

Focus

# Reliably estimating the effect of toxicants on fertilization success in marine broadcast spawners

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## Abstract

Recently, there has been a dramatic increase in the number of ecotoxicological studies examining the effects of toxicants on fertilization success in marine broadcast spawners and it appears that this life-history stage is one of the most vulnerable to toxicants. Most of the studies examining this issue use single sperm concentrations in their assays. Here, I discuss recent advances in fertilization ecology that suggest this technique has some severe limitations resulting in unreliable estimations of the size and direction of toxicant effects. I present an alternative assay technique and two metrics ( $F_{\max}$  and  $[\text{Sperm}]_{\max}$ ) that will reliably estimate the size of a toxicant's effect on fertilization success. This technique has the added advantage of making comparisons among species and studies easier without an impractical increase in effort.

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## 1. Introduction

Most marine invertebrates reproduce by releasing eggs and sperm into the water column, whereupon they meet and fertilization takes place externally. For over twenty years, ecotoxicologists have examined the effects of anthropogenic toxicants on gametes and fertilization in marine broadcast spawning invertebrates (Dinnel et al., 1987, 1989). Recently, the number of such studies has dramatically increased (Zuniga et al., 1995; Vaschenko et al., 1999; Negri and Heyward, 2000, 2001; Harrison and Ward, 2001; Ross and Bidwell, 2001; Novelli et al., 2002, 2003; Negri et al., 2005; Reichelt-Brushett and Harrison, 2005; Reichelt-Brushett and Michalek-Wagner, 2005; Victor and Richmond, 2005; Xie et al., 2005). Reasons for this increase include: (i) the realization that early life-history stages appear to be far more sensitive to toxicants than adult stages (e.g. Xie et al., 2005); (ii) the increased recognition that fertilization success in marine invertebrates is

rarely 100% and has the potential to limit population growth (Levitan and Petersen, 1995; Yund, 2000; Marshall, 2002); and (iii) assays on fertilization are quick and convenient relative to other larval assays (Ringwood, 1992). Most studies on the effects of toxicants and pollutants on fertilization show large reductions in fertilization success leading to concern regarding the longer term effects of pollutants on population replenishment in broadcast spawners (references above). These studies have played a valuable role in highlighting the importance of examining the most sensitive and important phases in the life-history of the study organism. However, recent advances in fertilization ecology suggest that the experimental methods used in the majority of these studies are inappropriate and severely limit our ability to make interspecific comparisons and generalizations.

Earlier ecotoxicological studies on fertilization in broadcast spawners used a range of sperm concentrations in their assays (e.g. Dinnel et al., 1987; Ringwood, 1992). However more recently, most studies use only a single sperm concentration for determining how a toxicant reduces fertilization success (e.g. Zuniga et al., 1995; Negri et al., 2005;

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Reichelt-Brushett and Harrison, 2005; Reichelt-Brushett and Michalek-Wagner, 2005; Xie et al., 2005). Whilst the use of a single sperm concentration is quick and convenient, there are a number of major problems associated with this approach which arise from the specific biology and kinetics of fertilization in free-spawners.

**2. Fertilization curves in broadcast spawners**

A number of factors such as egg size and egg concentration have been shown to affect fertilization success somewhat, but overwhelmingly, the most important factor is sperm concentration (Levitan et al., 1991; Levitan, 1991, 1996; Marshall et al., 2000). This is because, as the sperm concentration increases, the chances of eggs and sperm contacting each other also increases and fertilization success is proportional to sperm–egg contact rates (Vogel et al., 1982; Styan, 1998; Millar and Anderson, 2003). This fits well with the predictions of the original models of fertilization kinetics in marine broadcast spawners: fertilization success increases quickly with sperm concentration, until fertilization plateaus at 100% (Vogel et al., 1982; Fig. 1). However, more recently it has been recognized that there can also be too many sperm. When this happens, fertilization success does not actually reach 100%: instead, success drops sharply once the sperm concentration exceeds a certain point so that fertilization curves are bell-shaped (Styan, 1998; Marshall et al., 2000; Styan and Butler, 2000; Millar and Anderson, 2003; Fig. 1). This

