# THE NEUROHORMONAL INDUCTION OF THE RELEASE OF OOCYTES AND SPERM FROM *PECTEN MAXIMUS*

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## ABSTRACT

In order to induce *in vitro* maturation of *Pecten maximus* oocytes, experiments were carried out using different concentrations of the serotonin and dopamine agonists TFMPP, 8-OHDPAT, quinpirole and bromocriptine. The greatest germinal vesicle breakdown (GVBD) occurred using TFMPP. Metaphasic blocks were rarely observed. Oocytes treated with TFMPP at $10^{-3}$ and $5\times10^{-4}$M could be fertilized at low levels. The maximum fertilization rate recorded was 23%. A second series of experiments was conducted to determine the effects of serotonin, dopamine, the agonists cited above and $PGE_2$ on the release of oocytes and sperm from *P. maximus*. These inducers were injected into both the male and female parts of the gonads and spermiation was systematically tested approximately 20 minutes following injection. No oocyte release was recorded, except after one injection of 8-OHDPAT at $5\times10^{-4}$M; the effect of 8-OHDPAT on the release of oocytes was nevertheless insignificant.

## INTRODUCTION

Spawning of *Pecten maximus* in the laboratory is generally achieved by applying thermal shock to selected animals. The selection of animals is based on the stage of maturity as described by Cochard and Devauchelle (1993), with those selected usually at stage four and five. Thermal shock inductions lead to highly variable results in terms of quality of oocytes and rate of hatching. Variations can be attributed to factors such as characteristics of the broodstock (especially the exact stage of maturity), environmental conditions, method of stimulation, and incubation conditions (Devauchelle and Mingant 1991). To improve the success of spawning in the laboratory, a program was developed to study the effects of aminergic neurohormones and prostaglandins on the stimulation of spawning.

Knowledge of the mechanisms which control different events in the reproductive cycle of bivalves is poor in comparison to that of vertebrates or even crustaceans. Bivalves have no endocrine glands and sexual activity is under neuroendocrine control. Several authors have demonstrated or suggested relationships between reproductive processes and peptidergic neurohormones (Stefano and Catapane 1979; Grizel et al. 1991), steroids (Lubet and Mathieu 1990; Osada et al. 1992) and aminergic neurohormones.

Variations in aminergic neurohormone levels have been recorded during the reproductive cycle of bivalves. For example, dopamine content increases in neurons just before spawning in *Mytilus edulis*, *Crassostrea gigas*, *Patinopecten yessoensis* (Osada et al. 1987) and *Pecten maximus* (Paulet et al. 1993). However, *in vitro* dopamine treatments ($10^{-4}$M) do not reinitiate meiosis in *P. maximus* (Guerrier, unpublished data). On the other hand, serotonin levels in ganglia vary during the reproductive cycle of *P. maximus* (Paulet et al. 1993). Serotonin (5-HT) injections into the gonads are known to induce spawning in various bivalve species (Matsutani and Nomura 1982; Gibbons and Castagna, 1984; Hiraï et al. 1988). *In vitro*, serotonin treatments reinitiate oocyte meiosis in *Spisula solidissima* (Hiraï et al. 1988; Krantic

et al. 1991), *S. sachalinensis* (Varaksin et al. 1992), *Patinopecten yessoensis* (Osada et al. 1992), and *Ruditapes philippinarum* (Osani and Kuraishi 1988; Guerrier et al. 1993). Among serotonin agonists, 8-OHDPAT but not TFMPP was able to induce meiosis reinitiation in *Spisula solidissima* (Krantic et al. 1991), while both compounds proved to be equally as effective on *Ruditapes philippinarum* oocytes. In *Pecten maximus*, Guerrier (unpublished data) observed that the reserves at meiosis reinitiation were significantly higher with TFMPP or 8-OHDPAT ($10^{-4}$M) than with serotonin which, unexpectedly, seemed to be rather ineffective on this species. Prostaglandins have been found in bivalve gonads (Nomura et al. 1979). The role of prostaglandins in stimulating the release of oocytes from the ovary of *Patinopecten yessoensis* has been demonstrated by Matsutani and Nomura (1987) and Matsutani (in press).

Using this information, a series of *in vitro* experiments was designed to complete data on the effects of injections of several aminergic neurohormones on spawning of *P. maximus*. The effect of $PGE_2$ injections into scallop gonads was also tested.

