*Journal of Reproduction and Fertility* (2000) **120**, 73–81

# Production of tissue inhibitors of metalloproteinases (TIMPs) by pig ovarian cells *in vivo* and the effect of TIMP-1 on steroidogenesis *in vitro*

E. M. Shores and M. G. Hunter*

*Division of Animal Physiology, School of Biological Sciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire LE12 5RD, UK*

Precisely which ovarian cells produce tissue inhibitors of metalloproteinases (TIMPs) is unclear. Although granulosa cells are reported to produce TIMPs, thecal TIMP production has not been investigated nor has the influence of TIMPs on theca cells. Furthermore, although periovulatory follicles have been examined, little is known about smaller ovarian follicles. Follicles ⩾ 2 mm in diameter were collected from Large White hybrid gilts on the day before predicted oestrus ($n = 3$) or after hCG treatment ($n = 3$) and divided into 1 mm size classes. Small (2 to < 5 mm) follicles were kept intact, whereas follicles ⩾ 5 mm were separated into follicular fluid, granulosa and theca cell compartments. After homogenization, TIMP-1, -2 and -3 were detected by reverse zymography. Theca cells ($50 \times 10^3$ per well) were cultured with TIMP-1 (10, 100 or 200 ng ml$^{-1}$ with or without long-R3 insulin-like growth factor I (IGF-I)) in a serum-free system to investigate the effect on steroidogenesis and the number of cells. Both large and small pig follicles produced TIMPs and TIMP-1, -2 and -3 were detected in follicular fluid, granulosa and theca cell samples. There was a phase × tissue type interaction for the presence of both TIMP-1 and -2 ($P < 0.03$, $P < 0.05$, respectively), and TIMPs were detected in more granulosa and theca cell samples after hCG than during the follicular phase. The concentrations were influenced by the type of tissue (TIMP-1, $P < 0.005$; TIMP-2, $P < 0.005$, TIMP-3, $P > 0.05$), and the highest concentrations occurred in the theca tissue. There were tissue type × follicle size interactions for the presence of both TIMP-1 and -2 ($P < 0.001$). *In vitro*, TIMP-1 increased thecal steroidogenesis after 144 h (oestradiol, $P < 0.05$, progesterone, $P < 0.001$) but reduced the number of viable cells ($P < 0.001$). In conclusion, TIMP-1, -2 and -3 were present in large and small pig follicles and were produced by both granulosa and theca cells, although concentrations differed with the type of tissue. Production was regulated by factors including follicle size and phase of the oestrous cycle. In addition to controlling tissue remodelling, TIMP-1 may also regulate steroidogenesis.

## Introduction

The ovary is a dynamic structure in which the extracellular matrix (ECM) is continually remodelled to allow growth (Inderdeo *et al.*, 1996). Breakdown of the proteinaceous components of the ECM is performed by matrix metalloproteinases (MMPs), the activity of which is co-ordinated by inhibitors, including the tissue inhibitors of metalloproteinases (TIMPs) (Chun *et al.*, 1992). TIMP production appears to be under LH control (Mann *et al.*, 1991; Chun *et al.*, 1992) and protein or mRNA content increases in the follicular fluid and granulosa cells after the LH surge in many species (human: Curry *et al.*, 1988; rat: Mann *et al.*, 1991; sheep: Smith *et al.*, 1993; pig: Smith *et al.*, 1994). There is some dispute over which ovarian cells produce TIMPs. Several reports indicate that granulosa cells produce TIMPs in pigs (Smith *et al.*, 1994), sheep (Smith *et al.*, 1993), humans (Curry *et al.*, 1988) and rats (Mann *et al.*, 1991). However, some of these studies measured TIMP output by luteinized granulosa cells (Smith *et al.*, 1993, 1994). None of the reports considered theca cells. Conflicting reports state that TIMP mRNA is found within the theca layer (Chun *et al.*, 1992) or is not in the theca layer (Nomura *et al.*, 1989) of rat follicles. Much of the work has centred on the periovulatory period and the effect of the gonadotrophin surge on mRNA and protein content. In addition, in work on pig ovaries, only the largest follicles have been used (Smith *et al.*, 1994; Driancourt *et al.*, 1998), whereas little is known about the smaller follicles present in the ovary.

There are currently four confirmed TIMPs, designated 1–4. TIMP-1 and -2 are soluble in the ECM, TIMP-3 is bound to

*Correspondence.
Received 13 December 1999.


0022–4251/2000

the ECM, but the state of TIMP-4 has not been determined (McIntush and Smith, 1998). The most studied is TIMP-1, a 28 kDa polypeptide synthesized and secreted at a number of sites within the female reproductive system including granulosa cells, preovulatory follicles, luteal tissue, the uterus and oviduct (Buhi *et al.*, 1997). TIMP-1 may act as a growth factor (Hayakawa *et al.*, 1992; Smith *et al.*, 1994) or stimulate steroidogenesis *in vitro* (Boujrad *et al.*, 1995; Nothnick *et al.*, 1997) or regulate the bioavailability of growth factors such as insulin-like growth factor (IGF) (Fowlkes *et al.*, 1994; Besnard *et al.*, 1997). Less is known about the smaller TIMP-2 and TIMP-3 which are 21 kDa and 24 kDa, respectively (McIntush and Smith, 1998). Although some studies have examined granulosa cell TIMP production, very little is known about TIMP production by theca cells or the effect of TIMPs on theca cells.

The first aim of this study was to test the hypothesis that both large and small pig follicles produce TIMP-1, -2 and -3. It was also proposed that TIMP proteins are produced by both granulosa and theca cell layers and this was examined in larger ($\geqslant 5$ mm) follicles. Tissue was collected from animals before and after the LH surge and factors such as phase of the oestrous cycle, follicle size and type of cell that might influence the presence and amount of TIMP proteins were identified. Finally, studies were conducted *in vitro* to test the hypothesis that TIMP-1 influences steroidogenesis and the number of pig theca cells in serum-free culture.

## Materials and Methods

All chemicals were obtained from Sigma Chemical Company Ltd, Poole unless otherwise stated.

