ambient and OA seawater treatments. Average speeds were slightly but not significantly faster under OA conditions at this initial time-point: 56.89 ± 5.09 µm s^{-1} in ambient cf. 69.38 ± 5.62 µm s^{-1} in OA (Figure 1a). In ambient conditions average speeds remained high throughout the exposure period, only declining by the final time-point (t = 8 hours) to 33.43 ± 5.59 µm s^{-1} where they were significantly slower than at all earlier time-points (full stats output in Table S1-2). Under OA conditions average sperm swimming speeds had significantly declined by the earlier time-point of 4 hours exposure time, dropping to 22.94 ± 0.86 µm s^{-1}, which was significantly slower than at the two earlier time-points and resulted in a significant difference between treatments at this time-point.

A similar pattern was observed for percentage sperm motility. Sperm samples contained the highest percentage of motile sperm immediately after activation, and there was no significant difference between the two seawater treatments here, with 70.54 ± 6.19 % motile sperm in ambient cf. 69.09 ± 5.32 % in OA (Figure 1b). Under ambient seawater conditions sperm motility only declined slightly over time dropping to 52.68 ± 5.85 % after 8 hours, which meant that there were significantly less motile sperm at the end of the exposure than immediately after sperm activation (full stats output in Table S1-3). In the OA seawater treatment, a reduction in sperm motility was observed earlier, and showed a greater drop in the percentage of motile sperm than in ambient conditions. There were 36.64 ± 3.26 % motile sperm after 4 hours under OA: significantly fewer than at the two earlier time-points. Motility differences at 4 and 8 hours were significant between seawater treatments with significantly fewer motile sperm under OA.

Immediately after activation sperm swimming paths were recorded as 45.10 ± 4.08 linear in ambient and 42.66 ± 3.77 linear in OA seawater, with no significant difference between the two treatments here (Figure 1c). Sperm path linearity did not significantly change in the ambient treatment over the entire 8 hour exposure (full stats output in Table S1-4). However, reductions in sperm path linearity did take place under OA conditions 4 hours into the exposure, dropping to 21.67 ± 3.44. This was significantly different to the two earlier time-points for this treatment. There was no further change at the final time-point (t = 8) in either seawater treatment, and average sperm paths

were significantly less linear in OA conditions than in the ambient treatment at both 4 and 8 hours of exposure time.

Sperm physiology

The proportion of viable sperm remained high throughout the experiment in both treatments, with 89.9 ± 1.2 % live sperm in ambient conditions and 90.4 ± 1.5 % under OA (Figure 2a). There were no significant differences in viability between seawater treatments or over the course of the exposure (Table 2 and full stats output in Table S1-5).

We identified an interaction between the length of exposure and seawater conditions that significantly influenced sperm oxygen consumption (Table 2). Oxygen consumption was significantly higher in ambient conditions immediately after sperm activation than in the OA treatment (Table S1-6 for full stats output). However, by the 1 hour time-point we observed a switch, with sperm in the OA treatment now consuming oxygen at significantly higher rates than ambient sperm (Figure 2b). Sperm oxygen consumption declined in both seawater treatments 4 hours into the exposures. There was no further change in either treatment at the final time-point, and there were no further significant differences between treatments at these two later time-points.

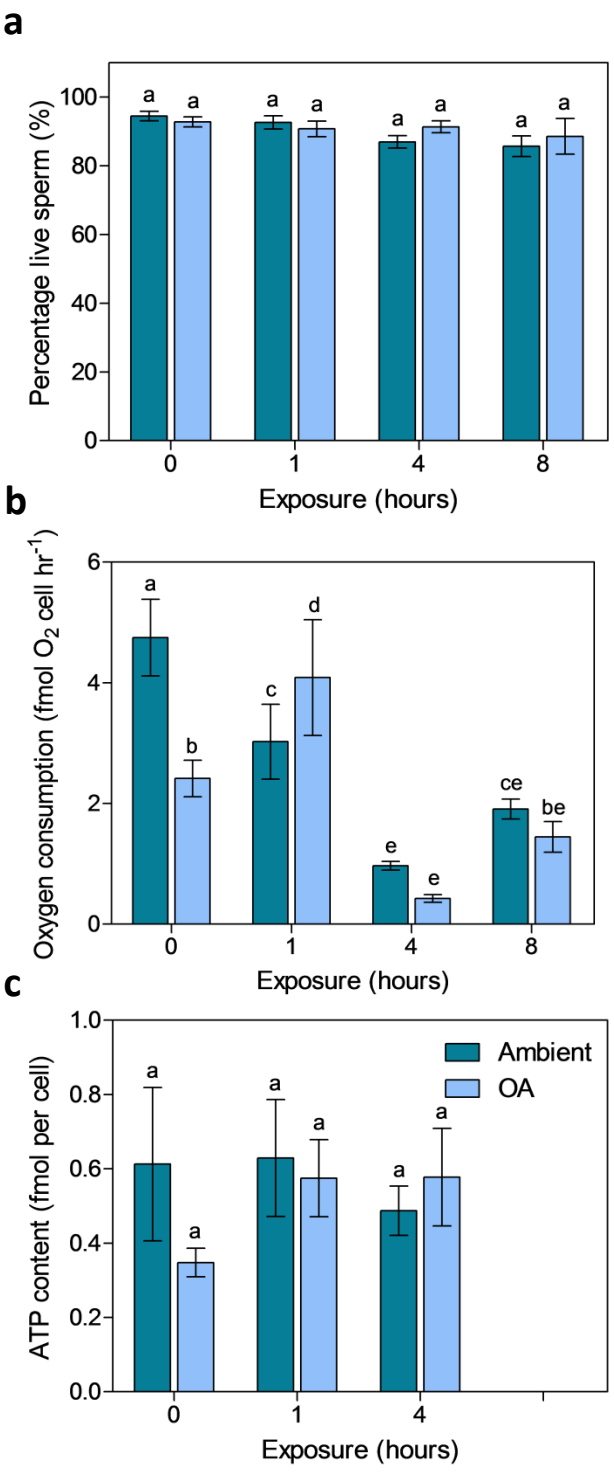

Figure 2. Impacts of ocean acidification on sperm physiology over an 8 hour exposure in *A.marina.* Sperm viability (a), oxygen consumption (b) and ATP content (c) for sperm incubated under ambient or simulated OA seawater conditions. Data are means ± S.E. Lower case letters signify the results of the two-way repeated measures ANOVA (the absence of shared letters represents a significant difference ($p \leq 0.05$) between either seawater treatments or between time-points within the same seawater treatment). n=6 males.

Due to a technical issue on the day of collection the 8 hour samples for ATP quantification were discarded. There was no significant change in sperm ATP content over the 4 hours data was collected for this trait in either seawater treatment. Over the 4 hour exposure the incubation contained 0.58 ± 0.09 fm ATP $sperm^{-1}$ in ambient seawater and 0.50 ± 0.06 fm ATP $sperm^{-1}$ in OA conditions (Table 2 and Figure 2c). Differences between seawater treatments were not significant (Table S1-7 for full stats output).

4.5 DISCUSSION

By taking a longitudinal cellular-lifetime approach to characterising the interactions between *Arenicola marina* sperm cells and their environment this work has shown that the length of time between spawning and fertilisation can strongly influence the impact of ocean acidification (OA) on sperm performance. We identified significant interactions between the length of exposure and seawater conditions that acted to reduce average sperm swimming speeds (-32.32 μm s^{-1}), decrease the percentage of motile (i.e. swimming) sperm (-25.28 %) and alter the linearity of sperm swimming paths (-13.49 %) following several hours under OA in comparison to sperm in ambient conditions. Immediately after sperm activation and following a one hour sperm exposure, there was no difference in *A. marina* sperm swimming performance between ambient and OA seawater conditions. However, four hours into the exposures we identified significant alterations to swimming behaviour in sperm incubated under OA, which were not mirrored by sperm under ambient conditions at this time-point. Swimming performance remained consistently high under ambient conditions for longer.

Previous studies have tended to use sperm immediately after dilution in seawater (i.e. 'experimental spawning') limiting sperm exposure time to OA. Fertilisation assays generally mix sperm and eggs immediately after sperm activation, and often at inappropriately high sperm concentrations where most fertilisation will ensue vigorously upon mixing. Motility analyses follow sperm over a few seconds and are normally performed within the initial minutes of sperm motility. The majority of investigations early in the sperm movement phase have identified sensitive motility responses to OA across externally fertilising marine invertebrate taxa. Most studies to date have found that the speed and/or number of motile sperm were reduced by at least one of the OA treatment levels investigated (Vihtakari *et al.*, 2013; Vihtakari *et al.*, 2016; Lewis *et al.*, 2012; Schlegel *et al.*, 2015; Schlegel *et al.*, 2012; Schlegel *et al.*, 2014; Havenhand *et al.*, 2008; Morita *et al.*, 2010; Uthicke *et al.*, 2013). So whilst the endpoint(s) affected and level of OA required to induce motility reductions may vary, there appears to be a common sensitivity.

Here we identified no change in *A. marina* sperm swimming parameters in our simulated OA conditions (1000 µatm pCO_2) immediately after sperm activation. Whilst, in our earlier work on the same population, experimental OA (1450 µatm pCO_2) significantly reduced average sperm swimming speeds following a ten minute exposure (Campbell *et al.*, 2014). Both average sperm swimming speeds and values of percentage sperm motility tended to be much higher in our previous work, potentially highlighting seasonal variation in sperm quality that may play some role in sensitivity to OA. Taken together, these two results align well with another study in the sea urchin *Centrostephanus rodgersii* (Schlegel *et al.*, 2015). The authors reported that sperm motility and swimming speeds were slightly enhanced in one OA treatment (950 µatm pCO_2), but significantly reduced at a greater OA treatment level (1550 µatm pCO_2) following a fifteen minute sperm exposure. It seems intuitive that there may be a range of seawater conditions at which sperm swimming performance is maximal. Small deviations in conditions are unlikely to result in any observable changes to swimming performance, which may even be slightly enhanced (Calabrese, 2008). However, swimming performance is likely to be negatively impacted by a change in seawater conditions that moves a sperm outside of this optimal range, which may be influenced by a male's developmental, parental and evolutionary history.

Our results clearly demonstrate the potential for perturbation of marine invertebrate sperm motility to develop over an exposure to OA conditions. Similar findings were identified in response to elevated temperatures for two sea urchin species; *Tripneustes gratilla* and *Echinometra mathaei* (Rahman *et al.*, 2009). The authors observed similar levels of sperm fertilising ability across temperature treatments immediately after sperm activation. But later in the exposures, sperm developed a reduced fertilising ability at elevated temperatures (25 or 30 °C) than in control conditions (20 °C). This provides tentative evidence that changes to sperm functioning can develop across broad taxonomic groups in response to environmental variables. A more complete understanding of fertilisation dynamics in nature would help to inform experimental design and ensure that the reproductive consequences of environmental change are assessed under ecological conditions that bear a strong relevance to the population in question.

The length of time between sperm release and successful fertilisation for natural populations of external fertilisers is likely to be highly variable as a consequence of reproductive strategy, population density, spawning synchrony and the local hydrological and topographical conditions at gamete release (Johnson *et al.*, 2012). Spawning asynchrony is frequently observed in externally fertilising taxa, hence unfertilised eggs regularly encounter sperm of various ages (i.e. time in seawater; Lotterhos and Levitan, 2010). Whilst sperm are still fertilisation competent, some of these encounters should lead to successful fertilisation. Fitzpatrick *et al.* (2012) provide indirect evidence for the selective advantage of longer-lived sperm in the mussel *Mytilus galloprovincialis* where males with slower swimming sperm had higher fitness when gamete concentrations were low. They interpreted this as evidence of the adaptive benefits of sperm that use their energy resources more slowly enabling them to search for diffuse eggs for longer. Their work highlights the potential for sperm that have experienced an extended exposure to the seawater conditions to significantly contribute to a male's fertilisation success. This, taken with the large sperm longevities recorded for many broadcast spawning species, suggests that overlooking the potential for OA to impact sperm functioning beyond the first few minutes of activity could limit our understanding of the reproductive consequences of OA. Cellular effects may take some time to manifest; there be regulatory mechanisms, cellular 'tipping points' or several steps in a chain of activity before observable changes to sperm functioning take place within ecologically relevant timeframes.

The decrease in the speed and number of motile sperm after four hours under OA was not accompanied by significant differences between treatments in sperm viability, oxygen consumption or ATP content. Therefore, it appears unlikely that changes in any of these physiological measures were directly responsible for the declines in swimming performance under OA. Sperm viability did not appear challenged during the exposures under our experimental conditions, confirming that sperm which stopped swimming were still viable. *A. marina* sperm are characterised by a remarkably long movement phase, and can retain a high fertilisation capacity for up to 60 hours (Williams and Bentley, 2002). It is therefore highly unlikely that sperm longevity influences fertilisation rates in this species. The lack of significant differences between treatments in sperm ATP content suggests that an exhaustion of cellular ATP was unlikely to be responsible for the

significant proportion of cells losing motility or swimming more slowly after four hours in OA conditions. This aligns with a study in another marine external fertiliser with a long sperm movement phase, the Pacific oyster (*Crassostrea gigas*; Suquet *et al.*, 2010). But this contrasts with reports from several species of marine fish where it appears that intracellular ATP content controls the short duration of sperm motility common to this group (Dreanno *et al.*, 1999; Perchec *et al.*, 1995; Christen *et al.*, 1987).