## MATERIAL AND METHODS

### IN VITRO MATURATION OF OOCYTES

Scallops were dredged in the Bay of Brest and conditioned in the hatchery. When the gonads reached stage four maturity (Cochard and Devauchelle 1993), they were removed from the scallop, cut and placed in a beaker filled with filtered (1 $\mu$) seawater. Free oocytes were filtered twice and placed in 50 ml filtered seawater. The supernatant oocytes were discarded, sinking oocytes were reserved for the experiments.

The inducers were tested at the following concentrations:

1. TFMPP (RBI S5): $10^{-4}$M, $2\times10^{-4}$M, $5\times10^{-4}$M, and $10^{-3}$M;
2. 8-OHDPAT (RBI S002): $10^{-4}$M and $5\times10^{-4}$M;
3. quinpirole (RBI Q102): $10^{-6}$M, $10^{-5}$M, and $10^{-4}$M;
4. bromocriptine (RBI B115): $10^{-6}$M, $10^{-5}$M, and $10^{-4}$M.

Tests were run on two to ten batches of 5000 oocytes placed in 5 ml tubes. Each batch originated from a different scallop. After 30, 60 and 90 min treatments, the oocytes were placed successively in formalin (6%) and GA buffer, two GA buffers, and finally one GA buffer containing Hoescht 33258 (Dubé 1988). The oocytes were then stored at 4°C and observed with an epifluorescent microscope. Controls were submitted to the same treatments. The GVBD (germinal vesicule breakdown, Fig. 1) stage, considered as the first step of meiosis reinitiation, was systematically checked. Metaphasic blocks (Fig. 2) and fertilization of oocytes were followed in three TFMPP and 8-OHDPAT experiments. In this case, the sperm was collected from scallops stimulated by thermal shock (maximum +10°C) or serotonin injection (Faure et al., this publication) and dispersed in the oocytes solution at the ratio of 30 spermatozoa per oocyte. Sperm concentrations were calculated in reference to the following relationship obtained at 220 nm absorbance: $y = (1.037 \times 10^{-6}X) + (- 20.21 \times 10.3)$. Oocytes and sperm were in contact for 60 minutes, after which the percentage of fertilized oocytes (Fig. 3) was estimated and the embryos fixed as described above.

### STIMULATION OF SPAWNING BY INJECTIONS OF INDUCERS INTO SCALLOP GONADS

Scallops collected from the Bay of Brest were submitted to injections when their gonads were at stage four, either immediately following collection or after an artificial conditioning period in the hatchery. Injections were made in the male and female parts of the gonads and the total

volume injected was 0.4 ml of filtered (1 $\mu$) seawater for the controls, or 0.4 ml of filtered seawater containing single or mixed inducers. Concentrations of the inducers were determined according to the results of the *in vitro* tests:

1. serotonin (RBI S011): $10^{-3}$M and $5\times10^{-4}$M;
2. dopamine (D019): $10^{-6}$M, $10^{-5}$ M, $10^{-4}$ M, and $10^{-3}$ M;
3. TFMPP: $2\times10^{-4}$M, $5\times10^{-4}$M, $10^{-3}$M, and $5\times10^{-3}$M;
4. 8-OHDPAT: $5\times10^{-4}$M and $10^{-3}$ M;
5. quinpirole: $10^{-6}$M, $10^{-5}$M, $10^{-4}$M, and $10^{-3}$M;
6. bromocriptine: $10^{-4}$M.

In addition, the effect of $PGE_2$ (SIGMA P4172) was tested at $5\times10^{-7}$ M, $5\times10^{-6}$M and $10^{-6}$M.

Five to thirty scallops were used to test each preparation and the percentage of sperm and oocyte releasing animals was recorded for each.

## RESULTS

### IN VITRO MATURATION OF OOCYTES

#### Induction of Germinal Vesicle Breakdown (GVBD)

Quinpirole and bromocriptine used at concentrations of $10^{-4}$ to $10^{-6}$M induced GVBD at low but non-significant levels compared to controls (Table 1). No effect of duration of treatment was observed. However, the difference in GVBD values between controls and treatments showed that bromocriptine ($10^{-4}$M) treatments were more effective than quinpirole treatments ($P = 0.05$) (Fig. 4).

