The Effect of ACTH during Oestrus on the Reproduction in the Sow

with Special Reference to Duration of Oestrus, Ovulation, Hormonal Patterns, Gametes and Early Embryo Development

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To my family with love

About pigs…

"The time has come," the Walrus said,
"To talk of many things:
Of shoes and ships and sealing wax,
Of cabbages and kings,
And why the sea is boiling hot,
And whether pigs have wings."

Lewis Carroll (1832 – 1898), Through the Looking-Glass (1872)

The work of teaching and organizing the others fell naturally upon the pigs, who were generally recognized as being the cleverest of the animals.

George Orwell (1903 – 1950), Animal Farm (1945)

I understand the inventor of the bagpipes was inspired when he saw a man carrying an indignant, asthmatic pig under his arm. Unfortunately, the manmade sound never equalled the purity of the sound achieved by the pig.

Alfred Hitchcock (1899 – 1980).

In the Ngong Forest I have also seen, on a narrow path through thick growth, in the middle of a very hot day, the Giant Forest Hog, a rare person to meet.

Karen Blixen (1885 – 1962), Out of Africa (1937)

Whoever has looked into the eye of a shrewd old sow should feel humility. It is a bright clear eye, more like the eye of a human than the eye of an animal. It looks at you quite directly, even with what might be called a piercing gaze. The look sizes you up, appraises you.

Louis Bromfield (1896 – 1956)

Ode to the Pig: His Tail
My tail is not impressive
But it's elegant and neat.
In length it's not excessive-
I can't curl it round my feet-
But it's awfully expressive,
And its weight is not excessive,
And I don't think it's conceit,
Or foolishly possessive
If I state with some agressive-
ness that it's the final master touch
That makes a pig complete.

Walter R. Brooks, (1886 – 1958)

Never eat more than you can lift.
Miss Piggy

Abstract

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Regrouping of weaned sows gives rise to stressful situations during the critical time when the sows must resume oestrous activity after lactation. The aim of these studies was to simulate the social stress seen after regrouping through repeated injections of synthetic ACTH during oestrus in the sow. The period of treatment lasted for about 48 h from the onset of standing oestrus. The following reproductive events were studied and compared between the ACTH-treated sows (ACTH group) and the control sows (C group): duration of standing oestrus, time of ovulation, hormonal patterns, the number of spermatozoa and their morphology as well as the intraluminal environment of the uterine tubal junction (UTJ) and of isthmus shortly after ovulation, oocyte/embryo transport in the oviduct, and embryo development at 48 or 60 h after ovulation.

The sows in the ACTH group stopped displaying signs of standing oestrus sooner after ovulation than the C group, but no effect was found on the time of ovulation. Cortisol and progesterone concentrations were elevated significantly in jugular blood samples taken after the ACTH injections. There were minor differences in oestradiol and LH concentrations between the groups. Overall, inhibin α concentrations were significantly higher during the treatment period in the ACTH group than in the C group.

There was a tendency towards a larger number of spermatozoa in the UTJ and oviduct among the sows in the ACTH group. A majority of sows in the ACTH group had moderately to exaggerated amounts of mucus in the intraluminal environment of the sperm reservoir.

The ACTH injections had no effect on embryo development. However, fewer oocytes/embryos were retrieved from the ACTH group than from the C group and there was a tendency towards faster embryo transportation to the uterus.

In conclusion, simulated stress caused significant loss of oocytes and embryos, shortened the duration of standing oestrus and changed the hormonal pattern of progesterone, and possibly of inhibin α, oestradiol and LH. There were also tendencies towards an altered intraluminal environment in the oviduct and UTJ and augmented transportation of spermatozoa and embryos through the female genital tract in the ACTH-treated sows.

Keywords: Pig, Sus Scrofa, ACTH, stress, oestrus, ovulation, endocrinology, sperm transport, embryo transport, embryo development.

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Pigs
Pigs are playful
Pigs are pink
Pigs are smarter
than you think.
Pigs are slippery
Pigs are stout
Pigs have noses
Called a snout.
Pigs are pudgy
Pigs are plump
Pigs can run
But never jump.
Pigs are loyal
Pigs are true
Pigs don't care for
Barbecue.