ATP is the cellular energy currency (Imamura *et al.*, 2009) and is critical to sperm movement where it is hydrolysed by dynein ATPase to power flagellar beating (Christen *et al.*, 1983). *A. marina* sperm synthesise ATP aerobically through oxidative phosphorylation (OXPHOS) and anaerobically via glycolysis, with ATP production shared by the two pathways when oxygen is available (Arrata *et al.*, 1978). As OXPHOS generates an 18-fold higher ATP yield than glycolysis (Kamp *et al.*, 1996), it is presumed to be the primary pathway when oxygen conditions are not limiting, such as in our exposures. As the energy demand of mature sperm has switched away from biosynthesis to motility, oxygen consumption is a good approximation for rates of aerobic ATP generation to supply dynein ATPase. We identified significant differences in oxygen consumption rates between sperm incubated in ambient and OA seawater treatments immediately after sperm activation and following a one hour sperm exposure indicating that rates of aerobic ATP generation varied between sperm in the two treatments early in the exposures. However, sperm ATP content remained unchanged throughout the four hours of data available for this trait, and there were no significant differences between seawater treatments at either early time-point.

Our sampling points may not have been frequent enough to pick up on the expected changes in sperm ATP content. Immediately after activation sperm in ambient conditions had significantly higher oxygen consumption rates, but sperm would have only just activated, initiated motility and started to consume ATP which may have been too early to detect any change in cellular ATP levels. One hour into the exposures there had been a switch with sperm in OA conditions now consuming oxygen at a significantly faster rate than ambient sperm, and this may have allowed sperm in the OA treatment to correct the hypothesised deficit in cellular ATP levels before this sampling time-point. Alternatively, sperm in the OA treatment may have employed higher rates of glycolysis

and/or cellular energy stores may have been liberated (Boulais *et al.*, 2015) to maintain a constant supply of ATP to dynein early in the movement phase and maintain cellular ATP levels. ATP produced aerobically in the sperm mitochondria needs to be transported along the entire length of the sperm flagellum to the sites of use. *A. marina* sperm employ a phosphocreatine shuttle to deliver aerobically produced ATP to dyneins along the flagellum in reactions catalysed by creatine kinase (Kamp *et al.*, 1995). Consequently, total cell ATP content may not be a good proxy for the ATP levels available to dynein ATPase to power swimming.

The more rapid decline we observed in the speed and number of motile sperm after several hours in OA conditions could have knock-on ecological implications for future *A. marina* populations due to their reproductive strategy. Males spawn onto the sediment surface at low tide, producing dense exposed sperm puddles. Females release eggs into their burrows 20-40 cm down into the sediment. The incoming tide activates and transports sperm towards female burrows, the sites of fertilisation and early larval development. Female worms irrigate their burrows via peristaltic contractions of their muscular body wall, which draws in sperm. But because the rate of female burrow irrigation is low, interaction between sperm and eggs may take place several hours after spawning and dilution in seawater (Williams and Bentley, 2002). Both sperm swimming speed and percentage sperm motility hold central roles in models of population fertilisation ecology in external fertilisers (Vogel *et al.*, 1982; Styan, 1998). Hence, the more rapid decline in both of these traits under OA has the potential to reduce fertilisation rates for sperm drawn inside the female burrow and into close proximity of eggs. Our previous work in this species linked a decline in sperm swimming speeds to reduced fertilisation success under OA conditions (Campbell *et al.*, 2014). The competitive quality of an ejaculate is mediated by the relative number and motile performance of functioning sperm (Gage *et al.*, 2004; Evans *et al.*, 2013; Campbell *et al.*, 2016).

Our data also showed that whilst there was no change in sperm linearity over the exposure in ambient conditions, in the OA treatment linearity declined following a four hour incubation. The ecological implications of alterations to sperm path linearity for external fertilisation are not currently well established. Marine invertebrate sperm swim

in circular or helical paths (Jikeli *et al.*, 2015) and physical models have revealed that this enables them to sense gradients in chemical cues released by eggs most effectively (Beltrán *et al.*, 2007). Following an initial searching phase, if a sperm detects such a gradient, it then navigates towards the egg in a phenomenon termed chemotaxis (Beltrán *et al.*, 2007) through an orchestrated series of turns (Nishigaki *et al.*, 2014). Egg chemical cues work over relatively short distances, within a few egg diameters of the source (approximately a few hundred microns), so in *A. marina* this would presumably take effect once inside a female's burrow. The reductions in sperm path linearity we observed after several hours under OA conditions may therefore have the potential to influence fertilisation rates through alterations to sperm searching efficiency however, more research is needed to fully establish this relationship.

There is currently no field data on the seawater conditions inside an *A. marina* burrow during fertilisation. As female worms irrigate their burrows with incoming tidal water, presumably replacing the contents with fresh seawater numerous times, we would expect the chemistry to roughly approximate the local coastal conditions. However, ecological data would aid in the design of OA-relevant experiments by informing control conditions and future projections from this baseline. The resulting changes in seawater chemistry are more complex in coastal systems, where there are a multitude of drivers of pH such as nutrient inputs, watershed processes and changes in ecosystem structure and metabolism (Duarte *et al.*, 2013). Coastal organisms are also exposed to additional anthropogenic stressors which include hypoxia, nutrient enrichment, decreased salinity and pollutants (Przeslawski *et al.*, 2015). Further work is required to better understand the conditions that are currently experienced by *A. marina* sperm alongside the consequences of the sperm performance declines we identified for fertilisation success, development and subsequent population recruitment for *A. marina* populations under future coastal conditions.

We have shown that sperm swimming performance declines in current male *A. marina* spawning into simulated future conditions. However, this does not address the potential for adaptation. Sperm-related traits can respond rapidly to selection (Hosken and Ward, 2001; Nandy *et al.*, 2013) but for an evolutionary response the trait needs to be heritable, there also needs to be additive genetic variance in the trait present within the

population and a selection gradient (Foo and Byrne, 2016). Evolutionary responses could be constrained by negative trade-offs between different ejaculate components or offspring traits preventing directional selection leading towards phenotypic values of an individual trait conferring a higher fitness.

4.6 CONCLUSIONS

We found that the length of time between spawning and fertilisation can strongly influence the impact of OA on sperm performance. Key fitness-related aspects of sperm functioning declined faster under OA conditions, remaining consistently high in ambient conditions for longer. The more rapid decline in the speed and number of functioning sperm under OA conditions could have population-level consequences in this species due to *A. marina*'s reproductive ecology. The mechanisms underlying these declines remain unclear but are not related to total sperm cell ATP content or changes in viability. Our results highlight the importance of incorporating a species' life history and a better understanding of fertilisation dynamics in nature when designing experiments to uncover environmental influences upon reproductive endpoints.

4.7 SUPPORTING INFORMATION

4.7.1 Supporting figures

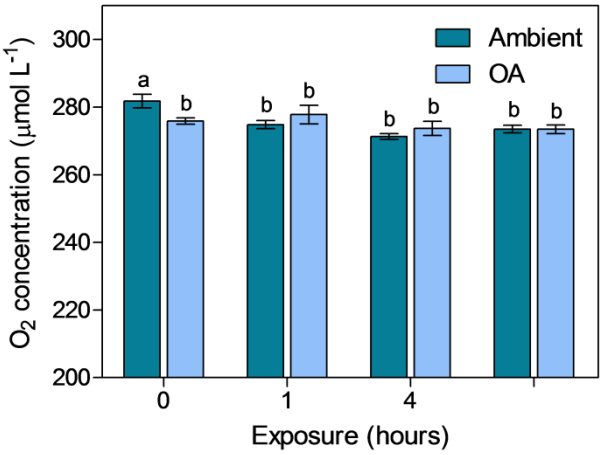

Figure S1-1. The oxygen concentration of sperm-seawater samples incubated over 8 hours under controlled pCO_2 conditions (n=6 males). Samples were taken at time-points during the incubation and oxygen concentration immediately determined via a fiber optic oxygen sensor. Lower case letters signify the results of the two-way repeated measures ANOVA (the absence of shared letters represents a significant difference [$p \leq 0.05$] between time-points). Significant treatment differences were only observed immediately after sperm activation signalling differences between the handling of treatment seawaters prior to the exposures, and these disappeared at all later time-points.

4.7.2 Supporting tables

Table S1-1. The results of pairwise multiple comparisons using the Holm-Sidak method for the two-way repeated measures ANOVA to assess the influence of seawater conditions and exposure length (hours) on the oxygen concentration of sperm samples.

Comparisons for factor	**Levels of comparison**	**Difference of means**	**t**	***p***
Exposure length within current ocean conditions	**0 vs 1**	**6.949**	**3.033**	**0.020**
	0 vs 4	**10.465**	**4.567**	**<0.001**
	0 vs 8	**8.295**	**3.620**	**0.005**
	1 vs 4	3.516	1.534	0.354
	1 vs 8	1.346	0.587	0.561
	4 vs 8	2.170	0.947	0.579
Exposure length within OA conditions	0 vs 1	1.923	0.839	0.650
	0 vs 4	2.142	0.935	0.735
	0 vs 8	2.417	1.055	0.760
	1 vs 4	4.065	1.774	0.363
	1 vs 8	4.340	1.894	0.345
	4 vs 8	0.275	0.120	0.905
Seawater conditions within exposure length 0	**Current vs OA**	**5.905**	**2.493**	**0.022**
Seawater conditions within exposure length 1	Current vs OA	2.966	1.252	0.226
Seawater conditions within exposure length 4	Current vs OA	2.417	1.020	0.321
Seawater conditions within exposure length 8	Current vs OA	0.028	0.012	0.991

Notes: Significant results are highlighted in bold ($p \leq 0.05$).

Table S1-2. The results of pairwise multiple comparisons using the Holm-Sidak method for the two-way repeated measures ANOVA to assess the influence of seawater conditions and exposure length (hours) on the average swimming speed of sperm.

Comparisons for factor	**Levels of comparison**	**Difference of means**	**t**	***p***
Exposure length within current ocean conditions	0 vs 1	3.995	0.567	0.923
	0 vs 4	1.628	0.231	0.819
	0 vs 8	**23.457**	**3.328**	**0.014**
	1 vs 4	2.367	0.336	0.932
	1 vs 8	**19.462**	**2.761**	**0.039**
	4 vs 8	**21.828**	**3.097**	**0.021**
Exposure length within OA conditions	0 vs 1	14.613	2.073	0.092
	0 vs 4	**46.440**	**6.588**	**<0.001**
	0 vs 8	**46.502**	**6.597**	**<0.001**
	1 vs 4	**31.827**	**4.515**	**<0.001**
	1 vs 8	**31.888**	**4.524**	**<0.001**
	4 vs 8	0.062	0.009	0.993
Seawater conditions within exposure length 0	Current vs OA	12.488	1.990	0.061
Seawater conditions within exposure length 1	Current vs OA	1.870	0.298	0.769
Seawater conditions within exposure length 4	**Current vs OA**	**32.323**	**5.150**	**<0.001**
Seawater conditions within exposure length 8	Current vs OA	10.557	1.682	0.109

Notes: Significant results are highlighted in bold ($p \leq 0.05$).

Table S1-3. The results of pairwise multiple comparisons using the Holm-Sidak method for the two-way repeated measures ANOVA to assess the influence of seawater conditions and exposure length (hours) on the percentage of motile sperm.

Comparisons for factor	**Levels of comparison**	**Difference of means**	**t**	***p***
Exposure length within current ocean conditions	0 vs 1	0.094	1.365	0.333
	0 vs 4	0.106	1.537	0.442
	0 vs 8	**0.202**	**2.914**	**0.042**
	1 vs 4	0.012	0.172	0.865
	1 vs 8	0.107	1.549	0.510
	4 vs 8	0.095	1.377	0.448
Exposure length within OA conditions	0 vs 1	0.114	1.652	0.208
	0 vs 4	**0.343**	**4.952**	**<0.001**
	0 vs 8	**0.411**	**5.948**	**<0.001**
	1 vs 4	**0.228**	**3.300**	**0.008**
	1 vs 8	**0.297**	**4.296**	**<0.001**
	4 vs 8	0.069	0.996	0.328
Seawater conditions within exposure length 0	Current vs OA	0.0244	0.390	0.701
Seawater conditions within exposure length 1	Current vs OA	0.044	0.707	0.488
Seawater conditions within exposure length 4	**Current vs OA**	**0.261**	**4.162**	**<0.001**
Seawater conditions within exposure length 8	**Current vs OA**	**0.234**	**3.742**	**0.001**

Notes: Significant results are highlighted in bold ($p \leq 0.05$).