The results of quinpirole, bromocriptine, TFMPP and 8-OHDPAT treatments at $10^{-4}$M, or TFMPP and 8-OHDPAT treatments at $5\times10^{-4}$M, did not differ significantly with duration of treatment (Table 2). GVBD increased when 8-OHDPAT, quinpirole and bromocriptine at $10^{-4}$M were used, but these results were not statistically different from those of the controls. In the other treatments, GVBD percentages significantly differed from controls. Treatment efficiencies increased with TFMPP at $10^{-4}$M, followed by 8-OHDPAT at $5\times10^{-4}$M (Fig. 5). Maximum GVBD percentages were recorded with TFMPP at $5\times10^{-4}$M. The differences between these three treatments were highly significant ($P = 0.001$).

The comparison of experiments run with TFMPP at $10^{-4}$M, $2\times10^{-4}$M, $5\times10^{-4}$M and $10^{-3}$M showed that average percentages of GVBD increased with increasing concentrations from $10^{-4}$M to $2\times10^{-4}$M (Fig. 6). The results were significantly different between $10^{-4}$M or $2\times10^{-4}$M and $5\times10^{-4}$M ($P = 0.001$) and between $10^{-4}$M and $10^{-3}$M ($P = 0.001$). No significant difference was recorded between treatments at $5\times10^{-4}$M and $10^{-3}$M, although the effectiveness of the latter concentration appeared to be lower. Variation in the duration of treatment did not significantly affect the results for each concentration tested (Fig. 7). Metaphasic blocks were rarely observed even when the occurrence of GVBD was high (Table 3).

The oocytes treated with TFMPP ($5\times10^{-4}$M and $10^{-3}$M) and 8-OHDPAT ($5\times10^{-4}$M) were fertilized at low rates. Fertilized oocytes were also found in two controls. These results were not significantly different (Fig. 8), partially due to the high variability of the results. However, fertilization rates appeared to be lower when the oocytes were treated for only 30 minutes.

STIMULATION OF SPAWNING BY INJECTIONS OF INDUCERS INTO SCALLOP GONADS

When single neurotransmitters were injected, sperm was released only with serotonin treatment (Table 4). Sperm was obtained about 20 minutes after injection. No significant release of oocytes occurred, the only release following an injection of 8-OHDPAT into a single scallop.

When mixtures of neurotransmitters were injected, no release of oocytes was observed. Although serotonin alone systematically induced sperm release, its effect decreased when the injected preparation contained quinpirole or TFMPP as well (Table 5). TFMPP injected with 8-OHDPAT or quinpirole was not effective in inducing spermiation (Table 6).

$PGE_2$ did not induce gamete release at any concentration tested (Table 7).

## DISCUSSION

In *Spisula solidissima*, which releases sperm and oocytes following serotonin injection (Krantic et al. 1993a), binding assays performed on semi-purified membrane fractions allowed charcterization of the physiological 5-HT receptors present on the oocyte plasma membrane (Krantic et al. 1993a,b). These presented the same pharmacological profile and kinetic parametes as observed for the biological response, i.e. GVBD. These original receptors differ from those of *Ruditapes philippinarum* which, in addition to serotonin, respond with the same efficacy to 8-OHDPAT and TFMPP (Gobet et al., in prep.). In the gonochoric *Patinopecten yessoensis*, serotonin injections were efficient in producing oocytes and sperm release (Matsutani and Nomura 1982) as well as in inducing the *in vitro* release of oocytes (Matsutani and Nomura 1987; Osada et al. 1992). For *Pecten maximus*, we found that serotonin and agonists do not induce oocyte release and sperm is obtained only following serotonin injection. In this species, as in *Ruditapes*, GVBD is obtained with both agonists 8-OHDPAT and TFMPP but not with 5-HT. Under these conditions, metaphase I arrested oocytes were not obtained in great number, a feature likely to depend on a defect in the degree of maturity (competence) of the oocytes directly dissected from the ovary. Moreover, it appeared that serotonin agonists inhibited the efficacy of serotonin in promoting spermiation. Since the levels of serotonin and dopamine in the nervous ganglia are correlated to reproductive cycle (Paulet et al. 1993), the results presented here suggest that further experiments need to be conducted to compare male and female specificity and sensitivity to inducers, including estrogens and prostaglandins which are known to increase serotonin response in *P. yessosensis* (Osada et al. 1992). In addition, it is not impossible that the difference between male and female parts are exacerbated in hermaphroditic species compared to gonochoric species.