### *Tissue collection*

Large White hybrid gilts were housed in groups of five or six animals and oestrus was detected with a vasectomized boar. After gilts had shown at least one oestrous cycle of normal duration they were killed at the appropriate stage of a subsequent cycle. All follicular phase ovaries ($n = 3$ animals) were collected 1 day before predicted oestrus (Hunter *et al.*, 1994; Miller *et al.*, 1998). Alternatively, a single i.m. injection of 500 iu hCG was given to mimic the LH surge and the animals ($n = 3$) were killed 24 h later (Driancourt *et al.*, 1998). The ovaries were removed, placed on ice and transferred to the laboratory within 30 min of removal.

### *TIMP extraction*

TIMP extraction was based on the method of Zhu and Woessner (1991). Follicles from 2 to < 5 mm in diameter were kept intact and placed in 0.5 ml calcium chloride buffer (10 mmol calcium chloride $l^{-1}$ with 0.25% (v/v) Triton-X100). All follicles of at least 5 mm in diameter were separated into follicular fluid, granulosa cell and theca cell samples. The follicular fluid was removed with a needle and syringe and frozen at –20°C. The follicle was cut open in 0.5 ml buffer and the granulosa cells were scraped out. Confirmation that all the granulosa cells are removed by this method was provided by Shores *et al.* (2000). The remaining follicle shell was regarded as the theca layer and was transferred to another 0.5 ml buffer. All theca layer and whole follicle samples were cut into smaller pieces to aid homogenization. Tissue was homogenized with a hand-held Omni homogenizer (10001, Camlab, Cambridge) for approximately 1 min until a uniform solution was obtained. Samples and buffers were kept on ice during processing and extracted samples were stored at –20°C.

### *Protein assays*

The total protein content of all samples ($n = 184$) was measured using a method adapted from Bradford (1976) for use on microtitre plates. The light absorbance was measured at 595 nm. Samples were compared with a BSA standard curve of range 0–1 mg $ml^{-1}$.

### *Reverse zymography for TIMP*

TIMP activity was detected by reverse zymography using a kit supplied by University Technologies International (Calgary) and adapted for use with mini-gels. Acrylamide SDS gels (12%, 0.75 mm thick) were prepared containing 1 mg gelatin $ml^{-1}$ together with an active MMP solution (6% v/v). Standards (TIMP-1+2 and TIMP-3) were loaded onto each gel. After electrophoresis at 4°C, the gel was rinsed in buffer (50 mmol Tris $l^{-1}$, 5 mmol calcium chloride $l^{-1}$, 2.5% (v/v) Triton-X100) for 2 h at room temperature. The gel was washed thoroughly with distilled water and incubated for 24 h at 37°C in prewarmed buffer without Triton-X100. Gels were stained with Coomassie blue.

### *Theca cell cultures with TIMP-1*

Theca cells were collected from large (6–9 mm in diameter) morphologically healthy follicles as described in Shores *et al.* (2000). Briefly, follicles were cut open and the granulosa cells were scraped away. Theca cell sheets were peeled away from the follicle shell and digested with enzymes (collagenase type II, hyaluronidase type I-S) to disperse the cells. The number of viable cells before culture was assessed using trypan blue exclusion.

Theca cells were seeded into 96-well plates at $50 \times 10^3$ viable cells per well. Each well contained Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium supplemented with 10 ng bovine insulin $ml^{-1}$ and 0.01 ng LH $ml^{-1}$ (USDA-pLH-B2). The optimization of theca cell culture conditions to maintain steroidogenesis and the number of viable cells is described in full in Shores *et al.* (2000). In the current study, wells received either the optimal dose of 100 ng long-R3 IGF-I $ml^{-1}$ (synthetic IGF-I analogue, Gropep Pty Ltd, Adelaide), or to investigate the effect of TIMP-1 under sub-optimal conditions, no IGF-1 was added. Recombinant TIMP-1 (Biogenesis, Poole) was applied to the cells at 10, 100 or 200 ng $ml^{-1}$. Three repeat cultures were performed, each with four replicates per treatment. Cultures were incubated

for 144 h at 37°C in a humidified atmosphere of 5% $CO_2$ in air. The medium was replaced every 48 h and stored at –20°C before assay for oestradiol and progesterone. The number of viable cells after 144 h was assessed by the uptake of neutral red dye as described by Campbell *et al.* (1996) and Picton *et al*. (1999). Hormone production was expressed per 1000 viable cells.

### *Hormone assays*

The concentrations of oestradiol and progesterone were measured in culture medium by radioimmunoassay as described by Grant *et al*. (1989). The concentrations of these hormones in samples collected for reverse zymography were also assessed. For follicles of ⩾ 5 mm, this assessment was carried out using follicular fluid samples and for follicles < 5 mm in diameter whole follicle homogenates were used. Inter- and intra-assay coefficients of variation were 12.2% and 6.4% for $n = 8$ oestradiol assays and 13.8% and 7.5% for $n = 8$ progesterone assays, respectively. The minimum detectable limits were 1.0 pg per tube for oestradiol and 12.5 pg per tube for progesterone.

### *Image and statistical analysis*

Gels were photographed with a digital camera (UVP, Cambridge) and the images analysed using Labworks 3.0 software (UVP). The absorbance of the dark bands on the gels was expressed as the integrated absorbance, which takes into account the area covered by the band. All values were corrected for the amount of total protein loaded onto the gel and were expressed in arbitrary units relative to the absorbance of the standards (standard = 1 unit) on the same gel.

Data collected from the reverse zymography gels were analysed in two forms. First the presence or absence of TIMP proteins in the follicular samples was assessed. TIMP protein was identified by the ability of the computer software to differentiate a band from the background stain. These data had a binomial distribution and were analysed by analysis of deviance using a generalized linear model (Genstat 5 Commitee, 1989). The influence of phase of the oestrous cycle (that is follicular phase or after hCG treatment), animal, individual follicle, follicle size and types of tissue (granulosa cells, theca cells or follicular fluid) and possible interactions between these factors were included in the model. Secondly, absorbance values were assessed. Only those samples that showed the presence of TIMP protein were included in this model. The data were normalized by log transformation and subjected to the above generalized linear model. Follicles of < 5 mm were kept intact and not separated into granulosa cell, theca cell and follicular fluid samples. Therefore, when the above analysis was applied to these smaller follicles the effect of the type of tissue was excluded from the model. Hormone data from the cell cultures were log transformed $(x + 1)$ to remove heterogeneity of variance before ANOVA using Genstat (Genstat 5 Committee, 1989). Pooled variance was used to calculate the standard error of the difference (SED) between two means. When indicated by ANOVA, treatment effects were compared using Bonferroni's test. For all analyses, treatment effects were considered significant at $P < 0.05$.