Table S1-4. The results of pairwise multiple comparisons using the Holm-Sidak method for the two-way repeated measures ANOVA to assess the influence of seawater conditions and exposure length (hours) on average sperm path linearity.

Comparisons for factor	**Levels of comparison**	**Difference of means**	**t**	***p***
Exposure length within current ocean conditions	0 vs 1	7.307	1.409	0.523
	0 vs 4	9.941	1.917	0.285
	0 vs 8	11.676	2.252	0.176
	1 vs 4	2.634	0.508	0.852
	1 vs 8	4.369	0.843	0.791
	4 vs 8	1.735	0.335	0.740
Exposure length within OA conditions	0 vs 1	5.381	1.038	0.308
	0 vs 4	**20.992**	**4.049**	**0.001**
	0 vs 8	**30.818**	**5.943**	**<0.001**
	1 vs 4	**15.611**	**3.011**	**0.016**
	1 vs 8	**25.437**	**4.906**	**<0.001**
	4 vs 8	9.826	1.895	0.131
Seawater conditions within exposure length 0	Current vs OA	2.440	0.465	0.647
Seawater conditions within exposure length 1	Current vs OA	0.514	0.0980	0.923
Seawater conditions within exposure length 4	**Current vs OA**	**13.492**	**2.569**	**0.018**
Seawater conditions within exposure length 8	**Current vs OA**	**21.582**	**4.110**	**<0.001**

Notes: Significant results are highlighted in bold ($p \leq 0.05$).

Table S1-5. The results of pairwise multiple comparisons using the Holm-Sidak method for the two-way repeated measures ANOVA to assess the influence of seawater conditions and exposure length (hours) on sperm viability.

Comparisons for factor	**Levels of comparison**	**Difference of means**	**t**	***p***
Exposure length within current ocean conditions	0 vs 1	0.020	0.454	0.880
	0 vs 4	0.085	1.915	0.246
	0 vs 8	0.095	2.148	0.230
	1 vs 4	0.065	1.687	0.284
	1 vs 8	0.075	1.956	0.278
	4 vs 8	0.010	0.269	0.790
Exposure length within OA conditions	0 vs 1	0.018	0.461	0.957
	0 vs 4	0.010	0.263	0.958
	0 vs 8	0.052	1.358	0.713
	1 vs 4	0.008	0.198	0.845
	1 vs 8	0.034	0.897	0.852
	4 vs 8	0.042	1.095	0.814
Seawater conditions within exposure length 0	Current vs OA	0.014	0.320	0.754
Seawater conditions within exposure length 1	Current vs OA	0.012	0.305	0.765
Seawater conditions within exposure length 4	Current vs OA	0.061	1.591	0.134
Seawater conditions within exposure length 8	Current vs OA	0.029	0.760	0.460

Notes: Significant results are highlighted in bold ($p \leq 0.05$).

Table S1-6. The results of pairwise multiple comparisons using the Holm-Sidak method for the two-way repeated measures ANOVA to assess the influence of seawater conditions and exposure length (hours) on sperm oxygen consumption.

Comparisons for factor	**Levels of comparison**	**Difference of means**	**t**	***p***
Exposure length within current ocean conditions	**0 vs 1**	**1.725**	**2.777**	**0.029**
	0 vs 4	**3.781**	**6.087**	**<0.001**
	0 vs 8	**2.840**	**4.571**	**<0.001**
	1 vs 4	**2.056**	**3.310**	**0.010**
	1 vs 8	1.115	1.794	0.161
	4 vs 8	0.942	1.516	0.141
Exposure length within OA conditions	**0 vs 1**	**1.673**	**2.693**	**0.036**
	0 vs 4	**1.990**	**3.203**	**0.014**
	0 vs 8	0.967	1.557	0.131
	1 vs 4	**3.663**	**5.896**	**<0.001**
	1 vs 8	**2.640**	**4.250**	**0.001**
	4 vs 8	1.022	1.645	0.210
Seawater conditions within exposure length 0	**Current vs OA**	**2.335**	**5.060**	**<0.001**
Seawater conditions within exposure length 1	**Current vs OA**	**1.063**	**2.305**	**0.034**
Seawater conditions within exposure length 4	Current vs OA	0.543	1.176	0.255
Seawater conditions within exposure length 8	Current vs OA	0.462	1.002	0.330

Notes: Significant results are highlighted in bold ($p \leq 0.05$).

Table S1-7. The results of pairwise multiple comparisons using the Holm-Sidak method for the two-way repeated measures ANOVA to assess the influence of seawater conditions and exposure length (hours) on sperm ATP content.

Comparisons for factor	**Levels of comparison**	**Difference of means**	**t**	***p***
Exposure length within current ocean conditions	0 vs 1	0.122	0.988	0.721
	0 vs 4	0.052	0.422	0.682
	1 vs 4	0.070	0.565	0.827
Exposure length within OA conditions	0 vs 1	0.018	0.461	0.957
	0 vs 4	0.010	0.263	0.958
	1 vs 4	0.008	0.198	0.845
Seawater conditions within exposure length 0	Current vs OA	0.265	1.752	0.106
Seawater conditions within exposure length 1	Current vs OA	0.054	0.359	0.726
Seawater conditions within exposure length 4	Current vs OA	0.090	0.598	0.561

Notes: Significant results are highlighted in bold ($p \leq 0.05$

4.7.3 Supporting methods

Sperm swimming analysis

Aliquots of sperm samples were transferred to Leja 20 mm standard counting chambers. Analysis took place immediately via a Microptic Sperm Class Analyser® (SCA: Microm, UK) fitted with a Nikon Eclipse 50i negative phase contrast microscope (100 x magnification) and a Peltier cooled stage which was operated at 15 ± 0.1°C. Images were captured at a rate of 100 frames s^{-1} with individual sperm tracked for 0.5 seconds. A minimum of 500 sperm were tracked in each sample and a range of motility parameters calculated for each individual sperm using the SCA® Motility and concentration module. We decided to analyse a large number of sperm in one technical replicate as previous work in marine external fertilisers found high within-sample repeatability in CASA parameters between technical replicates (Fitzpatrick *et al.*, 2012).

Sperm ATP content

The sperm samples were prepared for ATP quantification using the following methodology. Briefly, a 50 µl aliquot of each sperm sample was added to individual Eppendorfs containing 450 µl of sperm buffer (25 mM HEPES, 10 mM magnesium acetate, 3 mM sodium azide, 2 mM EDTA, pH 7.75), boiled for 1 minute and immersed in liquid nitrogen before storage at -80 °C for analysis at a later date.

Seawater chemistry

Analysis was carried out using a custom built system described by Friederich *et al.* (2002) and following the methodology detailed in Lewis *et al.* (2012). This analytical system allowed the measurement of seawater DIC with a precision of ± 3 µM. Total alkalinity and pCO_2 were calculated using CO2SYS (Pierrot *et al.*, 2006) according to Findlay *et al.* (2013) and the DIC, pH and salinity measurements using the NBS scale and Dickson standards.

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Ocean acidification changes the male fitness landscape

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Ocean acidification changes the male fitness landscape

5.1 ABSTRACT

Sperm competition is extremely common in many ecologically important marine taxa. Ocean acidification (OA) is driving rapid changes to the marine environments in which freely spawned sperm operate, yet the consequences of OA on sperm performance are poorly understood in the context of sperm competition. Here, we investigated the impacts of OA (+1000 µatm pCO_2) on sperm competitiveness for the sea urchin *Paracentrotus lividus*. Males with faster sperm had greater competitive fertilisation success in both seawater conditions. Similarly, males with more motile sperm had greater sperm competitiveness, but only under current pCO_2 levels. Under OA the strength of this association was significantly reduced and there were male sperm performance rank changes under OA, such that the best males in current conditions are not necessarily best under OA. Therefore, OA will likely change the male fitness landscape, providing a mechanism by which environmental change alters the genetic landscape of marine species.

5.2 INTRODUCTION

Seawater conditions are currently changing at a rate faster than at any other time for the past 300 million years (Hönisch *et al.*, 2012), as rising atmospheric carbon dioxide (CO_2) levels modify seawater chemistry and decrease ocean pH (Caldeira and Wickett, 2003; termed OA). The unprecedented rate of change is likely to place significant novel selection on marine taxa. Negative impacts of OA on growth, reproduction or survival have been observed in a broad range of species (Dupont *et al.*, 2010; Kroeker *et al.*, 2010) and OA is now widely considered a major threat to marine taxa worldwide (Kroeker *et al.*, 2013). The majority of marine species release sperm and eggs directly into the water column for external fertilisation, including several large taxa with key roles in ecosystem functioning (Volkenborn *et al.*, 2007; Lawrence *et al.*, 2001). The sperm of external fertilisers may be particularly vulnerable to OA due to the limited buffering capacity of internal-sperm pH against changes to seawater pH, together with the large number of pH-dependent steps taking place inside the sperm cell that are critical to fertilisation (Nishigaki *et al.*, 2014). Consistent with this, the majority of studies of external fertilisers have identified reductions in sperm swimming speeds and/or the proportion of motile sperm in at least one of their OA treatments (Havenhand *et al.*, 2008; Morita *et al.*, 2010; Schlegel *et al.*, 2012; Vihtakari *et al.*, 2013). However, some studies have found no effect (Havenhand and Schlegel, 2009) and even motility enhancement under OA (Caldwell *et al.*, 2011), which could indicate species-specific sperm sensitivity to OA or simply methodological differences between studies.

Sperm are under intense selection to achieve fertilisation, as in most species only a tiny fraction of the millions of sperm in an ejaculate succeed in fertilising an egg (Birkhead and Møller, 1998). But being fertilisation competent is often not enough, because in the vast majority of sexually reproducing species sperm from rival males compete for fertilisations (Parker, 1970). Sperm competition is practically ubiquitous across the animal kingdom (Birkhead and Møller, 1998) and results in strong selection on males to produce high quality ejaculates (Parker, 1970). Sperm number, length, swimming velocity and viability all influence sperm competitiveness in internal fertilisers and pair spawning fish (Simmons and Fitzpatrick, 2012). These characteristics should also influence sperm

competition outcomes in marine external fertilisers. However, this area has received little research attention and experimental evidence to link sperm phenotypes to competitive fertilisation success is scarce in this ecologically important group. Under non-competitive scenarios, sperm concentration (Benzie and Dixon, 1994), age (time in seawater post-spawning; Williams and Bentley, 2002), swimming velocity and longevity (Levitan, 2000) all influence male reproductive success. However, male external fertilisers rarely gain sole access to a batch of eggs in the sea as a consequence of life-history strategies which include spawning aggregations and the synchronous release of gametes (Levitan, 2005). Despite this, our understanding of the male traits influencing sperm competition outcomes remains poor in external fertilisers. Changes to seawater chemistry resulting from OA will fundamentally alter the fertilisation environments in which marine sperm operate. Therefore, it is becoming increasingly important to identify the factors influencing competitive fertilisation success in marine external fertilisers, and to identify any changes that could occur as a result of OA. Selection at the gamete stage can have far-reaching consequences for populations, carrying over to subsequent life stages (Schlegel *et al.*, 2012) and to date we have little information on sperm performance in future oceans. The importance of including environmental effects on sperm ecology in order to understand species evolutionary responses was recently highlighted by Reinhardt *et al.* (2015). Their review presented overwhelming evidence that the environment can influence sperm, and highlighted pH as one of the key environmental factors that can influence phenotypic sperm function across species. Here, we address this key area of research by exploring the repeatability of the outcome of sperm competition across two environments.

We conducted a series of paired competitive fertilisation trials under current ocean conditions (pH 8.18, 462 µatm pCO_2) and simulated future OA conditions (pH 7.71, 1468 µatm pCO_2) in the sea urchin *Paracentrotus lividus*. Male ejaculate characteristics were evaluated in each seawater treatment and, based upon average sperm swimming speeds (curvilinear velocity: VCL) in current seawater conditions, males were split into a 'fast' and a 'slow' group. Each 'fast' male was randomly paired with a 'slow' male generating n= 11 pairs (average speed difference ± 95 % confidence interval= 42.78 µm s^{-1} ± 12.51). Each pair competed to fertilise a batch of eggs (n= 10,000) in both current and OA seawater treatments at a total sperm concentration of 1 x 10^5 sperm ml^{-1} (and an equal

number from each male). The resulting larvae were genotyped using microsatellites to assign paternity.