Low values of GVBD were obtained with dopamine and dopamine agonists while $PGE_2$ alone did not induce gamete release. In order to interpret these results, further experiments need to be carried out with these inducers.

The originality of the current study is the comparison of *in vitro* and *in vivo* tests. This comparison demonstrated differences in the effectiveness of inducers when tested *in vitro* and *in vivo*, as measured by GVBD, metaphasic blocks, fertilization rates and gamete release. This helps in understanding the mechanisms which control these different events, often referred to as maturation and spawning processes. As explained and discussed by Abdelmajid et al. (1993), not only one chemical and/or neurotransmitter controls these very different events. The *in vitro* and *in vivo* parallel studies allows revelation of these differences, and will be maintained in further experiments.

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Table 1. Percentage of GVBD induced by quinpirole and bromocriptine compared to controls. Each value corresponds to three experiments made on oocytes of three different scallops.

| DURATION (minutes) | CONTROL | | QUINPIROLE | | | | | | BROMOCRIPTINE | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $10^{-6}$M | | $10^{-5}$M | | $10^{-4}$M | | $10^{-6}$M | | $10^{-5}$M | | $10^{-4}$M | |
| | $\bar{x}$ | σ | $\bar{x}$ | σ | $\bar{x}$ | σ | $\bar{x}$ | σ | $\bar{x}$ | σ | $\bar{x}$ | σ | $\bar{x}$ | σ |
| 30 | 12.3 | (4.0) | 19 | (12.8) | 19 | (8.6) | 24.3 | (7.6) | 19 | (7.5) | 25 | (16.3) | 22.3 | (10.1) |
| 60 | 16.3 | (11.4) | 25 | (15.6) | 18 | (6.48) | 21 | (12) | 21 | (9.4) | 26.7 | (15.2) | 28 | (17) |
| 90 | 17 | (11) | 25 | (24.1) | 26.7 | (13.0) | 18.7 | (10.3) | 19.7 | (6.5) | 23 | (11.6) | 31 | (16.1) |

Table 2. Percentage of GVBD induced by quinpirole, bromocriptine, 8-OHDPAT and TFMPP at different concentrations.

| DURATION (minutes) | CONTROLS | | $10^{-4}$ M (n = 3) | | | | | | $5\times10^{-4}$ M (n = 3) | | | | $10^{-4}$ M (n = 3) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | QUINPIROLE | | BROMOCRIPTINE | | 8 OHDPAT | | 8 OHDPAT | | TFMPP | | CONTROLS | | TFMPP | |
| | $\bar{x}$ | σ | $\bar{x}$ | σ | $\bar{x}$ | σ | $\bar{x}$ | σ | $\bar{x}$ | σ | $\bar{x}$ | σ | $\bar{x}$ | σ | $\bar{x}$ | σ |
| 30 | 12.3 | (4) | 24.3 | (7.6) | 22.3 | (10.1) | 22.3 | (12.2) | 51 | (23) | 85.3 | (11.5) | 7 | (5) | 41 | (3) |
| 60 | 16.3 | (11.4) | 21.3 | (12) | 28 | (17.0) | 22.3 | (7.6) | 74.7 | (9.8) | 97 | (2.2) | 12.5 | (8.5) | 54 | (9) |
| 90 | 17 | (11) | 18.7 | (10.3) | 31 | (16.1) | 21.3 | (5.3) | 73.7 | (10.1) | 98.7 | (1.2) | 12 | (4) | 53 | (8) |

Table 3. Percentages of GVBD and metaphasic blocks recorded after oocytes treatments with TFMPP at $10^{-4}$M and $10^{-3}$M.

| DURATION (minutes) | % GVBD | | | | | | % METAPHASIC BLOCKS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CONTROL | | TFMPP $10^{-4}$M | | TFMPP $10^{-3}$M | | CONTROL | | TFMPP $10^{-4}$M | | TFMPP $10^{-3}$M | |
| | $\bar{x}$ | σ | $\bar{x}$ | σ | $\bar{x}$ | σ | $\bar{x}$ | σ | $\bar{x}$ | σ | $\bar{x}$ | σ |
| 30 | 6.3 | (4.2) | 31.3 | (13.9) | 87 | (8) | 0 | | 1 | (1.4) | 0 | |
| 60 | 11.3 | (7.1) | 40 | (21.1) | 94 | (4) | 0 | | 1 | (1.4) | 0 | |