## Results

### *Steroid concentration in follicular fluid*

Administration of hCG decreased oestradiol concentrations (follicular phase 247.3 ng $ml^{-1}$; after hCG 59.7 ng $ml^{-1}$; SED 15.3 ng $ml^{-1}$, $P < 0.001$) and increased progesterone concentrations (follicular phase 208.0 ng $ml^{-1}$; after hCG 231.9 ng $ml^{-1}$; SED 10.6 ng $ml^{-1}$, $P < 0.05$) in follicular fluid. Hormone concentrations were also used to confirm that animals in the follicular phase group had not undergone an endogenous LH surge (Fig. 1).

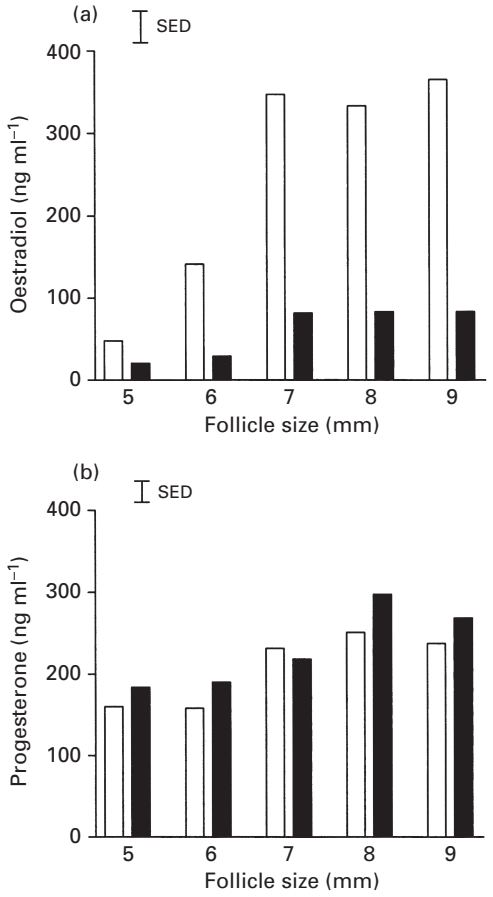


**Fig. 1.** Concentration of (a) oestradiol and (b) progesterone in the follicular fluid of follicles collected from gilts during the follicular phase (□) or after hCG treatment (■). Values represent the mean of three to nine follicles per size class per treatment, total $n = 35$.

*Reverse zymography for TIMPs in follicles ≥ 5 mm: positive samples*

TIMP-1, -2 and -3 were detected in granulosa cell, theca cell and follicular fluid samples in all follicle size classes collected during the follicular phase and after hCG treatment. The individual animal had no effect on the presence or absence of TIMPs. Samples were randomized across gels and a typical reverse zymography gel is presented (Fig. 2).

The presence of TIMP-1 protein in follicles ⩾ 5 mm was influenced by a phase × tissue type interaction ($P < 0.05$, Fig. 3a). In granulosa and theca cells, the percentage of positive samples increased after hCG. Follicular fluid had a lower percentage of positive samples after hCG compared with samples collected during the follicular phase. The presence of TIMP-1 protein was influenced by size ($P < 0.05$) and by a tissue type × follicle size interaction ($P < 0.001$; Table 1).

The presence of TIMP-2 in follicles ⩾ 5 mm was influenced by the type of tissue ($P < 0.01$): there were more positive samples in granulosa (59%) and theca cells (63%) than in follicular fluid samples (38%). The phase × tissue type interaction ($P \leqslant 0.05$, Fig. 3b) was the same as for TIMP-1. Finally, a tissue type × size interaction ($P < 0.001$) was present (Table 1). None of the factors considered here influenced the percentage of samples positive for TIMP-3 in follicles ⩾ 5 mm (Table 1, Fig. 3c).

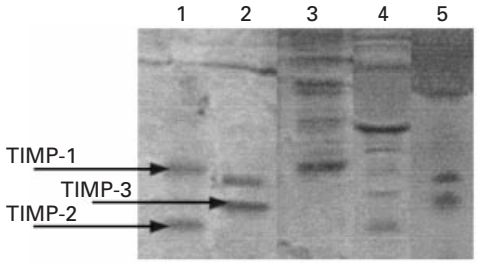


**Fig. 2.** Reverse zymography gel for the detection of tissue inhibitors of metalloproteinase 1 (TIMP-1), -2 and -3. Lanes 1 and 2 are the standards TIMP 1+2 and TIMP-3, respectively. This composite figure shows typical positive samples for TIMP-1, TIMP-2 and TIMP-3 in lanes 3–5, respectively.

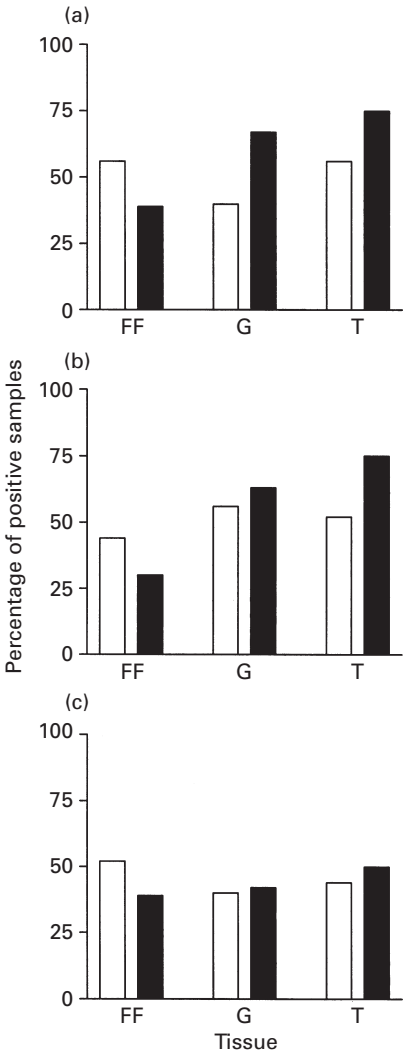


**Fig. 3.** The percentage of positive samples observed in follicular fluid (FF), granulosa (G) and theca (T) cell samples collected from pig follicles ⩾ 5 mm in diameter. Animals were in the follicular phase (□, $n = 25$ samples per type of tissue) or had been treated with hCG to mimic the LH surge (■, $n = 24$ samples per tissue type). Samples were analysed by reverse zymography to detect the presence of (a) tissue inhibitor of metalloproteinase 1 (TIMP-1), (b) TIMP-2 and (c) TIMP-3 protein.