5.3 METHODS

Assessment of ejaculate characteristics

Adult urchins (Dunmannus Seafood Ltd., Ireland) were induced to spawn via KCl injection (Levitan, 2000) with sperm collected dry prior to use. Sperm was activated in each seawater condition and incubated for 10 minutes at 14 ± 0.1°C (see Supplementary Methods for details of seawater pCO_2 manipulation and Table S1-6). Ejaculate characteristics were then measured within each seawater treatment using Computer Assisted Sperm Analysis (CASA) and the methodology described in Campbell *et al.* (2014) (see also Supplementary Methods online). Immotile sperm were defined as sperm swimming below threshold values of 10 µm s^{-1} curvilinear velocity (VCL) and 3.2 µm s^{-1} straight line velocity (VSL). The average sperm swimming speed (VCL) was then calculated for all motile sperm within a sample. Averages of two additional CASA parameters were calculated for all motile sperm within a sample; sperm path linearity (LIN) and sperm path straightness (STR). LIN and STR are measures of the linearity of sperm swimming paths and are calculated by the CASA software (see Supplementary Methods online). Higher values of either LIN or STR indicate more linear sperm swimming paths i.e. a sperm is progressively motile.

The sperm swimming data was visually checked for normality, which was further confirmed via Shapiro-Wilk normality tests. The influence of OA on ejaculate traits was assessed using paired t-tests and variance in traits compared across seawater treatments using Levene's median tests. As the percentage sperm motility data significantly deviated from a normal distribution, the influence of OA on this ejaculate trait was assessed via the non-parametric equivalent of the paired t-test: the Wilcoxon signed-rank test. Males were ranked by ascending sperm performance in current and OA seawater conditions and the strength of correlation between male ranks in the two seawater treatments was examined using the Spearman's rank correlation coefficient.

Competitive fertilisations and larval paternity assignment

Each pair of males competed to fertilise the eggs (n=10,000) of a single female in the two seawater treatments. Eggs were obtained from a total of 6 females. Competitive fertilisation trials were repeated with the eggs of additional females (n=3) to reduce the influence of fertilisation biases generated by differences in gamete compatibility. Sperm from each pair was activated in seawater of the appropriate treatment, mixed, and immediately added to the eggs and treatment seawater at a final sperm concentration pf 1×10^5 sperm ml^{-1}. This sperm concentration was selected to avoid conditions of both sperm limitation and polyspermy based upon data collected for another sea urchin species *Strongylocentrotus franciscanus* (Levitan, 1993; Levitan *et al.*, 2007). Fertilisation beakers were incubated overnight at 14 ± 0.1°C. The resulting larvae were reared at 18 ± 0.1°C in current seawater conditions from 1 to 3 days post fertilisation before larvae (23 ± 0.78 per trial: average ± 95 % confidence interval) and each set of potential parents were genotyped on the basis of microsatellite loci (Calderón *et al.*, 2009), and larval paternity assigned using Cervus v. 3.0.7 (Marshall *et al.*, 1998) at a greater than 95 % level of confidence (n=1273) [see also Supplementary Methods and Table S1-7 online]. Trials involving some combinations of pairs and females had to be discarded if microsatellite genotyping could not clearly assign paternity (due to the presence of shared alleles or possible null alleles). This meant that the resulting larvae of trials with the eggs of either two or three females were genotyped for each pair (see Table S1-8 online for raw data).

Statistical modelling

The faster male of a pair (based upon average sperm swimming speed (VCL) in current conditions) was selected as the 'focal' male and his competitor as the 'rival' male. Relative ejaculate traits were calculated as the focal male minus the rival male. We explored the influence of relative male ejaculate traits and seawater conditions on competitive fertilisation success in our paired trials using a generalized linear-mixed effects modelling (GLMM) approach. GLMM fixed effects were scaled around their mean value and the binomial error family and probit link were selected for the model structure. We accounted for the random effect of pair identity and included a second random term:

a dispersion parameter, to account for overdispersion. Models were built using the following basic fixed effects structure:

Y ~ SC + M + S

Where Y= the proportion of larvae sired by the focal male, SC= seawater conditions, M= the relative proportion of motile sperm and S= relative average sperm swimming speed (VCL).

Once we had constructed a model containing main effects only, we built a series of models containing combinations of main effects and two-way interactions and finally a model containing all three two-way interactions between fixed effects. This resulted in the following GLMM fixed effects structures:

Y ~ SC * M + S

Y ~ SC + M * S

Y ~ SC * M + S

Y ~ SC * M + SC * S

Y ~ SC * M + M * S

Y ~ M * S + SC* S

Y ~ SC * M + M * S + SC * S

We repeated the construction of GLMMs containing different fixed effects structures where we substituted relative average sperm swimming speed (VCL) for three alternative relative speed terms to investigate whether faster sub-populations of sperm within a male's ejaculate were associated with competitive fertilisation success. These included the average sperm swimming speed (VCL) of the fastest 1, 5 and 10 % of motile sperm (see Supplementary Methods online). This resulted in a total of 32 GLMMs.

We compared the performance of each constructed GLMM against our model selection criteria to identify the 'best fitting' model most supported by the paternity share data (see Tables S1-2 to S1-5 online for outputs). Our primary selection criterion was a minimised Akaike information criterion corrected for small sample sizes (AICc; Burnham,

2002). A maximised rounded Akaike weight [w_i(AICc)] was the secondary selection criterion, which we directly interpreted as a conditional probability for each model. We only considered models with a Δ_i(AICc) of less than 2 i.e. the difference between the AICc value of the i^{th} model in the selection and the minimum AICc value. Models with AICc values within 2 of the 'best fitting' model were considered statistically equivalent. However, the model which performed maximally against our selection criteria was selected to generate the graphs in Figure 2 and test the significance of seawater conditions and relative male ejaculate traits on competitive fertilisation success in our paired trials.

Statistical analyses were conducted in R version 3.0.2 (R Core Team, 2013). Modelling was undertaken in the 'lme4' R package using the 'glmer' command. Model dispersion was checked in R (see Supplementary Methods online for R code). GLMM performance against our selection criteria was compared using the 'MuMIn' R package and the 'model.sel' command. The conditional and marginal coefficient of determination was calculated for the 'best fitting' GLMM using the 'MuMIn' R package and the 'r.squaredGLMM' command. The fitted values and 95 % confidence intervals found in Figure 2 were generated using the 'best fitting' GLMM, the 'effects' R package and the 'effect' command. This allowed the influence of one significant interaction or main effect on paternity shares to be plotted, whilst minimising the influence of other terms marginal to the effect in question. All graphs were produced in GraphPad (GraphPad Prism 6)

5.4 RESULTS AND DISCUSSION

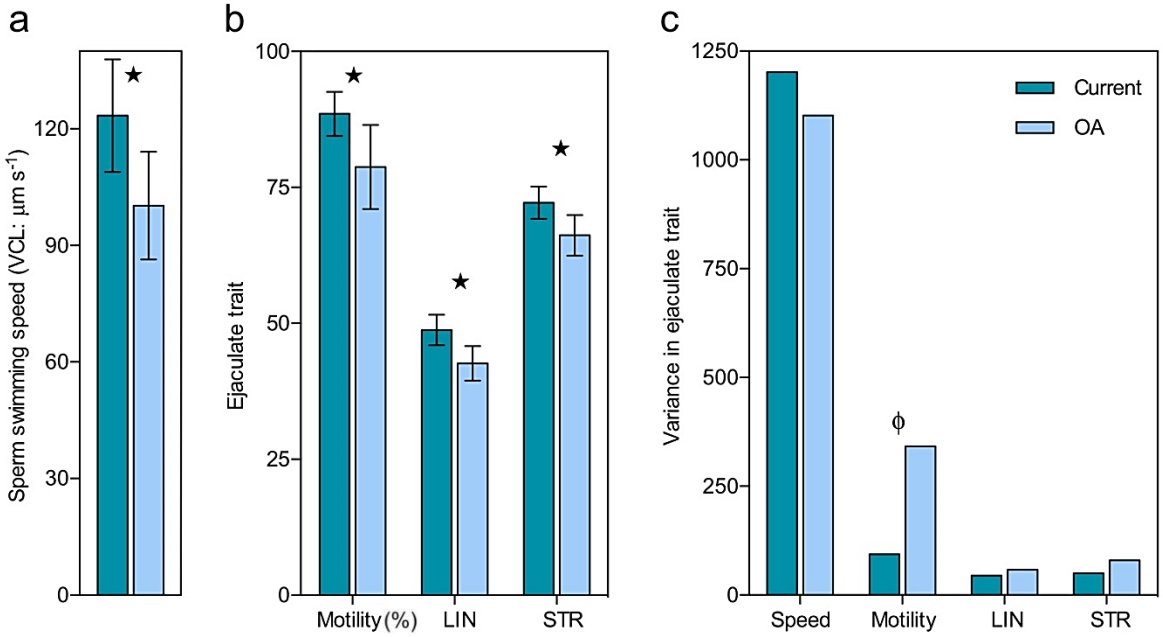

Figure 1. Ejaculate traits in current and OA seawater conditions. (a-b) Male sea urchin (n =22) ejaculate performance in seawater treatments (group means ± 95 % confidence intervals) and (c) the variance observed in each ejaculate trait. * Indicates the significant reduction in all ejaculate traits under OA conditions and ϕ indicates the significant increase in variance in sperm motility under OA conditions ($p \leq 0.05$). LIN =average sperm path linearity and STR =average sperm path straightness.

Marine sperm are generally stored immotile in the gonad. Upon release into seawater, the change in extrinsic environmental conditions triggers a chain of events leading to activation and the initiation of swimming. Intracellular pH plays a crucial role in the activation of marine invertebrate sperm swimming (Morisawa *et al.*, 1999) and determines the activity of enzymes which produce ATP to power swimming and drive flagella beating which propels a sperm forward (Nishigaki *et al.*, 2014). pH can

differentially affect freshwater sperm (Alavi and Cosson, 2005) but the natural ranges of pH for freshwater are much wider than relevant for seawater or OA. Internal sperm pH is presumed to be vulnerable to changes in seawater pH as sperm are single cells and have a greatly reduced cytoplasm which is thought to limit their pH buffering capacity. Consistent with this we found that our experimental OA conditions significantly changed sperm performance. Average swimming speeds were reduced by 18.8 % (Fig. 1a. Paired t-test: t = 3.692, df = 21, p = 0.001), and 9.8 % fewer sperm in an ejaculate were classed as motile (Fig. 1b. Wilcoxon signed-rank test: V = 3.088, df = 21, p = 0.015). Both ejaculate traits are important in models of population fertilisation ecology in external fertilisers (Styan, 1998). The reductions we observed could potentially reduce population level fertilisation rates with negative implications for population size and/or persistence.

Marine invertebrate sperm swim in circular or helical paths (Jikeli *et al.*, 2015). Physical models have revealed that this enables them to sense gradients in chemical cues released by unfertilised eggs most effectively (Friedrich and Jülicher, 2008). Following an initial search phase, if a sperm detects such a gradient, it then navigates towards the source in a phenomenon known as chemotaxis (Kaupp *et al.*, 2008). We assessed sperm swimming behaviour using computer assisted sperm analysis (CASA) and found significant reductions under OA in two CASA derived parameters which measure the linearity of sperm swimming paths; average path linearity (LIN: Fig. 1b. Paired t-test: t = 3.037, df = 21, p = 0.006) and average path straightness (STR: Fig. 1b. Paired t-test: t = 3.320, df = 21, p = 0.003). Whilst the exact repercussions of these reductions under OA are currently unknown, any change has the potential to influence fertilisation rates through alterations in sperm searching efficiency. Fitzpatrick *et al.* (2012) demonstrated the selective importance of curved sperm swimming paths for maximising fertilisation success, providing further evidence that this character influences male fitness. Although variance remained unchanged across seawater treatments in most ejaculate traits, there was a significant increase in variance in the percentage of motile sperm in a male's ejaculate under OA (Fig. 1c. Levene's test: F = 13.264, df = 41, p = 0.001. Table S1-1 online). So, in addition to an overall reduction in percentage sperm motility across males, differences between males were also amplified under OA conditions which could have implications for male competitiveness.

Table 1. Parameter estimates for the 'best fitting' GLMM of the influence of relative ejaculate traits (calculated as the focal male – his rival male) and seawater conditions upon the proportion of larvae sired by the focal male in paired competitive fertilisation trials [significant terms are highlighted in bold ($p \leq 0.05$)]. The model controlled for the random effects of pair identity and a dispersion parameter.