Table 4. Number of *Pecten maximus* individuals releasing gametes after injection of single neurotransmitters tested at different concentrations.

| INDUCER | [C] M | NUMBER OF TESTED ANIMALS | SPERMIANT ANIMALS % (n) | ANIMALS PRODUCING OOCYTES % (n) |
|---|---|---|---|---|
| SEROTONIN | $5\times10^{-4}$ | 25 | 84(21) | 0 |
| | $10^{-3}$ | 15 | 87(13) | |
| DOPAMINE | $10^{-6}$ | 5 | 0 | 0 |
| | $10^{-5}$ | 5 | 0 | 0 |
| | $10^{-4}$ | 5 | 0 | 0 |
| | $10^{-3}$ | 5 | 0 | 0 |
| TFMPP | $2\times10^{-4}$ | 14 | 0 | 0 |
| | $5\times10^{-4}$ | 24 | 0 | 0 |
| | $10^{-3}$ | 10 | 0 | 0 |
| | $5\times10^{-3}$ | 10 | 0 | 0 |
| 8-OHDPAT | $5\times10^{-4}$ | 15 | 0 | 7(1) |
| | $10^{-3}$ | 5 | 0 | 0 |
| QUINPIROLE | $10^{-6}$ | 5 | 0 | 0 |
| | $10^{-5}$ | 5 | 0 | 0 |
| | $10^{-4}$ | 5 | 0 | 0 |
| | $10^{-3}$ | 5 | 0 | 0 |
| BROMOCRIPTINE | $10^{-4}$ | 10 | 0 | 0 |
| SEAWATER | | 30 | 0 | 0 |

Table 5. Number of *Pecten maximus* individuals releasing sperm and oocytes after injection of a mixture of neurotransmitters compared to serotonin injections.

| EXP | INDUCER | [C] | NUMBER OF TESTED ANIMALS | NUMBER OF SPERMIANT ANIMALS | NUMBER OF ANIMALS PRODUCING OOCYTES |
|---|---|---|---|---|---|
| 1 | SEROTONIN | $5\times10^{-4}$ | 5 | 4 | 0 |
| | SEROTONIN + TFMPP | $5\times10^{-4}$ x 2 | 5 | 3 | 0 |
| 2 | SEROTONIN | $5\times10^{-4}$ | 5 | 3 | 0 |
| | SEROTONIN + 8-OHDPAT | $5\times10^{-4}$ x 2 | 5 | 1 | 0 |
| 3 | SEROTONIN | $5\times10^{-4}$ | 5 | 3 | 0 |
| | SEROTONIN + QUINPIROLE | $5\times10^{-4}$ x 2 | 5 | 3 | 0 |

Table 6. Number of *Pecten maximus* individuals releasing sperm and oocytes after injections of mixtures of neurotransmitters (TFMPP + quinpirole and TFMPP + 8-OHDPAT).

| EXP | INDUCER | [C] | NUMBER OF TESTED ANIMALS | NUMBER OF SPERMIANT ANIMALS | NUMBER OF ANIMALS PRODUCING OOCYTES |
|---|---|---|---|---|---|
| 1 | SEROTONIN | $5\times10^{-4}$ | 5 | 4 | 0 |
| | TFMPP + 8-OHDPAT | $5\times10^{-4}$ x 2 | 5 | 0 | 0 |
| 2 | SEROTONIN | $5\times10^{-4}$ | 5 | 3 | 0 |
| | TFMPP + QUINPIROLE | $5\times10^{-4}$ x 2 | 5 | 1 | 0 |

Table 7. Number of *Pecten maximus* individuals releasing sperm and oocytes after $PGE_2$ injections compared to serotonin injections.

| INDUCER | [C] | NUMBER OF TESTED ANIMALS | NUMBER OF SPERMIANT ANIMALS | NUMBER OF ANIMALS PRODUCING OOCYTES |
|---|---|---|---|---|
| SEROTONIN | $10^{-3}$ | 10 | 9 | 0 |
| $PGE_2$ | $5\times10^{-7}$ | 10 | 0 | 0 |
| | $10^{-6}$ | 10 | 0 | 0 |
| | $5\times10^{-6}$ | 10 | 0 | 0 |