**Table 1.** The percentage of positive samples for tissue inhibitors of metalloproteinases (TIMP) 1, 2 and 3 from follicular fluid, granulosa cell and theca cell samples collected from pig follicles ⩾ 5 mm

| Size (mm) | Follicular fluid | | | Granulosa cells | | | Theca cells | | |
|---|---|---|---|---|---|---|---|---|---|
| | TIMP-1 | TIMP-2 | TIMP-3 | TIMP-1 | TIMP-2 | TIMP-3 | TIMP-1 | TIMP-2 | TIMP-3 |
| 5 to < 6 | 50 | 80 | 40 | 54 | 55 | 27 | 9 | 36 | 36 |
| 6 to < 7 | 29 | 57 | 57 | 29 | 71 | 57 | 71 | 71 | 43 |
| 7 to < 8 | 46 | 15 | 54 | 46 | 62 | 46 | 85 | 54 | 54 |
| 8 to < 9 | 60 | 30 | 30 | 80 | 70 | 60 | 70 | 90 | 40 |
| 9 to < 10 | 50 | 13 | 50 | 50 | 38 | 13 | 100 | 75 | 63 |

There was a tissue type × size interaction for TIMP-1 and TIMP-2 ($P < 0.001$).
$n$ = 3–7 for each size class, total number of samples = 147.

*Reverse zymography for TIMPs in follicles ≥ 5 mm: amount of TIMPs*

TIMP-1 concentrations were influenced by the type of tissue ($P < 0.002$, Table 2). Follicular fluid had the lowest concentrations of TIMP-1, threefold lower than granulosa cells. Theca cells had the highest TIMP-1 content, over 3.5-fold more than granulosa cells and over tenfold higher than follicular fluid. TIMP-1 concentrations were also influenced by the phase of the oestrous cycle in which the samples were collected ($P < 0.01$) and were on average twofold higher after hCG than during the follicular phase (Table 3). TIMP-2 concentrations were influenced by the type of tissue ($P < 0.005$, Table 2) but not by the phase of the oestrous cycle. As for TIMP-1, follicular fluid had the lowest TIMP-2 content, threefold lower than granulosa cells. Theca cells had the highest TIMP-2 concentrations, 2.7-fold more than granulosa cells and over eightfold higher than follicular fluid.

A trend towards an influence of the type of tissue on the amount of TIMP-3 was observed but this result was not significant (Table 2). The phase of the oestrous cycle did not affect TIMP-3 concentrations. Theca cells showed the highest concentrations of TIMP-3, which were threefold higher than those in granulosa cells and follicular fluid samples.

*Reverse zymography for TIMPs in small follicles (< 5 mm)*

TIMP proteins were detected in samples from smaller intact follicles during the follicular phase and after hCG treatment (data not shown). TIMP-2 and -3 were detected in at least one follicle in each size class before and after hCG treatment. TIMP-1 was present in 3 to < 4 and 4 to < 5 mm follicles but not in 2 to < 3 mm follicles collected during the follicular phase. However, TIMP-1 was detected in follicles from all size classes after hCG treatment.

The phase of the oestrous cycle did not affect the presence of any of the TIMPs. Follicle size did not influence the presence of TIMP-2 and -3 in these smaller follicles. There was a tendency for the number of TIMP-1 positive samples to increase with follicle size but this result was not significant. The amounts of TIMP proteins in samples from smaller follicles were also measured. There was no effect of phase of the oestrous cycle, follicle size or any interactions on the amount of any of the TIMP proteins (data not shown).

*Theca cell cultures with TIMP-1*

At 48 h oestradiol and progesterone concentrations were unchanged by TIMP-1 in the presence or absence of 100 ng IGF-1 $ml^{-1}$. TIMP-1 with IGF-1 increased oestradiol production by 1.7-fold after 144 h ($P < 0.05$, Fig. 4b). In the absence of IGF-1 there was a trend toward increased oestradiol output in the presence of higher doses of TIMP-1, but this effect was not significant (Fig. 4a). After 144 h, TIMP-1 increased progesterone production by theca cells but only in the absence of IGF-1. Without IGF-1, 200 ng TIMP-1 $ml^{-1}$ increased progesterone output 1.6-fold above control values ($P < 0.001$, Fig. 4c). When IGF-1 was added to the medium, TIMP-1 dose did not alter progesterone production (Fig. 4d). All doses of TIMP-1 reduced the number of viable cells to approximately 75% of control values irrespective of the presence of IGF-1 (without IGF-1: $P < 0.001$, Fig. 4e; with IGF-1: $P < 0.05$, Fig. 4f).

**Table 2.** The amount of tissue inhibitors of metalloproteinases (TIMP) (arbitrary units) in pig follicles ⩾ 5 mm divided according to the type of tissue

| | Follicular fluid | *n* | Granulosa cells | *n* | Theca cells | *n* |
|---|---|---|---|---|---|---|
| TIMP-1 | 0.31 ± 0.02 | 23 | 0.91 ± 0.03 | 25 | 3.27 ± 0.02 | 30 |
| TIMP-2 | 0.40 ± 0.04 | 19 | 1.21 ± 0.02 | 25 | 3.27 ± 0.01 | 28 |
| TIMP-3 | 0.61 ± 0.03 | 22 | 0.40 ± 0.03 | 21 | 1.50 ± 0.03 | 24 |

Values are mean (± SEM) of all follicles before and after hCG treatment.
*n* = 1–7 per animal.