Fixed effects	Estimate (± SE)	df	Z value	Pr(>Z)
Intercept	0.037 ± 0.110	7	0.335	0.738
Relative sperm swimming speed (VCL)	**0.368 ± 0.094**	**7**	**3.897**	**<0.001**
Seawater conditions (OA)	**0.502 ± 0.159**	**7**	**3.159**	**0.002**
Relative percentage of motile sperm	**0.474 ± 0.165**	**7**	**2.868**	**0.004**
Relative percentage of motile sperm * seawater conditions (OA)	**-0.496 ± 0.194**	**7**	**-2.559**	**0.010**

We selected the generalized linear mixed-effects model (GLMM) which was most supported by the observed paternity share dataset (see Methods and Tables S1-2 to S1-5 online). The 'best fitting' model contained the following fixed effects; relative average sperm swimming speed (VCL) and an interaction between the seawater conditions and relative percentage sperm motility. This model was slightly underdispersed (model dispersion: 0.541). Results from our competitive fertilisation trials revealed a positive relationship between sperm swimming speed and paternity shares (Fig. 2a. GLMM: $p <$ 0.001. Table 1). Males with a speed advantage over their rival fertilised a greater proportion of the batch of eggs, and their relative reproductive success increased with larger speed advantages. Similar results have been reported in pair spawning fish (Evans *et al.*, 2013; Gage *et al.*, 2004) and internal fertilisers (Boschetto *et al.*, 2011; Gasparini *et al.*, 2010) but see (Lüpold *et al.*, 2012) providing tentative evidence that sperm swimming speed might provide a selective advantage under sperm competition across reproductive

modes. This positive relationship between speed and competitive fertilisation success held across seawater conditions. This provides previously missing empirical support for a long-held paradigm; that faster swimming speeds enhance sperm competitiveness in external fertilisers. Models containing the average swimming speed of the fastest 1, 5 or 10 % of motile sperm were not most supported by the observed paternity shares. Hence, the results from our trials do not provide support for an association between the faster sub-populations of sperm within a male's ejaculate and competitive fertilisation success.

The competitive fertilisation trials also revealed that paternity share was influenced by the percentage of motile sperm in a male's ejaculate (Fig. 2b). In current ocean conditions this meant ejaculates with more motile sperm secured greater paternity, a result that seems intuitive and aligns with the body of literature on sperm concentration and fertilisation success collected under non-competitive scenarios (Benzie and Dixon, 1994; Lillie, 1915). Interestingly, we identified a significant interaction between seawater conditions and the influence of percentage sperm motility on paternity (GLMM: p = 0.010. Table 1). Under OA conditions, the relationship between the percentage of motile sperm in an ejaculate and a male's competitive fertilisation success significantly weakened (Fig. 2c). The modelled data revealed that whilst males with more motile sperm than their rival still fertilised the majority of a batch of eggs (> 50 %) in OA conditions, the positive relationship between this ejaculate feature and paternity is lost. The consequences of this are far from clear, but changing the relationship between an ejaculate trait and male reproductive success under OA means OA is altering selection on males and their ejaculates.

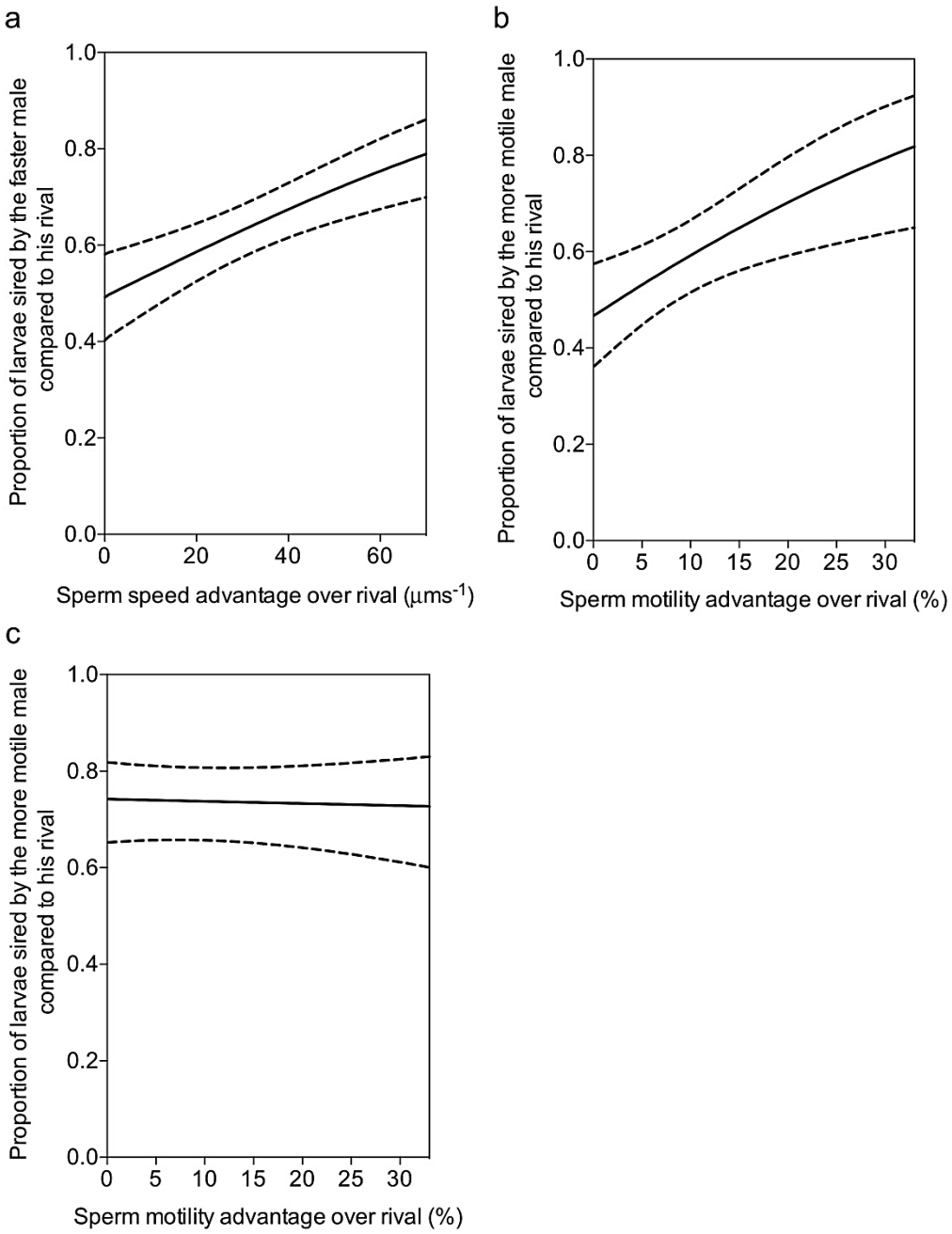

Figure 2. The GLMM modelled influence of relative male ejaculate traits on competitive fertilisation success. The modelled relationship between (a) average sperm swimming speed (VCL) and secondly the percentage of motile sperm in a male's ejaculate under current seawater conditions (b), and under future OA conditions (c), and the proportion of larvae sired by the focal male in paired competitive fertilisation trials (n =11 pairs) in the sea urchin *Paracentrotus lividus*. Predictions ±95 % confidence intervals were calculated using the 'best fitting' GLMM with all other model parameters kept at their observed median values.

We compared male performance ranks across seawater treatments to investigate whether relative ejaculate performance in current seawater conditions correlated with performance under OA. We found a significant correlation between male ranks by the percentage of motile sperm in their ejaculate between current and OA seawater conditions (Fig. 3a. Spearman's rank correlation: $r_s = 0.730$, df = 20, $p = <0.001$). Despite this correlation, there were many rank order changes illustrated by the crossing over of lines in Figure 3a. When males were ranked by the average speed of the motile sperm in their ejaculate, there was a similar association (Fig. 3b. Spearman's rank correlation: $r_s = 0.614$, df = 20, $p = 0.003$), but once again there were many rank order changes. This, together with the results of the competitive fertilisation trials, suggests that the identity of high fitness males could change as OA progresses. The positive relationship between the percentage of motile sperm in a male's ejaculate and competitive fertilisation success was lost under OA conditions. So, despite the correlation in male ranks for this ejaculate trait across seawater conditions, males with the greatest percentage of motile sperm might not secure the highest relative reproductive success under OA. The positive relationship between sperm swimming speed and paternity shares won in competition held across seawater conditions, but despite this association, there were substantial rank order switches which could generate changes in the identity of males securing reproductive success in future oceans.

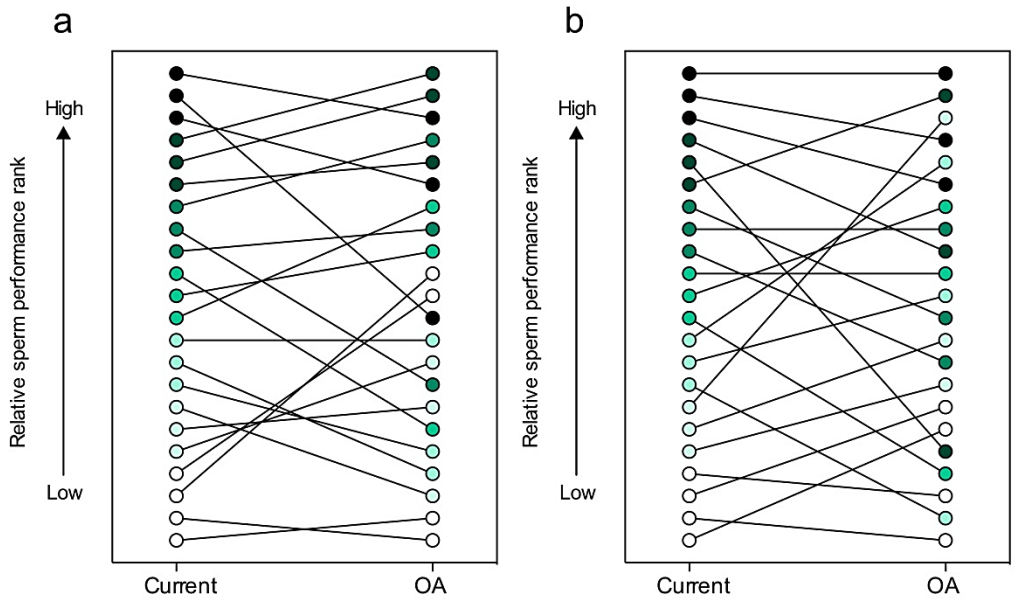

Figure 3. Male sperm performance ranks in current and OA seawater conditions. Male sea urchins (n =22) ranked by (a) the percentage of motile sperm in an ejaculate and (b) average sperm swimming speed (VCL). Points are coloured by rank in ambient seawater (darker colours represent higher ranks in ambient conditions i.e. faster speed or greater motility) and male ranks in the two seawater treatments are connected by lines.

A significant proportion of the paternity variation we observed could not be explained by a model composed of relative male ejaculate characteristics and our treatment seawater conditions. Both the fixed and random terms included in the 'best fitting' GLMM only explained 11.04 % of variation in the observed paternity shares. Therefore, there must be other parameters which contributed to sperm competition outcomes under the conditions of our fertilisation trials, which were not measured in our study. One accepted source of variation in sea urchin fertilisation rates is differences in gamete compatibility (Evans and Marshall, 2005). Gamete recognition proteins help sperm and eggs to identify one another and fuse (Vacquier, 1998). Eggs can show strong affinities to sperm with particular recognition protein genotypes, generating differences in male fertilisation rates (Palumbi, 1999), but we did not assess this here. In addition to roles in sperm

activation and determining the activity of enzymes involved in sperm swimming, intracellular sperm pH is known to be involved in other processes essential for fertilisation in marine invertebrates. These include sperm response to egg chemical cues, which either act to enhance sperm movement or aid sperm navigation towards an egg (Darszon *et al.*, 2008), and the acrosome reaction (Vacquier and Moy, 1997). Thus there is clear potential for additional OA impacts beyond those we measured here.

Seawater pCO_2 is more variable in coastal waters than open oceans (Hofmann *et al.*, 2011), and is often elevated in benthic habitats compared to surface waters where values of up to 2500 µatm have been recorded (Melzner *et al.*, 2013). High resolution data on current seawater pH and pCO_2 values for coastal benthic environments is limited and rarely linked to the location and timing of marine invertebrate spawning events, as it can be challenging to observe these often unpredictable and rare events. There is no current data on the seawater carbonate chemistry during a *P. lividus* population spawning event to provide us with details of the environmental conditions that their sperm currently compete within and inform our OA treatment conditions. Given this, we selected an OA treatment level for our study based upon the lower range of pH values projected under the Representative Concentration Pathway (RCP) 8.5 scenario for the year 2100 (Stocker *et al.*, 2013) in line with other studies on coastal benthic species. The conditions for sperm competition in complex natural fertilisation environments will be far more variable than in our simplified laboratory setup (Johnson *et al.*, 2012; Levitan, 1998), but our results provide a valuable first insight into the reproductive consequences of OA for external fertilisers under conditions of sperm competition. Populations of *P. lividus* may spawn into seawater conditions approximating our OA treatment within a relatively short timeframe (~100 years), which given the generation time of this species could limit the potential for evolutionary responses.