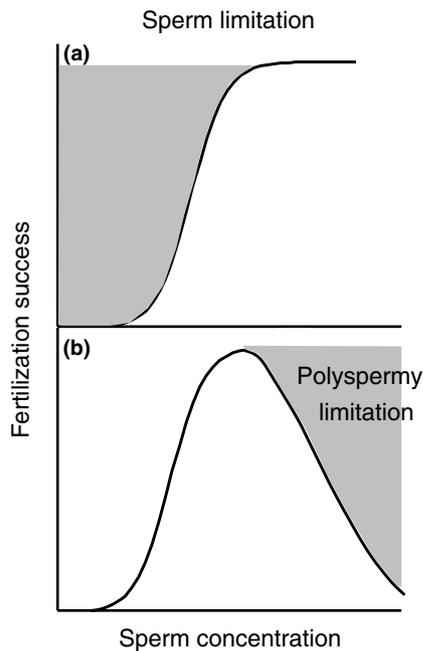


Fig. 1. Schematic of the relationship between sperm concentration and fertilization success. Panel (a) shows the traditional view of fertilization success and panel (b) shows the current view including the effects of polyspermy. Note in panel (b) that on the left side of the curve, fertilization success is limited by sperm availability and on the right side, limited by polyspermy.

decrease in fertilization success at high sperm concentrations is due to polyspermy—multiple sperm enter the egg before it creates a block to excess sperm, and the egg becomes unviable (the timing of blocks varies among phyla). The fact fertilization success can increase or decrease with a reduction in egg–sperm contact rates makes the use of single sperm concentrations in ecotoxicological assays inappropriate. To illustrate, consider the examples shown in Fig. 2. In each of the three situations, the addition of a toxicant reduces the number of egg–sperm interactions (either by killing sperm or reducing sperm–egg contact efficiency). Depending on the sperm concentration and ‘where’ that concentration is on the fertilization curve for that species, very different results will be found, both in magnitude and direction (Fig. 2). Indeed, Dinnel et al. (1987) found that the effects of their toxicant were much greater at low sperm concentrations than at high sperm concentrations. Thus, despite the

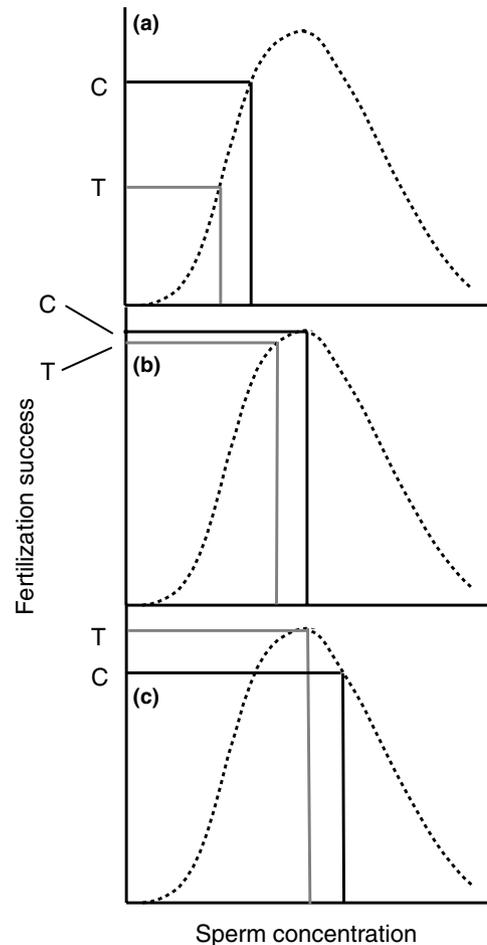


Fig. 2. Some of the potential problems with using a single sperm concentration to examine the effects of toxicants on fertilization—despite the actual the effect in each panel being identical, the direction and magnitude of the effect appears to be different depending on what sperm concentration is used. In panel (a), an low sperm concentration is used so it appears that the treatment causes a large reduction in fertilization success, in panel (b) an intermediate sperm concentration is used and so there appears to be little effect of the treatment and in panel (c) a high sperm concentration is used so it appears that the treatment has a benefit for fertilization because polyspermy has been reduced.

toxicant having identical effects (i.e. reducing sperm–egg contact rates by a constant amount) one could easily misinterpret the effects, or at the least underestimate the magnitude of the effects.