Charles Vincent Ghigna (b. 1946)

Contents

Appendix

List of original papers I-IV

The thesis is based on the following papers, which will be referred to in the text by Roman numerals:

I. Brandt, Y., Lang, A., Madej, A., Rodríguez-Martínez, H. & Einarsson, S. 2006. Impact of ACTH administration on the oviductal sperm reservoir in sows: The local endocrine environment and distribution of spermatozoa. *Animal Reproduction Science 92*, 107–122.

II. Brandt, Y., Lang, A., Madej, A., Rodríguez-Martínez, H. & Einarsson, S. 2006. Impact of ACTH during oestrus on the ultrastructure of the spermatozoa and their environment in the tubal reservoir of the postovulatory sow. *Animal Reproduction Science*, in press (e-published ahead of print 2005).

III. Brandt, Y., Lundeheim, N., Madej, A., Rodríguez-Martínez, H. & Einarsson, S. 2006. Effects of ACTH injections during estrus on concentrations and patterns of progesterone, estradiol, LH, and inhibin α and time of ovulation in the sow. Accepted for publication in *Domestic Animal Endocrinology*.

IV. Brandt, Y., Madej, A., Rodríguez-Martínez, H. & Einarsson, S. 2006. Effects of exogenous ACTH during oestrus on early embryo development and oviductal transport in the sow. Accepted for publication in *Reproduction in Domestic Animals*.

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Abbreviations

ACTH	adrenocorticotropic hormone
AI	artificial insemination
AIJ	ampullary-isthmic junction
BTS	Beltsville thawing solution
CL	corpora lutea
CLSM	confocal laser scanning microscopy
CRH	corticotropin-releasing hormone
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
LH	luteinising hormone
LM	light microscopy
PBS	phosphate buffered saline
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
SD	standard deviation
SEM	scanning electron microscopy
SR	sperm reservoir
TEM	transmission electron microscopy
UTJ	uterine-tubal junction
ZP	zona pellucida

Introduction

Background

The domesticated pig (*Sus scrofa*) has been the companion of humankind for over 9000 years (Jensen, 1995). However, the history of the pig can be traced much further. The presumed pig ancestors emerged in the Eocene (about 22 million years ago) and the first true pigs appeared in the Oligocene (about 12 million years ago) (Graves, 1984). Pigs in the wild (the family *Suidae*) are gregarious and live in small matriarchic groups consisting of about 3–4 adult females and their offspring. The group can cover distances of between 10 and 20 square kilometres depending on the feed supply. The adult boars live alone and are only allowed near the group during the mating season, which occurs in the autumn (Graves, 1984; Jensen, 1995). Pigs continued to reproduce in the wild in parallel to the domestication, and on many occasions domesticated pigs have also been reintroduced in the wild (feral pigs).

As pigs were domesticated, they were also bred to fit the demands of their owners. For example, the domesticated pig is poly-oestral (the gilts and sows can come into heat all year around, not only during autumn). There can be no doubt that the breeds were modified by crosses of carefully selected individuals, which presented the desired characteristics (Darwin, 1859). Over the centuries, the pig became increasingly adapted to a life with humans. However, the early household pigs were seldom kept in large groups and were most likely roaming more or less freely around their home farms. With the coming of specialisation in the beginning of the 1960s, large-scale intensive breeding units were formed at a quick pace, compared to the slow progress of development before this (Daelemans, 1984; Jensen, 1984). Although the exterior of the domesticated pig is quite different from that of the wild boar, their instincts and needs are still strikingly similar. If domesticated pigs are released into the wild, their behaviour will not differ substantially from that of the wild boars (Jensen, 1995).