We have provided novel evidence that OA influences competitive interactions between males during fertilisation. We found that OA conditions reduced fundamental sperm performance parameters, caused some switching of male ranks by relative sperm performance and changed the influence of an ejaculate characteristic on sperm competitiveness. These changes are likely to be the tip of the iceberg with additional cascading effects yet to be identified. Importantly, the identity of competitive males and

the male trait combinations important for fitness are likely to change with OA, and hence we might expect a shift in the fitness landscape for males under future ocean conditions.

5.5 ACKNOWLEDGEMENTS

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5.6 SUPPLEMENTARY INFORMATION

5.6.1 Supplementary tables

Table S1-1. Statistical output of Levene's test for homogeneity of variance in male ejaculate traits across seawater conditions. Significant terms are highlighted in bold ($p \leq 0.05$).

Ejaculate trait	df	F	Pr(>F)
Percentage of motile sperm	**41**	**13.264**	**0.001**
Average sperm swimming speed (VCL) of all motile sperm	41	0.005	0.945
Average straightness of sperm path (STR)	41	1.411	0.242
Average linearity of sperm path (LIN)	41	1.624	0.210

Table S1-2. Performance of GLMMs containing the average sperm swimming speed (VCL) of all motile sperm as the relative speed term against our selection criteria. Models are ordered by ascending AICc values (a minimised AICc value was the primary selection criterion) and model dispersion is reported. The model with the highest performance against our selection criteria is highlighted in bold. The performance of models from tables S1-2 to S1-5 were compared simultaneously against our criteria, hence the sum of wi(AICc) for all 32 models is equal to 1. [SW= seawater conditions, M= the relative proportion of motile sperm and S= relative average sperm swimming speed (VCL) of all motile sperm]

Model fixed effects	df	AICc	Δ_i(AICc)	w_i(AICc)	Dispersion
SW * M + S	**7**	**167.4**	**0.00**	**0.273**	**0.541**
SW + M + S	6	168.5	1.16	0.153	0.360
SW * M + M * S	8	172.2	4.79	0.025	0.603
SW * M + SW * S	8	172.3	4.94	0.023	0.583
SW + M * S	7	172.4	5.04	0.022	0.392
SW * S + M	7	172.7	5.36	0.019	0.391
SW * S + M * S	8	177.4	10.07	0.002	0.422
SW * M + M * S + SW * S	9	177.9	10.51	0.001	0.653

Table S1-3. Performance of GLMMs containing the average sperm swimming speed (VCL) of the fastest 10 % of motile sperm as the relative speed term against our selection criteria. Models are ordered by ascending AICc values (a minimised AICc value was the primary selection criterion) and dispersion is reported. The performance of models from tables S1-2 to S1-5 were compared simultaneously against our criteria, hence the sum of wi(AICc) for all 32 models is equal to 1. [SC= seawater conditions, M= the relative proportion of motile sperm and S= relative average sperm swimming speed (VCL) of the fastest 10 % of motile sperm]

Model fixed effects	df	AICc	Δ_i(AICc)	w_i(AICc)	Dispersion
SC + M + S	6	168.9	1.56	0.125	0.316
SC * M + S	7	169.6	2.22	0.090	0.429
SC + M * S	7	173.0	5.67	0.016	0.337
SC * S + M	7	173.3	5.95	0.014	0.337
SC * M + SC * S	8	174.6	7.22	0.010	0.457
SC * M + M * S	8	174.7	7.29	0.007	0.459
SC * C + M * S	8	178.1	10.75	0.001	0.361
SC * M + M * S + SC * S	9	180.5	13.14	0.000	0.492

Table S1-4. Performance of GLMMs containing the average sperm swimming speed (VCL) of the fastest 5 % of motile sperm as the relative speed term against our selection criteria. Models are ordered by ascending AICc values (a minimised AICc value was the primary selection criterion) and dispersion is reported. The performance of models from tables S1-2 to S1-5 were compared simultaneously against our criteria, hence the sum of wi(AICc) for all 32 models is equal to 1. [SC= seawater conditions, M= the relative proportion of motile sperm and S= relative average sperm swimming speed (VCL) of the fastest 5 % of motile sperm]

Model fixed effects	df	AICc	Δ_i(AICc)	w_i(AICc)	Dispersion
SC + M + S	6	169.6	2.19	0.092	0.304
SC * M + S	7	170.9	3.56	0.046	0.390
SC * S + M	7	173.8	6.46	0.011	0.329
SC + M * S	7	173.9	6.49	0.011	0.322
SC * M + M * S	8	176.0	8.60	0.004	0.422
SC * M + A * S	8	176.0	8.60	0.004	0.421
SC * S + M * S	8	178.8	11.47	0.001	0.349
SC * M + M * S + SC * S	9	181.8	14.46	0.000	0.462

Table S1-5. Performance of GLMMs containing the average sperm swimming speed (VCL) of the fastest 1 % of motile sperm as the relative speed term against our selection criteria. Models are ordered by ascending AICc values (a minimised AICc value was the primary selection criterion) and dispersion is reported. The performance of models from tables S1-2 to S1-5 were compared simultaneously against our criteria, hence the sum of wi(AICc) for all 32 models is equal to 1. [SC= seawater conditions, M= the relative proportion of motile sperm and S= relative average sperm swimming speed (VCL) of the fastest 1 % of motile sperm]

Model fixed effects	df	AICc	Δ_i(AICc)	w_i(AICc)	Dispersion
SC + M + S	6	171.9	4.49	0.029	0.274
SC * M + S	7	173.9	6.57	0.010	0.330
SC * S + M	7	174.7	7.37	0.007	0.321
SC + M * S	7	176.3	8.89	0.003	0.292
SC * M + SC * S	8	178.5	11.10	0.001	0.369
SC * M + M * S	8	178.9	11.56	0.001	0.358
SC * S + M * S	8	179.4	12.02	0.001	0.363
SC * M + M * S + SC * S	9	183.8	16.44	0.000	0.423

Table S1-6. Measured and calculated (CO2SYS; Pierrot *et al.*, 2006) seawater parameters for experimental seawater conditions [experiments took place on 12.06.13 (a) and 12.06.14 (b)].

Conditions	Measured parameters				Calculated parameters					
	T (°C)	S	**pH**	**DIC (µmol kg^{-1})**	**TA (µmol kg^{1})**	***p*CO$_2$ (µatm)**	**HCO$_3^-$ (µmol kg^{-1})**	**CO$_3^{2-}$ (µmol kg^{-1})**	**Ω Ca**	**Ω Ag**
Current[a]	14 ± 0.1	35	8.19	2513.0	2770.8	446.5	2299.0	196.8	4.69	3.01
Current[b]	14 ± 0.1	34.9	8.16	2512.7	2754.9	476.0	2308.3	186.1	4.43	2.84
OA[a]	14 ± 0.1	35	7.73	2638.0	2688.9	1407.0	2509.4	74.4	1.77	1.14
OA[b]	14 ± 0.1	34.9	7.69	2593.0	2628.3	1528.7	2467.9	66.2	1.58	1.01

Table S1-7. Details of microsatellite markers used in the study (n=22 adult sea urchins).

Marker	Size range of alleles in base pairs (study observations combined with published data(Calderón *et al.*, 2009))	Estimated number of alleles identified in study population
PHIST	338-473	26
PIB	82-438	11
PIC	331-421	16
PIL	187-277	15
PI15	96-190	22
PIT	160-272	13

Table S1-8. Raw data table of the percentage of larvae sired by the focal male in paired competitive fertilisation trials and relative ejaculate traits in current and OA conditions.

Seawater conditions	Pair ID	Relative average sperm swimming speed (VCL: μms^{-1})	Relative percentage sperm motility (%)	Female ID	Percentage of genotyped larvae sired by the focal male (%)
Current	1	70.00	6.42	2	73.08
				4	89.29
	2	40.95	16.07	1	50.00
				3	68.00
				4	69.23
	3	62.75	3.69	1	64.29
				2	42.86
				4	81.82
	4	56.94	13.15	2	52.38
				3	66.67
				4	69.57
	5	55.61	9.63	1	62.50
				2	57.14
				4	68.00
	6	17.72	0.46	1	36.00
				3	39.13
				4	44.83
	7	20.1	3.45	2	20.00
				3	37.50
				4	60.00
	8	1.66	26.34	5	72.22
				6	52.63
	9	43.14	6.26	5	55.00
				6	68.18
	10	48.95	23.83	5	86.36
				6	85.00
	11	52.73	-5.20	5	90.48
				6	91.30
OA	1	-52.09	-17.35	2	47.83
				3	26.92
	2	36.00	9.18	1	95.24
				3	78.95
				4	100.00
	3	34.77	-1.76	1	69.57
				2	79.17
				4	79.17
	4	-10.09	-22.13	2	57.14
				3	46.43
				4	71.43
	5	45.62	26.76	1	47.83
				2	52.94
				4	61.11
	6	-35.94	-2.15	1	34.62
				3	73.08
				4	21.74
	7	38.88	1.88	2	69.57
				3	79.17
				4	50.00
	8	23.32	10.87	5	61.90
				6	27.27
	9	-4.92	18.93	5	47.62
				6	38.89
	10	14.99	33.69	5	85.00
				6	94.44
	11	35.5	13.80	5	76.19
				6	91.30

5.6.2 Supplementary methods

Seawater pCO_2 manipulation

Artificial seawater (Aquamarine Ltd) was filtered to 1 µm and made up to a salinity of 35 salinity units (psu). Additional salinity measurements were made using a Mettler Toledo SG7 SevenGo pro conductivity meter to an accuracy of ± 0.1 psu. Seawater pCO_2 was manipulated using a computerised control system (NBS scale, AquaMedic, Germany) which regulated pH via a solenoid valve and CO_2 injector in conjunction with vigorous aeration. Additional seawater pH measurements were taken using a Metrohm (827 pH lab) pH_{NBS} electrode and NBS buffers and once at the desired pH (± 0.02) seawater was stored in sealed containers lacking an airspace for use within 1 hour of collection. A seawater pH value of 7.70 was targeted in the OA treatment to represent near-future OA as projected according to scenario RCP 8.5 IPCC WGI AR5 (Stocker *et al.*, 2013; Meinshausen *et al.*, 2011). The artificial seawater used in this study had a relatively high alkalinity compared to natural seawater, and this additional buffering capacity resulting in a slightly higher pCO_2 (1468 µatm) in order to reach the Intergovernmental Panel on Climate Change (IPCC) relevant pH value than would be required for open water natural seawater. However, this level of pCO_2 is relevant for coastal values (Melzner *et al.*, 2013). Dissolved inorganic carbon (DIC) analysis was carried out upon seawater samples collected during each experiment using a custom built system described by Friederich *et al.* (2002) and following the methodology found in Lewis *et al.* (2012). This system allowed the measurement of seawater DIC with a precision of ± 3 µM. Additional seawater parameters; total alkalinity and pCO_2, were calculated using CO2SYS (Pierrot *et al.*, 2006) according to Findlay *et al.* (2013) and using the pH, salinity and DIC measurements along with the NBS scale and Dickson standards.

Assessment of ejaculate characteristics

Following incubation a small sub-sample of diluted sperm was transferred to Leja 20 mm standard counting chambers for analysis. Motility assessment took place using a Microptic Sperm Class Analyser (SCA®: Microm, UK) fitted with a Nikon Eclipse 50i

negative phase contrast microscope (100 x magnification) and a Peltier cooled stage which was operated at 14 ± 0.1 °C. Images were captured at a rate of 100 frames s^{-1} with individual sperm tracked for 0.5 seconds. A minimum of 500 sperm were tracked in each sample and a range of CASA derived motility parameters calculated for each sperm tracked using the SCA® Motility and concentration module. Samples were analysed once (one technical replicate) but we analysed a large number of sperm per sample (> 500) as previous work has indicated a high within-sample repeatability for CASA parameters using this approach (Fitzpatrick *et al.*, 2012). Threshold values of > 10 µm s^{-1} curvilinear velocity (VCL) and > 3.2 µm s^{-1} straight line velocity (VSL) were used to remove the influence of immotile sperm moving via capillary drift from subsequent analysis and to determine the percentage of motile sperm within each sample (representative of the ejaculate). We also calculated several additional speed parameters for each male; the average VCL of the fastest 1, 5 and 10 % of sperm. To achieve this sperm were ordered by ascending speed and split into speed percentiles. Sperm path linearity (LIN) and sperm path straightness (STR) were calculated by the SCA® CASA System [LIN: VSL/VCL and STR: VSL/ average sperm path velocity (VAP)].