One potential solution to the above problem is to do a series of sperm concentrations for a single species, determine a concentration that is on the left side (i.e. sperm limiting side) of the curve and then conduct a single sperm assay at that concentration (a technique originally suggested by Dinnel et al., 1987). However, this still has a number of problems. First, toxicants can affect different aspects of the fertilization process and these effects can manifest in identical ways – i.e. a drop in fertilization success. It is tempting to think that specific mode of action of the toxicant is irrelevant for making ecological predictions but further consideration suggests otherwise. If a toxicant kills sperm or reduces the proportion of sperm–egg contacts that result in fertilization, (e.g. Franchet et al., 1999), then one would predict that low density populations (where fertilization success is sperm limited) will be strongly affected by the toxicant in the field. On the other hand, if the toxicant impedes polyspermy blocks (e.g. Franchet et al., 1997), high density populations where polyspermy is a problem (e.g. Brawley, 1992; Franke et al., 2002; Marshall, 2002), would suffer much larger reductions in fertilization success than sperm limited populations.

The second problem with using a single, ‘ideal’ sperm concentration is that it makes comparison among different studies/species impossible. Different species require different concentrations of sperm in order to achieve equivalent fertilization success (Levitan, 2002). This means that different sperm concentrations for different species are necessary. However, this creates an important problem. Sperm age more slowly at higher concentrations (termed the respiratory dilution effect, Levitan, 1995) and so if two studies use different sperm concentrations, then they will also effectively be using two different exposure times because in one study, the eggs will be exposed to live sperm for longer. Given that fertilization success is strongly affected by the amount of time gametes are exposed for, this can make comparisons among studies highly problematic.

### 3. Appropriate measures of fertilization

Given the problems I have described above, what is the solution? Any metric and assay on the ecotoxicological effects on fertilization should contain the following components:

1. A resistant, independent metric of the effect of toxicant on fertilization (i.e. one that does not depend on ‘where’ on the fertilization curve one is for determining effect sizes).
2. A metric that provides information regarding what aspect of fertilization is being affected.
3. An assay that does not confound sperm–egg contact times among different species.

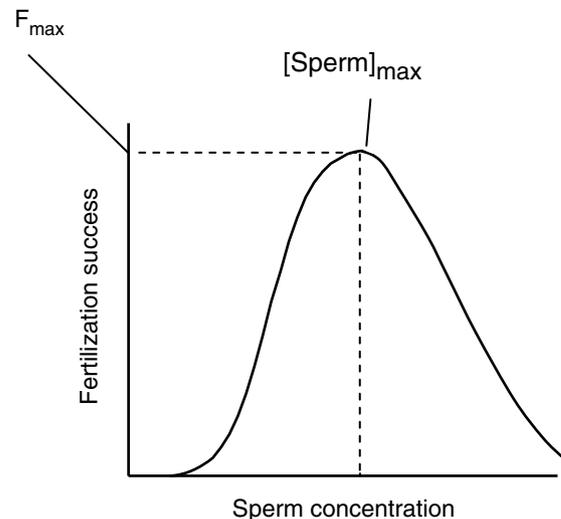


Fig. 3. Schematic showing the graphical representation of  $F_{\max}$  and  $[\text{Sperm}]_{\max}$ .

Fortunately, there is a straightforward assay and pair of metrics that meet these demands. Rather than using a single sperm concentration for a fertilization assay, I suggest the use of serial ten-fold dilutions, starting from the maximum sperm concentration obtainable down to  $\sim 1$  sperm  $\mu\text{L}^{-1}$ . From this assay, a fertilization curve can be constructed graphically for each of the experimental conditions (Fig. 3). Using this curve, one can then estimate the maximum fertilization success ( $F_{\max}$ ) that was achieved and the sperm concentration that maximized fertilization success ( $[\text{Sperm}]_{\max}$ ). These two measures effectively summarize the important components of the fertilization curve without the time-consuming parameterization of a fertilization kinetics model. Thus far, the use of  $F_{\max}$  and  $[\text{Sperm}]_{\max}$  has been restricted to ecological studies of fertilization (Marshall et al., 2000; Styan et al., 2005) but I suggest that they would have great utility in ecotoxicological studies generally.