So how does our domesticated, but more or less wild, pig cope when it is confined within a limited area, without the possibility of searching for food, fleeing if need be, or having social contact with other pigs? It is reasonable to assume that a pig in that kind of environment must suffer from chronic stress (Barnett, Cronin & Winfield, 1981; Hemsworth *et al.*, 1986; von Borell & Ladewig, 1989). Adaptive responses are partly genetically determined (Vellucci, 1997) and the breeders' need for high-producing pigs led to an extensive selection of individuals that could survive and reproduce in this environment. However, due to the stress, the animals might not reach their full genetic potential (Dobson & Smith, 2000). In the most intensive form of pig breeding, the pigs are kept individually in tether stalls. In this system, the pig can only stand up, sit, and lie down. Stereotypic behaviour among sows is common in these systems compared to those with loose-housed sows (Vestergaard, 1984; Varley & Stedman, 1994; Soede *et al.*, 1997), and can be interpreted as a way of coping with a stressful environment. Due to ethical consideration, this system is now regulated and

banned in an increasing number of countries, *e.g.* in the EU (Council Directive 2001/88/EC amending Directive 91/630/EEC Laying Down Minimum Standards for the Protection of Pigs). The systems developed to replace the tether stalls are often a return to group-housing, at least for the non-lactating sows. However, to achieve the pig breeders' economic goals, the groups are in most cases much larger than groups formed by pigs in the wild. Among wild pigs, confrontation between unfamiliar female pigs is a rare event (Kongsted, 2004), whereas there are numerous confrontations among group-housed sows (Pedersen *et al*., 1993). Depending on *e.g.* group size, about 2–7 days are required for aggression to subside and for a new group of sows to become relatively stable (Tsuma *et al*., 1996; Arey, 1999). In most systems regrouping is commonly done after weaning, which causes the most intensive period of stressful confrontations to coincide with many important reproductive events such as the onset of standing oestrus, insemination, ovulation, and early embryo transport and development.

Stress

Stress can be defined as the inability of an animal to cope with its environment (Dobson & Smith, 2000). A stressful stimulus triggers a complex response in the body. The pattern of responses depends on the nature of the stress, its intensity and duration, as well as the nature and current state of health of the individual (Buckingham *et al*., 1997). Perception of a stressful stimulus causes an acute response by the immediate release of noradrenaline and adrenaline (catecholamines) from sympathetic nerve endings and the adrenal medulla. This release of catecholamines prepares the organism for "fight or flight", an increase in both physical and mental activity. Repeated exposure to a stressor often results in adaptation or habituation. The time for this to occur depends on the type of stress, as well as on the severity and duration thereof (Vellucci, 1997). Via a complex array of nervous pathways, the perception of a stressful stimulus also triggers the release of CRH (corticotropin-releasing hormone) from the hypothalamus. CRH acts on the anterior pituitary gland to release ACTH (adrenocorticotropic hormone). ACTH in turn causes the adrenal cortex to produce and release glucocorticoids (such as cortisol), which have diverse metabolic, behavioural, and immunomodulating effects on the body (Buckingham *et al*., 1997). ACTH also causes the release of other hormones from the adrenal glands, *e.g.* progesterone (Bolaños, Molina & Forsberg, 1997) and inhibin α (Vänttinen *et al.* 2002). It might also affect the levels of other hormones, such as prostaglandins, probably due to enhanced conversion of arachidonic acid to prostaglandin (Laychock & Rubin, 1975). One of the major problems confronting stress researchers is how to measure stress in animals. One possible way is to use the biological cost of stress as an indicator (Moberg, 1993).

Stress and reproduction

A connection between stress and reproductive function has been known for many decades. The effects of stress on reproduction depend on the critical timing of stress in relation to the stage of the oestrous cycle, the genetic predisposition to stress, and the type of stress (Hennessy & Williamson, 1983). The effect of stress

on reproduction is also influenced by the duration of the responses induced by various stressors. It is well-accepted that prolonged or chronic stress results in inhibition of reproduction, but the effects of transient or acute stress are not clear (Tilbrook, Turner & Clarke, 2002).