**Table 3.** The amount of tissue inhibitors of metalloproteinases 1 (TIMP-1) (arbitrary units) in pig follicles ⩾ 5 mm showing differences between the types of tissue in the magnitude of the increase after hCG treatment

| Tissue | Follicular phase | *n* | After hCG | *n* | Fold increase |
|---|---|---|---|---|---|
| Follicular fluid | 0.26 ± 0.04 | 14 | 0.39 ± 0.06 | 9 | 1.5 |
| Granulosa cells | 0.44 ± 0.05 | 9 | 1.38 ± 0.05 | 16 | 3.1 |
| Theca cells | 2.00 ± 0.04 | 13 | 4.80 ± 0.03 | 17 | 2.4 |

Errors are SEM.
*n* = 1–7 per animal.

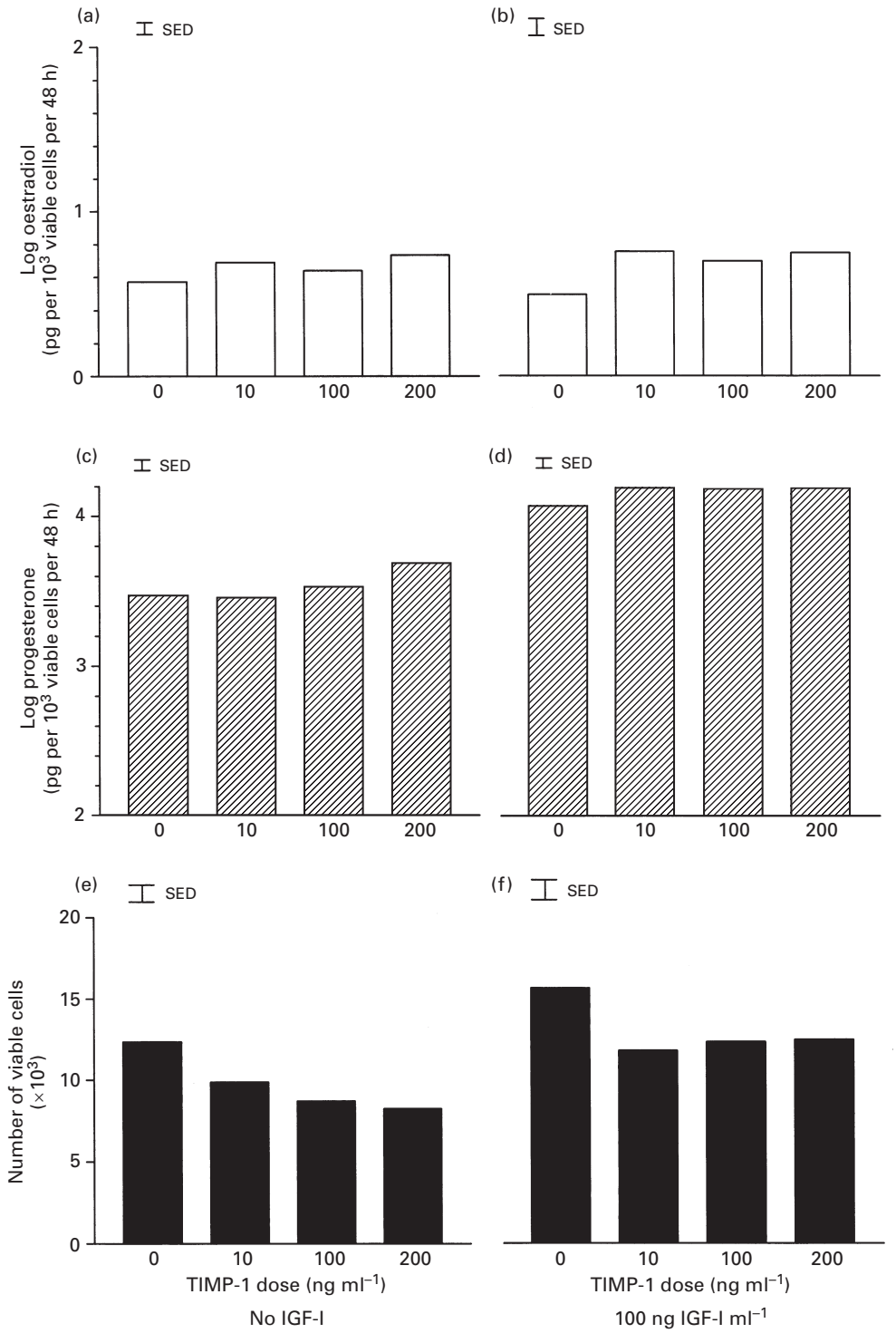


**Fig. 4.** The effect of tissue inhibitor of metalloproteinase 1 (TIMP-1) dose on oestradiol (□, a,b) and progesterone (▨, c,d) production and on the number of viable cells (■, e,f) of pig theca cells after 144 h in serum-free culture. The cells were cultured in the absence of insulin-like growth factor I (IGF-I) (a,c,e) or in the presence of 100 ng IGF-I $ml^{-1}$ (b,d,f). The hormone data were log transformed before ANOVA. The graphs show the mean of three separate cultures each containing four wells per treatment.

## Discussion

TIMP-1, -2 and -3 proteins were detected in both small and large pig follicles. The detection of TIMPs in small follicles by reverse zymography is a new finding as previous reports have concentrated on the large preovulatory follicles (Smith *et al.*, 1994; Driancourt *et al.*, 1998). In the current study, small follicles were unresponsive to hCG in terms of steroidogenesis (data not shown) and TIMP production. This finding may reflect a lack of sufficient granulosa cell LH receptors in these follicles (Chun *et al.*, 1992). No other studies have examined TIMPs in small follicles and it was anticipated that concentrations might be low; therefore, these follicles were kept intact to maximize the likelihood of detecting any TIMPs present. The small antral follicles (⩾ 2 mm) used in the present study were not grossly atretic (based on transparency and visible vascularity as described by Maxson *et al.*, 1985), but it is likely that any of these small follicles present in the ovary during the late follicular phase is in some stage of atresia. If atresia is regarded as a tissue remodelling process, it is likely that atretic follicles would contain MMPs to degrade the follicular ECM. Therefore it is also possible that atretic follicles contain TIMPs to co-ordinate the process and prevent degradation of the surrounding stroma. The present study has confirmed the presence of TIMPs within these small, probably atretic follicles.