Competitive fertilisations and larval paternity assignment

Fertilisations were allowed to proceed for 30 minutes in 20 ml of treatment seawater before fertilisation beakers were topped up to 350 ml with current seawater and incubated overnight at 14 ± 0.1 °C. Beakers were gently aerated from twelve hours post fertilisation and the incubation temperature increased to 18 ± 0.1 °C to enhance larval development. Larvae were fed an appropriate ration of *Isochrysis* algal paste on days 2 and 3. Larval development was terminated at the end of day 3 when cultures were filtered through a 60 µm mesh and larvae were re-suspended in 95 % ethanol and stored in microcentrifuge tubes at -20 °C alongside a 1 cm^2 section of gonad tissue dissected from each potential parent. Detailed methodology on paternity assignment can be found in Levitan (2008). In short DNA was extracted from individual larvae or adult gonad tissue, diluted and then amplified using fluorescently labelled primers via the polymerase chain reaction (PCR). Following amplification PCR products were genotyped using an Applied Biosciences 3730xl DNA Analyzer. The reaction products were visualised and

scored for six potential microsatellite loci (see Table S1-7 for information on microsatellite markers) using GeneMapper v. 3.5 software (Applied Biosystems, CA, USA). In order to assign paternity through the identification of inherited markers, a minimum of 2 of the most diagnostic loci for each potential set of parents were selected and screened for in the larvae.

R code

To calculate dispersion of a GLMM object:

```
overdisp_fun <- function(model) {
  ## number of variance parameters in
  ##   an n-by-n variance-covariance matrix
  vpars <- function(m) {
    nrow(m)*(nrow(m)+1)/2
  }
  model.df <- sum(sapply(VarCorr(model),vpars))+length(fixef(model))
  rdf <- nrow(model.frame(model))-model.df
  rp <- residuals(model,type="pearson")
  Pearson.chisq <- sum(rp^2)
  prat <- Pearson.chisq/rdf
  pval <- pchisq(Pearson.chisq, df=rdf, lower.tail=FALSE)
  c(chisq=Pearson.chisq,ratio=prat,rdf=rdf,p=pval)
}
#command to calculate the dispersion of a GLMM object
overdisp_fun(GLMMobject)
```

5.7 REFERENCES

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Discussion 6

General discussion

General discussion

In this body of work, I have explored some of the potential impacts of ocean acidification (OA) and other anthropogenic drivers of marine environmental change on freely spawned sperm functioning. I found that simulated OA conditions elicited a range of responses in externally fertilising sperm.

Sperm swimming responses to OA

I will begin by discussing functional sperm responses to OA in swimming performance. In the ecologically important coastal polychaete, *Arenicola marina*, I found that simulated OA conditions (pH 7.77, 1400 µatm pCO_2) reduced sperm swimming speeds (- 19 %) early in the motile phase. Percentage sperm motility was more robust to changes in seawater chemistry, varying by only ± 1 % across current conditions and elevated pCO_2 treatments up to 3000 µatm. In another set of exposures for the same study population of *A. marina* collected during a subsequent reproductive season, simulated OA conditions (pH 7.77, 1000 µatm pCO_2) did not induce any changes to the number or speed of motile sperm immediately after sperm activation. In exposures conducted for my final experimental chapter I observed significant reductions in early sperm swimming performance in the keystone sea urchin species, *Paracentrotus lividus* under simulated OA conditions (pH 7.71, 1468 µatm pCO_2). Both the number of motile sperm (-11 %) and the average swimming speed of sperm (-19 %) within an ejaculate were reduced under experimental OA.

Taken together, these findings provide tentative evidence of a threshold of sensitivity to elevated seawater pCO_2 for the sperm swimming performance of coastal echinoderms and polychaetes. Comparable OA treatments elicited identical percentage reductions in sperm swimming speed in both *A. marina* and *P. lividus*, whereas percentage sperm motility appeared more sensitive to this treatment level in *P. lividus*. Alternately, the differences in sperm swimming response to OA between the same *A. marina* study population in different reproductive seasons may not be related to the OA treatment

level, but may have been mediated through seasonal changes in food availability and temperature (Pansch *et al.*, 2014), or through the timing of gamete collection i.e. 'spawning readiness'. A better understanding of seasonal and annual variation in the carbonate chemistry, temperature and food availability that these populations are naturally exposed to and how it influences sperm sensitivities to OA would be required to really interpret these differences. This would involve extensive water sampling over many years however, which was well beyond the scope of this project.

Sperm physiological responses to OA

The systematic map (Chapter 2) highlighted a paucity of physiological investigations of sperm under OA conditions. This knowledge gap obstructs an understanding of the mechanisms underpinning sensitivity and tolerance, inhibiting our ability to predict responses beyond the small number of populations studied to date. I observed variable sperm physiological responses to OA in the experiments conducted as part of this thesis. The majority of the physiological parameters I investigated in *A. marina* sperm were not sensitive to OA as a single stressor (e.g. ATP content, viability, DNA damage). However, sperm oxygen consumption rates were sensitive to seawater pH/ pCO_2 conditions. Rates of oxygen consumption are a good proxy for aerobic ATP generation in the sperm mitochondria for motility, as mature sperm have lost their biosynthetic potential. I observed significantly higher oxygen consumption in *A. marina* sperm immediately after activation in current ocean conditions than in the simulated OA treatment (pH 7.77, 1000 µatm pCO_2). But one hour into the sperm exposures there was a switch, with sperm under OA now consuming oxygen at significantly higher rates than current sperm. My findings align well with one of the only other physiological investigations of sperm to OA. Schlegel *et al.* (2015) identified significant alterations to sperm mitochondrial membrane potential (MMP) in the sea urchin, *Centrostephanus rodgersii* at a similar OA treatment level (pH 7.80, 950 µatm). The MMP is generated through the activity of the mitochondrial respiratory chain; a major component of aerobic ATP generation. Taken together our findings strongly suggest that mitochondrial energy metabolism is sensitive to the changes in seawater chemistry associated with OA in both polychaete and sea urchin sperm. The consequences of these physiological alterations are currently unclear

but may be related to the motility declines I observed later in the exposures. There needs to be further experimental work to uncover the mechanisms underlying these alterations and any functional implications of disruption.

I would suggest future research is directed towards a thorough investigation of sperm enzyme activities (ATP synthase, creatine kinase, enzymes involved in the mitochondrial respiratory chain and dynein ATPase) alongside concentrations of cellular energy stores and shuttle molecules (creatine and phosphocreatine) in closely related species that vary in their tolerance of OA. Such work could help to uncover the physiological basis for motility perturbation. The development of single cell techniques, such as the recent advances that have enabled the measurement of ATP consumption of the eukaryotic flagellum of a single sea urchin sperm cell (Chen et al., 2015), will aid such work. Hopefully there will be further technological developments enabling the quantification of ATP levels in cellular compartments of sperm. This would increase our resolution to understand the production, delivery and use of ATP for sperm motility and quantify the concentration available to flagellar dyneins in current and projected future conditions.

Sperm viability did not appear to be challenged during the eight hour *A. marina* sperm exposures, which are presented in Chapter 4. *A. marina* has a remarkably long movement phase (Williams and Bentley, 2002) and it is highly unlikely that sperm longevity influences fertilisation rates in this species. Whilst a small number of studies have investigated the influence of seawater temperature rise on sperm longevity in a handful of sea urchin species (Rahman *et al.*, 2009; Binet and Doyle, 2013), there have been no corresponding studies for OA. A sperm's life span is presumed to be a function of its utilisation of endogenous energy reserves (usually phospholipids; Harumi *et al.*, 1990). Swimming fast and swimming for a long time both require energy and a trade-off between these two sperm traits appears likely. An inverse relationship between speed and longevity across male ejaculates was empirically demonstrated by Levitan (2000) in the sea urchin *Lytechinus variegatus*. Several authors have proposed that alterations to sperm swimming speeds under OA conditions may have knock-on consequences for sperm longevity i.e. reductions (if present) in sperm swimming speed might enable sperm to swim for longer periods. My findings do not provide support for this hypothesis.

I observed substantial reductions in swimming speed in *A. marina* sperm incubated for several hours under OA conditions (pH 7.77, 1000 µatm pCO_2). Rather than enabling sperm to swim for longer, these reductions were accompanied by significant numbers of sperm losing motility. There was also no change in sperm ATP content at this time-point (four hours into the exposures) suggesting that sperm that became immotile under OA conditions had not run out of energy reserves, leading me to query the mechanisms underlying the cessation of motility in this species. This finding agrees with work on *Crassostrea gigas* sperm, another marine external fertiliser with a remarkably long movement phase (Suquet *et al.*, 2010). The authors found that following a 20 hour movement phase, sperm that lost motility still contained 94 % of their initial energy reserves. Further experiments are required to establish the influence of the changing ocean chemistry for sperm longevity in species with varying swimming durations, alongside any interactions with projected warming.

Competitive fertilisation success under OA

In my final set of experiments (Chapter 5) I explored the implications of changes to sperm swimming performance under OA conditions (pH 7.71, 1468 µatm pCO_2) for competitive fertilisation success in *P. lividus*. I found that in current ocean conditions, both the number and speed of motile sperm in an ejaculate positively influenced sperm competitiveness, providing previously missing empirical support for this group. Whilst the relationship between sperm swimming speed and competitive fertilisation success held under the experimental OA conditions, most interesting was the change in the relationship between percentage sperm motility and male success at securing paternity in the paired competitive fertilisation trials under OA. This, in addition to changes in male sperm performance ranks, suggests that the 'best' males now may not necessarily be the best under OA. This has potential implications for male reproductive success in future oceans, through changes in the proportion of offspring contributed by each individual under the changed environmental conditions. Sperm-related traits have been shown to respond rapidly to selection (Hosken and Ward, 2001; Nandy *et al.*, 2013) but for an evolutionary ejaculate response to OA there are certain requirements; (I) that sperm competitiveness under OA conditions has an additive genetic basis and (II) that the genes

that code for traits influencing competitive fertilisation success can be transmitted from father to son. The heritability of sperm traits in marine external fertilisers is largely unknown due to difficulties culturing multiple generations under laboratory conditions. Marine invertebrates tend to have relatively long generation times, meaning that several years may pass before the F1 generation reproduce. They also tend to have biphasic life histories encompassing a larval stage in the seawater column, and an adult stage in the benthos, which requires the provision of settlement cues to ensure larvae successfully settle and metamorphose. A recent study in field crickets provides some phenotypic support for father to son transmission of sperm competitive ability (McNamara *et al.*, 2014), but this has yet to be established in a marine species. Selection might act differently on some of the several different life stages marine invertebrates undergo (Kroeker *et al.*, 2013b). Hence, strong selection at an early stage could act to reduce phenotypic plasticity through carry-over effects to later stages (Sunday *et al.*, 2014). Such a process might act to reduce population persistence in the face of further environmental change and the potential consequences need to be thoroughly assessed.

On further reflection, an alternative explanation of the results presented in Chapter 5 of this thesis is that OA may act to strengthen selection on percentage sperm motility (see Figure 2b-c on page 222). Whilst the positive relationship between the percentage of motile sperm in a male's ejaculate and competitive fertilisation success found in ambient conditions was lost under OA, this was replaced with a new flattened relationship that was fixed at high paternity shares. So males with an advantage over their rival in the concentration of motile sperm in an ejaculate had higher competitive success under OA, but with no gain in fitness at larger percent motility advantages. One disadvantage to the study is that male ejaculate traits were only measured once in each seawater condition, hence between-sample variability could not be quantified. The possibility exists that the crossing over of lines in Figure 3 (Chapter 5 page 224), representing the change in relative male ranks by ascending values of ejaculate traits, may be driven solely by within-ejaculate variation and may not represent a change that is driven by OA. In this case, we identified a strong correlation in relative male ranks between current and OA conditions, which would increase our power to predict the high fitness males under OA.

Internal sperm pH

The introductory review (Chapter 1) highlighted countless biochemical processes involving sperm internal pH (pHi) for a typical freely spawned sperm. This review also revealed numerous enzymes essential to sperm function, whose activity was strongly influenced by the pH conditions inside a sperm cell. A fundamental research question that currently remains unanswered is whether reductions in external seawater pH influence sperm pHi, or whether sperm are capable of regulating their internal pH against seawater pH change. During my PhD I developed techniques to monitor sperm pHi during short-term changes in external pH to assess pHi regulatory ability. I developed techniques to monitor sperm pHi using the cell permeable fluorescent dye SNARF-1-AM, whilst continuously perfusing cells with seawater. Emissions ratios of the dye at two wavelengths, used as an indicator of internal cell pH, were monitored before, during and after a switch in the perfusion medium from ambient seawater to simulated OA conditions and back again. Unfortunately, these techniques required significant development and this work is currently ongoing. Data for two sea urchin species, *P. lividus* and *Psammechinus miliaris*, is still being processed and it is too early to discuss the results. I also have associated sperm swimming data at the range of seawater conditions at which I measured sperm pHi response. I will compare sensitivities between these two species in both sperm swimming and sperm pHi response. I aim to publish my results within the next 6 months, and this work will hopefully significantly further our understanding of the mechanisms of sperm functioning under OA.