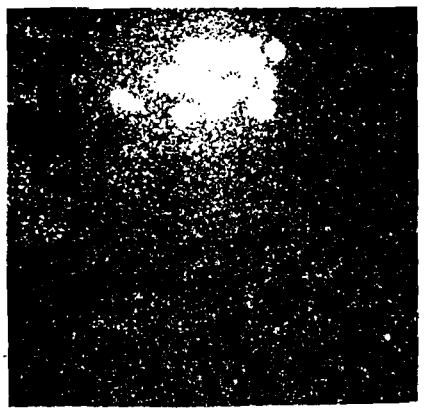

Fig. 1. GVBD stage. The nuclear membrane has disappeared. The chromosomes are still dispersed and condensed.

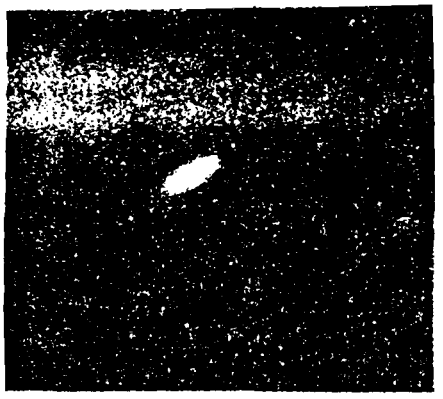

Fig. 2. Metaphasic blocks stage. Homologous chromosomes are placed on both sides of an equatorial plane.

Fig. 3. Fertilization stage. The male pronucleus is visible in the oocyte.

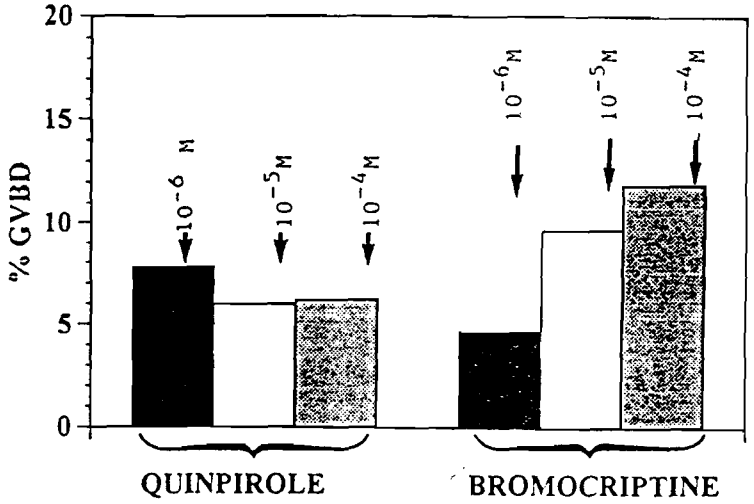


Fig. 4. Percentage of GVBD induced by quinpirole and bromocriptine treatments. In the figure, the values are expressed as the differences observed between GVBD percentages recorded in the controls and in the experimental tests.

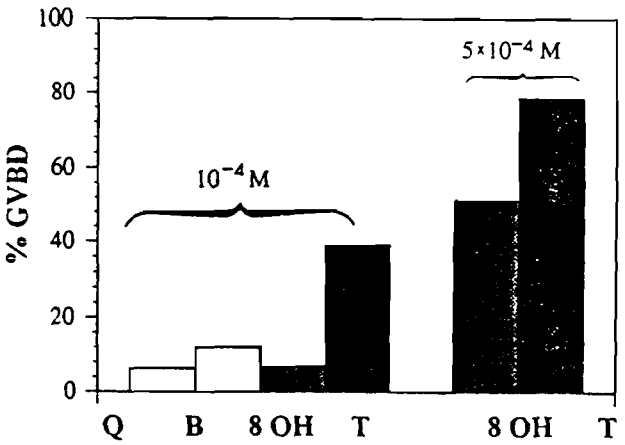


Fig. 5. Percentage of GVBD induced by quinpirole (Q), bromocriptine (B), 8-OHDPAT (8 OH), and TFMPP (T) at two concentrations: $10^{-4}$M and $5\times10^{-4}$M. In the figure, the values are expressed as the differences observed between GVBD percentages recorded in the controls and in the experimental tests.