Using these two metrics, one can determine the precise nature of the effect of the toxicant, use a standardized effect size and control for any differences in sperm concentration requirements among species. To illustrate, consider three examples (Fig. 4). These examples are based on a simplified fertilization kinetics model (Eq. (16) in Styan, 1998) and to explore the various effects of a toxicant on fertilization, I allowed sperm concentration, time until the polyspermy block occurs and egg viability to vary. For Fig. 4(a), the treatment shifts  $[\text{Sperm}]_{\max}$  to the left and  $F_{\max}$  downward. This indicates that the treatment has reduced the effectiveness of the polyspermy block (for an explanation of why this is case, see Millar and Anderson, 2003). In Fig. 4(b), the treatment shifts  $[\text{Sperm}]_{\max}$  to the left but  $F_{\max}$  is unchanged. This suggests that either sperm are being killed or the efficiency of sperm–egg interactions has been reduced (the ecological consequences are identical regardless of which is affected). Finally, if  $F_{\max}$  decreases but

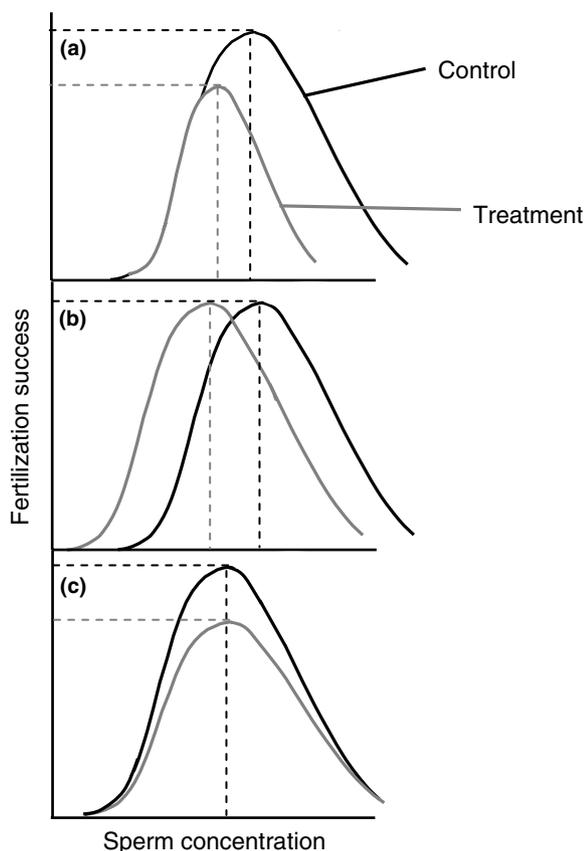


Fig. 4. Schematic showing the potential effect of toxicants on fertilization success and the subsequent predicted shift in  $F_{\max}$  and  $[\text{Sperm}]_{\max}$ . Panel (a) shows the predicted effect if the toxicant affects the efficiency of polyspermy blocks and Panel (b) shows the predicted effect if the toxicant affects sperm viability or sperm–egg interactions. Panel (c) shows the predicted effect if the toxicant affects egg viability only.

$[\text{Sperm}]_{\max}$  is unchanged, this suggests the toxicant is affecting egg viability only (Fig. 4c). As an additional improvement, I would also suggest that future assays use a constant, standard sperm–egg contact time of 1 h is used before rinsing the eggs in filtered seawater. By doing so, sperm–egg contact times will be kept more similar among different studies (although it should be noted that there may still be intrinsic, interspecific differences in sperm longevity). Finally, given that sperm and egg compatibility is highly variable among different individuals (Evans and Marshall, 2005), I suggest that the sperm of multiple males be used for each assay to reduce variation among experimental runs.

I believe that the use of the technique and metrics that I describe above will allow for more rigorous comparisons among different studies. These metrics are not sensitive to differences in egg viability among different studies (a potential problem if naturally spawned eggs are not used). Furthermore, they make no assumptions regarding the likely concentration of sperm in the field for any one species, rather they show the effect of the toxicant of interest across what is likely to be the full spectrum of concentrations. Most ecotoxicological studies use standardized effect sizes

so as to facilitate comparison. As I have discussed above, the use of single sperm concentrations will yield apparently variable effect sizes depending on the sperm concentration that is used making comparisons problematic.  $F_{\max}$  and  $[\text{Sperm}]_{\max}$  do not suffer from this problem and thus, these measures would lend themselves well to standardized measures such as  $EC_{50}$ . It should be noted that the use of  $F_{\max}$  and  $[\text{Sperm}]_{\max}$  does result in more processing etc. (each curve generates only one replicate for each measure) and this may be impractical in some instances. Nevertheless these metrics appear to me to be the simplest, easiest means of obtaining a reliable estimate of toxicant effects on fertilization.

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