A second aim of the present study was to compare TIMP production by the different cell layers. Therefore, follicles of at least 5 mm in diameter were separated into follicular fluid, granulosa and theca cell samples. Several studies have attempted to localize TIMPs and MMPs within the ovary by immunohistochemistry or *in situ* hybridization. McIntush *et al.* (1996) were unable to detect TIMP-1 before the LH surge and found only a few immunostained granulosa cells in ovulatory follicles of sheep after the LH surge. Bagavandoss (1998) detected TIMP-1 by immunofluorescence in the theca layer of rat follicles before the LH surge. Inderdeo *et al.* (1996) were unable to detect TIMP-1 protein in granulosa and theca layers of mouse follicles by *in situ* hybridization. Nevertheless, using northern blot analysis they were able to detect and observe increases in TIMP-1 mRNA, which peaked just before oestrus.

Therefore, there was still uncertainty about which types of cell express TIMP proteins before and after the LH surge. The current study answers this question in the pig by demonstrating the presence of TIMPs in granulosa cell, theca cell and follicular fluid compartments of large follicles (⩾ 5 mm) before and after the LH surge. This is the first report of the presence of TIMPs in the theca layer in pigs since previous reports have examined follicular fluid (Driancourt *et al.*, 1998) or follicular fluid and granulosa cells (Smith *et al.*, 1994). Reverse zymography showed that TIMPs occurred in follicles of all sizes but that there was considerable variation between individual follicles of the same size. The reasons for this variation are unclear. However, the variation cannot be due to experimental error. All samples were processed in the same way. Samples collected from an individual animal, processed in the same way at the same time showed some positive and some negative samples in the same size class. Samples were randomized across gels and the ability to detect TIMPs in a sample was not affected by which individual gel was used. The variation in the presence and concentrations of TIMPs in follicles at apparently the same stage of maturity could be another example of the heterogeneity of the ovulatory population observed by Grant *et al.* (1989) and Hunter *et al.* (1989). Antral follicle growth requires remodelling of the basement membrane and hence the co-ordinated activity of TIMPs and MMPs. Growth occurs by a process of ECM breakdown followed by ECM construction. At the exact moment the follicles were collected each individual follicle could be undergoing net ECM breakdown or construction. Which process is occurring would depend on the relative amounts of TIMPs and MMPs. In follicles undergoing ECM construction TIMP concentrations could be higher than in those undergoing matrix breakdown. TIMP positive samples may reflect net ECM construction. Matrix remodelling is a continuous process, which might explain why follicles of the same size are not all TIMP positive.

The percentage of TIMP-1 positive samples in granulosa and theca cell homogenates increased after hCG treatment, as did the amount of TIMP-1 protein present. Smith *et al.* (1994) also noted increased production of TIMP-1 after the LH surge in follicular shells (granulosa and theca cells) and granulosa cells in pigs. Work in humans (O'Sullivan *et al.*, 1997) and rats (Mann *et al.*, 1991) also indicates that LH increases TIMP-1 production by granulosa cells and intact ovary homogenates. Driancourt *et al.* (1998) observed increased TIMP-1 concentrations after hCG treatment of pigs, but this increase was not statistically significant. In that study they examined only follicular fluid. The current study showed that the increase in TIMP-1 concentrations induced by hCG was smaller in follicular fluid than in granulosa and theca cell samples. This difference may account for the apparent discrepancies between the studies of Smith *et al.* (1994) and Driancourt *et al.* (1998).

There was a decrease in the percentage of TIMP positive follicular fluid samples after hCG, in contrast to the increases observed in granulosa and theca cell samples. The reason for this decrease is unclear, but may reflect a retention of TIMPs within the cell layers, where they are required to control MMP activity as the follicle undergoes extensive tissue remodelling to form the corpus luteum. TIMP-1 (and TIMP-2) concentrations were highest in the theca layer and lowest in the follicular fluid. Theca cells are embedded in a considerable ECM, which requires extensive proteolysis to degrade. It is reasonable to predict that the theca layer has more MMP activity than follicular fluid or the granulosa layer, where the matrix is more scant. Previous reports show that TIMP and MMP activities increase concomitantly within tissues (Curry *et al.*, 1988; Chun *et al.*, 1992; Song *et al.*, 1999). Therefore, the theca layer could be expected to have high concentrations of TIMPs. The percentage of TIMP-1 positive samples from all types of tissue increased as follicle size increased. This trend was strongest in the theca layer. This increase probably reflects preparation for ovulation as the follicle increases in size and maturity. Proteolytic degradation of the follicular cell layers by MMPs is required to release the oocyte. TIMPs are required to control this

degradation and prevent stray MMPs from degrading the rest of the ovary.

The percentage of TIMP-2 positive granulosa and theca cell samples increased after hCG, although it did not affect the amount of TIMP-2 in these positive samples. Driancourt *et al.* (1998) also reported no change in TIMP-2 concentrations after hCG. TIMP-2 expression is thought to be largely constitutive (Leco *et al.*, 1994; Inderdeo *et al.*, 1996). However, treatment conditions have affected TIMP-2 concentrations in some types of cell (reviewed by Denhardt *et al.*, 1993). In the present study, pig ovarian cells responded to hCG with an increase in TIMP-2 positive samples.

TIMP-3 was detected in granulosa and theca cell samples and follicular fluid, and the amount was greatest in the theca cells. TIMP-3 has also been observed in mouse granulosa and theca cell layers (Inderdeo *et al.*, 1996). However, none of the factors examined in the current study affected either the percentage of positive samples or the amount of TIMP-3.

Other studies have concentrated on preovulatory follicles but the current study showed that TIMPs occur in all follicles from 2 mm in diameter. It is possible that TIMPs 'protect' small follicles from degradation by MMPs secreted by neighbouring ovulatory follicles (Chun *et al.*, 1992). TIMPs may also protect oocytes and aid in their maturation (Funahashi and Day, 1997) and early embryo development (McIntush and Smith, 1998). It would be interesting to examine TIMP production by the primordial and preantral follicles present in the ovary for what could be many oestrous cycles before they are stimulated to grow. Healthy bovine preantral follicles produce TIMPs *in vitro* (McCaffery *et al.*, 1999).