Several studies report impaired reproductive performance in group-housed sows compared to individually housed sows, attributed mainly to stressful social interactions between the sows and their surroundings. A study by Lynch, O'Grady & Kearney (1984) found that group-housed sows had much poorer reproductive performance than individually penned sows. In a Danish on-farm study, significantly fewer piglets per litter were born among non-lactating group-housed sows compared to individually housed sows (Fisker, 1995). Bokma (1990) reported that there was a higher incidence of return to oestrus when sows were introduced in a group during the first week after service compared to after 22 days after service. However, there are also contradicting results from a study by Hansen & Vestergaard (1984) where no difference was found between group housing and individual housing regarding the number of piglets born, and in a study by Barnett & Hemsworth (1991) where the reproductive performance of gilts was reduced in individual housing compared to group-housing. The effect of stress has been studied on a variety of reproductive events (see below), but still numerous questions remain unsolved, especially as regards stress in relation to regrouping.

Models for stress

Assessing the impact of stress demands a highly controlled environment. The ideal model for stress should include all factors in the stress-axis (*i.e.* activate the early parts in the axis), be repeatable, and not include stressors that are difficult to quantify and where the individual response may differ greatly (*e.g.* through restraint, social interactions within groups, painful injections or repeated bleeding). The model used should also be acceptable from an ethical point of view. Treatment duration and treatment intervals have varied between studies but the reproductive parameters seem to be most susceptible to stress during the follicular phase (Scholten & Liptrap, 1978; Barb *et al.*, 1982; Macfarlane *et al*., 2000; Lang *et al*., 2003), after mating and ovulation (Wettermann & Bazer, 1985), and during the maternal recognition of pregnancy (Arnold *et al*., 1982; Wettermann & Bazer, 1985; Tsuma *et al.*, 1996; Arey & Edwards, 1998).

In several places in the world, the environmental temperature can become very high. Heat stress is easily created under experimental conditions and is associated with reduced reproductive performance (Wettermann & Bazer, 1985). Since mixing of pigs is an event that gives rise to stressful situations, it has been used to assess stress. However, the environment and number of individuals in the groups varies as does the impact of stress on the individuals (Mendl, Zanella & Broom, 1992; Pedersen *et al.*, 1993; Arey & Edwards, 1998). Space restriction, *e.g.* tether stalls, and over-crowding are stressful for pigs (von Borell & Ladewig, 1989; Hemsworth *et al.*, 1986), but individual responses and ability to cope with environmental situations may vary with stress-susceptibility (Marple *et al.*, 1972). Cortisol and adrenalin concentrations are elevated in pigs that are transported

(Dalin *et al*., 1993). However, the amount of stress seen during transport is difficult to interpret due to environmental factors and the individual animal's previous experiences (Fazio & Ferlazzo, 2003). Food deprivation has been proven to be stressful in pigs (Anderson, 1975) and has been used in many studies despite the fact that many consequences of food deprivation result from the catabolic state, not only from stress. A well-tried attempt to mimic stress is through injections of synthetic or entire ACTH or glucocorticoids. To reduce the number of other stressors, animals can be fitted with indwelling vein catheters that allow for injections and blood sampling without restraining procedures. The use of ACTH injections as a model for stress only covers part of the events in the stress-axis, but nevertheless it has revealed many important peripheral and central mechanisms for stress-related disorders (Einarsson, Madej & Tsuma, 1996). In an effort to better simulate stress according to this model, a study was conducted at the Department of Clinical Sciences, SLU, where sows were intravenously injected with CRH (Lang *et al.*, 2004b). Unfortunately, CRH only affected cortisol concentrations in some individuals, possibly due to an insufficient CRH dosage or the timing of the CRH injections in the oestrous cycle.

In this thesis, stress was simulated by injections of synthetic ACTH every 2 or 4 h. The ACTH dose was chosen to achieve cortisol concentrations similar to those seen during regrouping of sows. The sows were injected with ACTH for a maximum of 48 h to simulate the most intensive period of stressful social interactions seen after regrouping. The treatment started at the onset of standing oestrus, a period not previously investigated with regard to the impact of ACTH, and also a period likely to be affected by stressful episodes during regrouping after weaning.