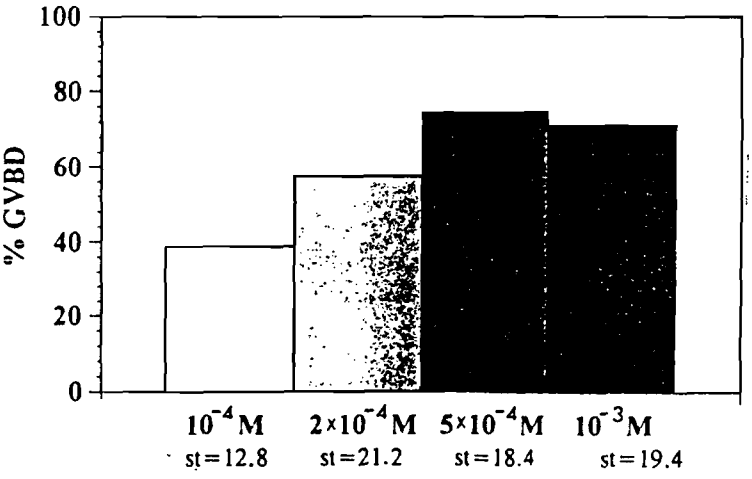


Fig. 6. Percentage of GVBD induced by TFMPP at four concentrations: $10^{-4}$M, $2\times10^{-4}$M, $5\times10^{-4}$M, and $10^{-3}$M tested for 30, 60, and 90 minutes. In the figure, the values are expressed as the differences observed between GVBD percentages recorded in the controls and in the experimental tests. The average value of the controls was 5.7 (st = 3.4). Variation of treatment duration had no significant effect.

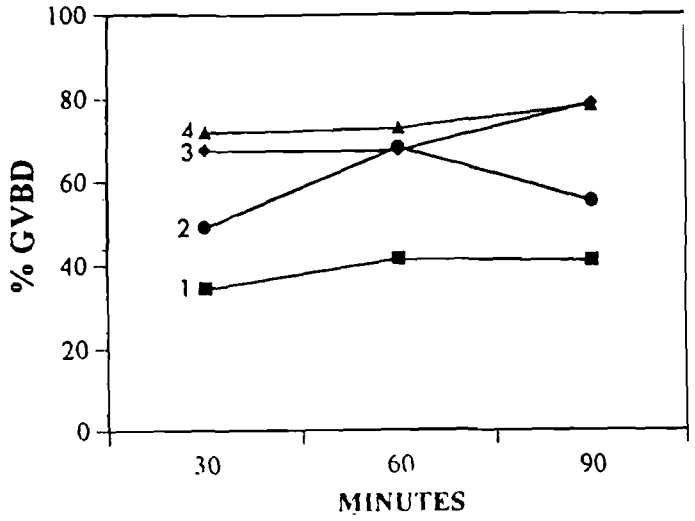


Fig. 7. Percentage of GVBD induced by TFMPP treatments during 30, 60 and 90 minutes at $10^{-4}$M (1), $2\times10^{-4}$M (2), $5\times10^{-4}$M (3), and $10^{-3}$M (4).

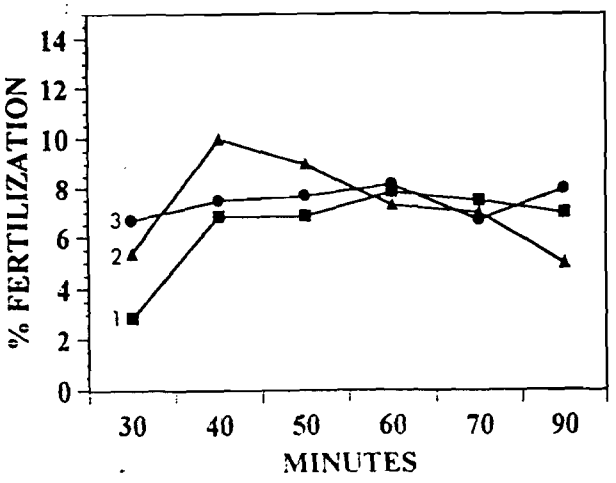


Fig. 8. Fertilization rates of oocytes treated with TFMPP at $10^{-3}$M (1) and $5\times10^{-4}$M (2), and 8-OHDPAT at $5\times10^{-4}$M (3) for 30, 60 and 90 minutes. The average value of the controls was 1.33 (st = 1.9). Three tests were carried out on oocytes issued from three scallops. The maximum individual fertilization rate was 23% and variation was high.