Another fundamental research question that is currently unanswered; is whether sperm are equally sensitive to each aspect of the changes in seawater chemistry projected under OA, or whether they are more sensitive to perturbation by changes in one of the carbonate system variables. Oceanic uptake of CO_2 results in several co-varying inorganic carbonate system variables, of these there has been an explicit focus by studies on pH. Waldbusser *et al.* (2015) applied unique chemical manipulations of seawater to decouple the carbonate system parameter-covariance and identified that mussel larvae were most sensitive to reductions in the saturation state of calcium carbonate rather than to pH or pCO_2. I think a similar approach could be successfully applied to sperm, to disentangle the influence of elevated concentrations of hydrogen ions and pCO_2 levels in OA- sperm

motility perturbation for sensitive species. Sung *et al.* (2014) used a less sophisticated approach to manipulate seawater chemistry. The authors found that *Strongylocentrotus nudus* fertilisation was more sensitive to seawater pCO_2 than pH, and that this was mediated via effects on sperm, but not sperm motility which was robust to changes in both seawater parameters.

Variation in sperm phenotype

Sperm are the most diverse cell type known, with dramatic evolutionary divergence in sperm from nearly all taxa (Pitnick *et al.*, 2009). Sperm phenotype varies between species, populations, males, ejaculates of the same male and within the same ejaculate. I found that coping with this variation was a key challenge to sperm research. Within-ejaculate phenotypic variation is always present and may be substantial (Immler *et al.*, 2008). Only a small number of sperm succeed in reaching and fertilising an egg, and current techniques do not allow a thorough investigation of the phenotypes of successful sperm. Techniques to sequence the genome of a single cell have recently been developed (multiple annealing and looping-based amplification cycles; Zong *et al.*, 2012) and applied to single human sperm cells to investigate the genetic diversity created by meiotic recombination (Lu *et al.*, 2012). These techniques hold great promise for future research into sperm. We currently lack an understanding of the adaptive significance of the variation within a single ejaculate despite recent scientific interest (Higginson and Pitnick, 2011). The ecological conditions characterising marine fertilisation environments are likely to be highly variable on both spatial and temporal scales, with a large proportion of this variability unpredictable for the adult male. Recent work has indicated that the sperm traits that confer superior fitness through enhanced fertilisation success may be highly context dependent (Johnson *et al.*, 2012). The highly heterogeneous nature of fertilisation environments encountered by free spawned sperm may result in a spatial and temporal mosaic of selective pressures under which no single sperm phenotype does best. The disruptive selection generated by such a mosaic might be responsible for maintaining within-ejaculate variability in sperm phenotypes in a male 'bet-hedging' strategy (Johnson *et al.*, 2012; Manier and Palumbi, 2008). Single cell

techniques might help uncover the phenotypes that do best under each set of conditions to help understand which cells are most robust to OA.

The distribution of swimming speeds within a single ejaculate follows a negatively skewed distribution, with the majority of sperm slow swimming, some sperm that swim fast and a very small number of sperm that swim extremely fast. Several studies have attempted to group sperm into 'sub-populations' based upon their swimming behaviour (Quintero-Moreno *et al.*, 2003; Abaigar *et al.*, 1999) often using automated or semi-automated methods to select the number of sub-populations and the thresholds of characteristics that define them. Mean values of ejaculate traits may be less informative in predicting paternity than some maximum values (Holt and Van Look, 2004). In Chapter 5 I ran a series of paired competitive fertilisation trials in *P. lividus* under current and simulated future ocean conditions. When modelling the relationship between relative male ejaculate traits and seawater conditions on paternity shares, I found that model fit was not improved by substituting average sperm swimming speed for the fastest 1, 5 or 10 % of sperm in an ejaculate. Hence, the results from these trials do not provide support for an association between the faster sub-populations of sperm within a male's ejaculate and competitive fertilisation success under the trial conditions.

Spatial and temporal pH dynamics

Today, surface ocean pH varies by 0.6 units around the globe (Stocker *et al.*, 2013), and seasonal and diurnal pH fluctuations can range by as much as 0.5- 1.0 unit respectively (Melzner *et al.*, 2013; Duarte *et al.*, 2013). Hence, in many systems present day pH variation may exceed the projected global decrease in open ocean pH by 2100. Whilst, OA is the dominant driver of long-term pH change in the open ocean (Stocker *et al.*, 2013), there are a multitude of drivers influencing the pH of coastal systems complicating the detection and attribution of pH change resulting from anthropogenic CO_2 emissions (Duarte *et al.*, 2013). There is currently a chronic lack of high resolution pH data specific to the natural environment a population inhabits (Hofmann *et al.*, 2011). The technology to remotely monitor ocean salinity and temperature is widely accessible, relatively cheap and easy to deploy. Similar reliable technology has only recently become available for

seawater pH measurements (e.g. the SeaFET™ ocean pH sensor) and the technology is much less affordable. Huge leaps forward were made during an incentivised global challenge to create affordable, accurate and efficient technology to measure ocean chemistry at any depth. The Wendy Schmidt Ocean Health XPRIZE offered a 2 million dollar prize to the winning design generating substantial research effort and innovative design. The commercialisation of the new affordable technology would greatly aid efforts to monitor coastal pH dynamics.

Knowledge of spatial and temporal pH dynamics can inform the design of OA experiments by characterising 'current' conditions and providing some *a priori* expectations of an organisms tolerance based upon the natural range to which they are currently exposed. The pH dynamics of coastal systems are not captured adequately by current models projecting future change. Many of the species studied to date are inter-tidal or coastal species, and the difficulties in modelling coastal pH leaves scientists uncertain of future trajectories to inform experimental design. High resolution data on current seawater pH and pCO_2 values for coastal benthic environments is limited and rarely linked to the location and timing of marine invertebrate spawning events, as it can be challenging to observe these often unpredictable and rare events. There is very little current data on the seawater carbonate chemistry during population spawning events to provide us with details of the environmental conditions that freely spawned sperm currently experience to inform control conditions.

Marine fertilisation environments

The ecological conditions characterising the marine fertilisation environments in which freely spawned sperm operate are likely to be highly variable ranging in competition intensity, gamete age and sperm concentration (Johnson *et al.*, 2012; Levitan, 1995) both between and within populations. However, there is currently very little field data characterising ecological conditions during natural spawning events. There are numerous methodological considerations when conducting fertilisation assays. These include the selection of vessel type, seawater volume, the number of competing male ejaculates, sperm to egg ratio(s) and gamete contact time. Similarly for sperm motility analyses

researchers must select the time(s) between sperm activation and motility observations and the presence or absence of egg-related compounds. Due to the paucity of information regarding the fertilisation dynamics of natural populations, it is currently unclear how accurately the methodologies selected by researchers mimic natural conditions. Such methodological decisions can influence the result of experimental manipulations to assess the influence of environmental change for free spawning species. Several investigations of external fertilisation success have found the influence of OA to be sperm concentration dependent (Gonzalez-Bernat *et al.*, 2013; Ericson *et al.*, 2010) i.e. the magnitude and/or presence of effects depended on the sperm to egg ratio, and this agrees with the wider ecotoxicology literature (Hollows *et al.*, 2007). The sperm traits that confer the greatest fitness may also vary depending on the ecological conditions. For example Fitzpatrick *et al.* (2012) found that slower swimming sperm were at a selective advantage at low sperm densities in the mussel *Mytilus galloprovincialis*, presumably through a trade-off with sperm longevity. Whilst several studies report the fitness benefits of faster swimming sperm in both competitive (Gage *et al.*, 2004; Evans *et al.*, 2013) and non-competitive contexts (Levitan, 2000; Kupriyanova and Havenhand, 2002) at higher sperm densities.

My key findings from Chapter 4 add to mounting evidence that methodological considerations can influence the outcome of perturbation experiments of external fertilisation. Disruption of *A. marina* sperm motility developed during the exposure to OA conditions. Hence, our conclusions on the influence of OA for *A. marina* sperm swimming performance would vary depending on the time-point selected for motility analysis. Because of *A. marina's* unusual reproductive strategy, the declines I observed in the number and speed of motile sperm under OA may have taken place within an ecologically relevant timeframe adding to mounting evidence that researchers should incorporate population-specific fertilisation ecology into the design and interpretation of perturbation experiments. There is clearly a need for sampling and monitoring programs to understand the ecological conditions characterising spawning events for current populations. Whilst these are likely to be highly variable, knowledge of the median and range of conditions currently experienced by the gametes of populations in question

would greatly improve our ability to uncover and predict the reproductive consequences of future environmental change.

The complex components of global environmental change

Environmental stressors rarely act in isolation, instead they co-occur with other natural or anthropogenic stressors, which may synergistically interact to negatively affect the health and fitness of organisms (Vinebrooke *et al.*, 2004; Kroeker *et al.*, 2013a). Recently there has increasingly been a shift towards multi-stressor studies, in the light of the complex components of global environmental change and the high likelihood of interactions amongst them (Harley *et al.*, 2006). However, the systematic map (Chapter 2) highlighted that multi-stressor studies are still in their infancy in regards to freely spawned sperm. To date there have been investigations into only a few of the array of potential stressor combinations, for a small number of species and experimental endpoints. This knowledge gap compromises our understanding of sperm functioning in future oceans. As sperm play a central and critical role in reproduction, understanding the impacts of environmental change on sperm function is fundamental to projecting population-level effects.

I commenced my experimental work for this thesis by addressing this knowledge gap and investigating potential interactions between two co-occurring stressors for *Arenicola marina* sperm along with other *A. marina* early life history stages (Chapter 3). My findings provide empirical evidence that the projected changes in ocean carbonate chemistry under OA could significantly alter the susceptibility of *A. marina* sperm and larvae to the common coastal pollutant copper. Intriguingly negative synergisms were not observed for all experimental endpoints, and the toxicity response depended on the early life stage and endpoint investigated. This work highlighted that sperm and larvae may be the most sensitive early life stages to enhanced copper toxicity as OA progresses, with heightened sperm DNA damage and decreased early larval survivorship in combined treatments.

I exposed each *A. marina* life history stage separately to the combined stressors to compare sensitivities and identify the most vulnerable stages. Future research endeavours should focus on environmentally realistic exposures conducted over successive life history stages. This should enable the identification of potential carry-over effects under ecologically relevant scenarios i.e. an accumulation of effects across successive stages that may result in later vulnerability to further stress as a consequence of exposure earlier in development (Pechenik, 2006). I would also advise future multi-generational studies to investigate the influence of parental environmental history on gamete and offspring performance under combined copper and OA scenarios as this may play some role in adaptive responses (Foo and Byrne, 2016). In a recent study, transgenerational responses to OA were assessed in the calcifying tube-worm *Hydroides elegans* comparing the influence of paternal and maternal pH environments for offspring fitness. This species has a relatively short generation time and has been observed to spawn 16 days post settlement at seawater temperatures above 20 °C (Arnold, 2002). Hence, it may be a feasible model species in which to test hypotheses on changing seawater conditions across multiple generations in a free spawning marine invertebrate.

Marine environments are complex, and there are a myriad of possible stressor combinations that could be tested in complex and hence unfeasible fully crossed multi-factorial experimental designs. One solution to this challenge, was adopted by Boyd *et al.* (2015) in their study of the physiological responses of a subantarctic diatom to complex future ocean scenarios. They proposed a collapsed factorial experimental design to investigate the combined effects of multiple co-occurring factors alongside the individual influence of a controlling environmental variable i.e. the driver predicted to be of scientific interest or to have the strongest effect based upon *a priori* information. For future ocean scenarios this involves testing projected future values of oxygen content, seawater pH/pCO_2, nutrient levels and light intensities in combination, whilst dissecting out the influence of a proposed dominant factor, which in the case of Boyd *et al.* (2015) was presumed to be temperature. The authors selected four treatments; current ocean conditions, future ocean conditions, future ocean conditions (without temperature) and future ocean conditions (only temperature). Of course the success of this method relies upon the accuracy of projections of future ocean conditions, as the influence of each

factor is not assessed individually or in combination with every other factor, so responses cannot be extrapolated outside of the treatment levels selected. I think this approach could be successfully applied to freely spawned sperm. This would require knowledge on the current conditions and levels of existing stressors characterising a population's natural habitat alongside accurate projections of future conditions. This technique would enable the evaluation of freely spawned sperm performance in simulated future conditions, and allow the individual influence of a dominant factor such as OA to be assessed.

6.1 CONCLUDING REMARKS

It is perhaps surprising given how important sperm are to marine invertebrate life histories, that we know so little about them, the conditions in which they currently function and the potential for the changing seawater chemistry projected under OA to impact their ability to function. This body of work has highlighted the importance of incorporating greater environmental realism into experiments designed to uncover the reproductive consequences of marine environmental change. Whilst tailoring experiments to a specific population limits the wide applicability of any conclusions, the variability inherent to natural environments, and hence marine fertilisation environments, necessitates this approach. There is evidently much work required in order to better understand the fertilisation dynamics of current populations of free spawners, and the ecological and environmental conditions characterising natural spawning events. Such information allows the characterisation of the conditions sperm currently experience on their journey towards an egg, which can then be used to inform appropriately designed controls and methodological decisions that may be decisive in the overall conclusions.

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