Since theca cells are embedded in ECM, it is possible that TIMPs regulate their function. TIMP-1 was added to theca cells cultured with and without IGF-1 to remove the possibility of the optimum culture system (Shores *et al.*, 2000) masking any effect of TIMP-1. TIMP-1 increased oestradiol and progesterone concentrations after 144 h. Boujrad *et al.* (1995) reported increased pregnenolone production by rat Leydig cells and increased progesterone synthesis by rat granulosa cells in response to TIMP-1. Increased oestradiol production by mouse granulosa cells in culture was noted by Nothnick *et al.* (1997). However, this group failed to observe any change in progesterone synthesis, perhaps due to the short culture period (24 h). The results of the current study indicate that theca cells respond to TIMP-1 in a similar way to granulosa cells, that is a general increase in steroidogenesis.

Relating the observations *in vitro* to the data collected *in vivo* indicates that the increased amounts of TIMP-1 after hCG treatment might be expected to increase steroidogenesis. However, although follicular fluid progesterone concentrations increased by about 10%, oestradiol concentrations decreased fourfold after hCG treatment. Nothnick *et al.* (1997) were unable to detect any difference in the serum concentrations of oestradiol and progesterone between TIMP-deficient and wild-type mice. Discrepancies between results obtained *in vivo* and *in vitro* indicate that TIMP plays a minor role in the regulation of steroidogenesis *in vivo*, perhaps functioning as a co-regulator, together with a wide array of other intraovarian factors already implicated in the control of steroidogenesis (for a review see Armstrong and Webb, 1997).

Follicular cells produce ECM components including fibronectin, collagens and laminin (Huet *et al.*, 1997). It was proposed that exogenous TIMP-1 would favour matrix build-up, leading to larger clumps of theca cells in the serum-free culture system. Work in this laboratory showed that the size of pig granulosa cell clumps was increased by oocyte conditioned medium (Brankin *et al.*, 1999). This finding indicates that exogenous factors can influence the size of cell clumps in culture, although no obvious increase was observed in the current study. Despite this finding, TIMP-1 may influence cell clump formation, perhaps by stabilizing the ECM leading to improved cell–cell communication, which in turn enhances steroidogenesis.

Hayakawa *et al.* (1992) reported the growth-promoting activity of TIMP-1 on several types of human and bovine cell (but not including any types of ovarian cell). This finding is in contrast to the current study in which TIMP-1 was detrimental to the number of viable theca cells present after 144 h. These differences may simply reflect species differences or differences in the types of cell in response to TIMP-1. However, it may be that TIMP-1 preferentially stimulates the differentiated function of the steroidogenic cells at the expense of proliferation. The general stimulation of steroidogenesis could be due to the sequence homology of TIMP-1 with steroid acute regulatory protein (StAR) (Hartung *et al.*, 1995). In addition, it would be interesting to investigate the effects of TIMP-2 and -3 on theca cell function, particularly TIMP-2 since the present study showed that the concentrations of this inhibitor varied with stage of the oestrous cycle.

In conclusion, the current study confirms that both large and small pig follicles produce TIMP-1, -2 and -3. This is the first report to use reverse zymography to show thecal TIMP production in pigs. TIMP concentrations were higher in the theca layer than in granulosa cells or follicular fluid. Studies *in vitro* demonstrated that TIMP-1 increased steroidogenesis but reduced the number of viable cells in pig theca cells. The current study confirms that TIMPs are produced within the ovary and that this production is regulated. TIMPs not only co-ordinate tissue remodelling but may also have a role in regulating steroidogenesis.

This work was supported by the BBSRC. The authors thank Jim Craigon for expert statistical advice and Martin Luck for critical review. They also thank D. G. Bolt, USDA, for the provision of pig gonadotrophins and D. R. Edwards for technical advice concerning the TIMP kit.

## References

**Armstrong DG and Webb R** (1997) Ovarian follicular dominance: the role of intraovarian growth factors and novel proteins *Reviews of Reproduction* **2** 139–146

**Bagavandoss P** (1998) Differential distribution of gelatinases and tissue inhibitor of metalloproteinase-1 in the rat ovary *Journal of Endocrinology* **158** 221–228

**Besnard N, Pisselet C, Monniaux D and Monget P** (1997) Proteolytic activity degrading insulin-like growth factor binding protein-2, -3, -4, and -5 in healthy growing and atretic follicles in the pig ovary *Biology of Reproduction* **56** 1050–1058

**Boujrad N, Ogwuegbu SO, Garnier M, Lee CH, Martin BM and Papadopoulos V** (1995) Identification of a stimulator of steroid hormone synthesis isolated from testis *Science* **268** 1609–1612

**Bradford MM** (1976) A rapid and sensitive method for quantitation of microgram quantities of protein using the principle of protein–dye binding *Analytical Biochemistry* **72** 248–254

**Brankin V, Webb R and Hunter MG** (1999) Factor(s) secreted by the porcine oocyte modulate granulosa cell growth and steroidogenesis *Journal of Reproduction and Fertility Abstract Series* **23** 66

**Buhi WC, Alvarez IM, Pickard AR, McIntush EW, Kouba AJ, Ashworth CJ and Smith MF** (1997) Expression of tissue inhibitor of metalloproteinase-1 protein and messenger ribonucleic acid by the oviduct of cyclic, early pregnant, and ovariectomized steroid-treated gilts *Biology of Reproduction* **57** 7–15

**Campbell BK, Scaramuzzi RJ and Webb R** (1996) Induction and maintenance of oestradiol and immunoreactive inhibin production with FSH by ovine granulosa cells cultured in serum-free media *Journal of Reproduction and Fertility* **106** 7–16

**Chun SY, Popliker M, Reich R and Tsafriri A** (1992) Localization of preovulatory expression of plasminogen activator inhibitor type-1 and tissue inhibitor of metalloproteinase type-1 mRNAs in the rat ovary *Biology of Reproduction* **47** 245–253

**Curry TE, Sanders SL, Pedigo NG, Estes RS, Wilson EA and Vernon MW** (1988) Identification and characterization of metalloproteinase inhibitor activity in human ovarian follicular fluid *Endocrinology* **123** 1611–1618

**Denhardt DT, Feng B, Edwards DR, Cocuzzi ET and Malyankar UM** (1993) Tissue inhibitor of metalloproteinases (TIMP, aka EPA): structure, control of expression and biological functions *Pharmacology and Therapeutics* **59** 329–341

**Driancourt MA, Quesnel H, Meduri G, Prunier A and Hermier D** (1998) Luteinization and proteolysis in ovarian follicles of Meishan and Large White gilts during the preovulatory period *Journal of Reproduction and Fertility* **114** 287–297

**Fowlkes JL, Suzuki K, Nagase H and Thrallkill KM** (1994) Proteolysis of insulin-like growth factors binding protein-3 during rat pregnancy: a role for matrix metalloproteinases *Endocrinology* **135** 2810–2813

**Funahashi H and Day BN** (1997) Advances in *in vitro* production of pig embryos *Journal of Reproduction and Fertility Supplement* **52** 271–283

**Genstat 5 Committee** (1989) *Genstat 5 Reference Manual* Clarendon Press, Oxford

**Grant SA, Hunter MG and Foxcroft GR** (1989) Morphological and biochemical characteristics during ovarian follicular development in the pig *Journal of Reproduction and Fertility* **86** 171–183

**Hartung S, Rust W, Balvers M and Ivell R** (1995) Molecular cloning and *in vivo* expression of the bovine steroidogenic acute regulatory protein *Biochemical and Biophysical Research Communications* **215** 646–653

**Hayakawa T, Yamashita K, Tanzawa K, Uchijima E and Iwata K** (1992) Growth-promoting activity of tissue inhibitor of metalloproteinases-1 (TIMP-1) for a wide range of cells. A possible new growth factor in serum *FEBS Letters* **298** 29–32

**Huet C, Monget P, Pisselet C and Monniaux D** (1997) Changes in extracellular matrix components and steroidogenic enzymes during growth and atresia of antral ovarian follicles in the sheep *Biology of Reproduction* **56** 1025–1034

**Hunter MG, Grant SA and Foxcroft GR** (1989) Histological evidence for heterogeneity in the development of preovulatory pig follicles *Journal of Reproduction and Fertility* **86** 165–170

**Hunter MG, Biggs C, Pickard AR and Faillace LS** (1994) Differences in follicular aromatase activity between Meishan and Large White hybrid gilts *Journal of Reproduction and Fertility* **101** 139–144

**Inderdeo DS, Edwards DR, Han VKM and Khokha R** (1996) Temporal and spatial expression of tissue inhibitors of metalloproteinases during the natural ovulatory cycle of the mouse *Biology of Reproduction* **55** 498–508

**Leco KJ, Khokha R, Pavloff N, Hawkes SP and Edwards DR** (1994) Tissue inhibitor of metalloproteinases-3 (TIMP-3) is an extracellular matrix-associated protein with a distinctive pattern of expression in mouse cells and tissues *Journal of Biological Chemistry* **269** 9352–9360

**McCaffery FH, Leask R, Riley SC and Telfer EE** (1999) The detection of matrix metalloproteinases and their inhibitors during the development of bovine preantral follicles *in vitro. Journal of Reproduction and Fertility Abstract Series* **24** 15

**McIntush EW and Smith MF** (1998) Matrix metalloproteinases and tissue inhibitors of metalloproteinases in ovarian function *Reviews of Reproduction* **3** 23–30

**McIntush EW, Pletz JD, Smith GW, Long DK, Sawyer HR and Smith MF** (1996) Immunolocalization of tissue inhibitor of metalloproteinases-1 within ovine periovulatory follicular and luteal tissues *Biology of Reproduction* **54** 871–878

**Mann JS, Kindy MS, Edwards DR and Curry TE** (1991) Hormonal regulation of matrix metalloproteinase inhibitors in rat granulosa cells and ovaries *Endocrinology* **128** 1825–1832

**Maxson WS, Haney AF and Schomberg DW** (1985) Steroidogenesis in porcine atretic follicles: loss of aromatase activity in isolated granulosa and theca *Biology of Reproduction* **33** 495–501

**Miller AT, Picton HM, Craigon J and Hunter MG** (1998) Follicle dynamics and aromatase activity in high-ovulating Meishan sows and in Large White hybrid contemporaries *Biology of Reproduction* **58** 1372–1378

**Nomura S, Hogan BL, Wills AJ, Heath JK and Edwards DR** (1989) Developmental expression of tissue inhibitor of metalloproteinase (TIMP) RNA *Development* **105** 575–583

**Nothnick WB, Soloway P and Curry TE** (1997) Assessment of the role of tissue inhibitor of metalloproteinase-1 (TIMP-1) during the periovulatory period in female mice lacking a functional TIMP-1 gene *Biology of Reproduction* **56** 1181–1188

**O'Sullivan MJB, Stamouli A, Thomas EJ and Richardson MC** (1997) Gonadotrophin regulation of production of tissue inhibitor of metalloproteinases-1 by luteinized human granulosa cells: a potential mechanism for luteal rescue *Molecular Human Reproduction* **3** 405–410

**Picton HM, Campbell BK and Hunter MG** (1999) Maintenance of oestradiol production and expression of cytochrome P450 aromatase enzyme mRNA in long-term serum-free cultures of pig granulosa cells *Journal of Reproduction and Fertility* **115** 67–77

**Shores EM, Picton HM and Hunter MG** (2000) Differential regulation of porcine theca cell steroidogenesis by LH, IGF-1 and granulosa cells in serum-free culture *Journal of Reproduction and Fertility* **118** 211–219

**Smith GW, Moor RM and Smith MF** (1993) Identification of a 30000 $M_r$ polypeptide secreted by cultured ovine granulosa cells and luteal tissue as a tissue inhibitor of metalloproteinases *Biology of Reproduction* **48** 125–132

**Smith MF, Kemper CN, Smith GW, Goetz TL and Jarrell VL** (1994) Production of tissue inhibitor of metalloproteinases-1 by porcine follicular and luteal cells *Journal of Animal Science* **72** 1004–1012

**Song L, Porter DG and Coomber BL** (1999) Production of gelatinases and tissue inhibitors of matrix metalloproteinases by equine ovarian stromal cells *in vitro. Biology of Reproduction* **60** 1–7

**Zhu C and Woessner JF** (1991) A tissue inhibitor of metalloproteinases and α-macroglobulins in the ovulating rat ovary: possible regulators of collagen matrix breakdown *Biology of Reproduction* **45** 334–342