Factors of importance around oestrus

After weaning the sow must resume cyclic activity. She must come into standing oestrus and be mated or inseminated in time before ovulation. The deposited semen should be transported from the cervix through the long uterine horns and oviducts to reach and fertilise the mature oocyte. The fertilised oocytes will undergo the first divisions and be transported through the isthmus of the oviduct to reach the uterus where the milieu must be prepared for the arrival of embryos. There is a close and complex relationship between the events occurring around oestrus and the hormonal patterns, and these delicate mechanisms can be disturbed by external factors such as stress and nutrition.

Oestrus and ovulation

Subsequent to the hormonal changes after weaning, standing oestrus usually occurs after around 4–7 days. The duration of the signs of standing oestrus varies between 24 and 72 h (Simonsson *et al*., 1997). Ovulation occurs approximately two-thirds into the total standing oestrus (Soede, Noordhuizen & Kemp, 1992; Mburu *et al*., 1995). Stress has been shown to cause disturbances in the display of oestrous signs and in duration of oestrus (Ehnert & Moberg, 1991). It is also possible that stress before oestrus can delay the onset thereof (Liptrap, 1970; Hennessy & Williamson, 1983; Lang *et al.*, 2003). If the sow does not show signs

of oestrus as expected, it is likely that she will be inseminated after ovulation, something which will have a negative effect on the rate of fertilisation (Kaeoket, Tantasuparuk & Kunavongkrit, 2005).

The recruitment of follicles usually starts within 48 h after weaning and in the oestrous cycle between day 14 and 16 (Foxcroft & Hunter, 1985). The follicular fluid is the medium where the ova will mature into oocytes. Follicular maturation is dependent on many factors, *e.g.* glucocorticoid concentration (Viveiros & Liptrap, 2000) and hormones such as oestradiol and progesterone (Moor & Dai, 2001). If there is a disturbance in the follicular development, this might affect oocyte quality (Ding & Foxcroft, 1994). If an oocyte is not mature enough there might also be disturbances in the pick-up process by the infundibulum at ovulation (Talbot, Shur & Myles, 2003). Ovarian cysts result from injections of ACTH during the follicular phase of the cycle (Liptrap, 1970; Scholten & Liptrap, 1978). The effect of lower doses of ACTH during standing oestrus on duration of standing oestrus and follicular development has not been studied.

Spermatozoa and their transport

Nowadays, the majority of Swedish sows are artificially inseminated (Simonsson *et al.*, 1997). During both natural mating and in most artificial inseminations (AI), the semen is deposited in the cervix of the sow. The spermatozoa are then passively transported within the reproductive tract of the sow, through the uterine horns to the oviducts. In the furrows of the junction between the uterus and oviduct (the uterine-tubal junction, UTJ) and in the first part of the isthmus, a sperm reservoir is formed. This is an area where spermatozoa can wait in a dormant state in relative safety until ovulation occurs. The reservoir is populated within minutes after insemination but the numbers of spermatozoa do not stabilise until a few hours after insemination (Viring, 1980; Viring & Einarsson, 1981; Mburu *et al.*, 1996). The spermatozoa can remain viable in the reservoir for approximately 24 h (Rodríguez-Martínez, 2001). The number, distribution and morphology of spermatozoa in the UTJ and the isthmus vary predictably in relation to ovulation in the sow (Hunter, Fléchon & Fléchon, 1987; Mburu, Rodríguez-Martínez & Einarsson, 1997). Around the time of ovulation, restricted numbers of spermatozoa from the reservoir leave the quiescent state and progress toward the upper isthmus segment neighbouring the ampullary-isthmic junction (AIJ), where fertilisation takes place (Mburu *et al.*, 1996). The signal for this relocation of spermatozoa in connection with ovulation has been postulated to be hormonal (Hunter, 2002), a change in temperature (Hunter & Nichol, 1986), and/or consist of some kind of signalling substances such as components in the follicular fluid or glycoproteins (Hunter, 2002). The transport and storage of spermatozoa in the sperm reservoir is closely related to the local environment (Jansen, 1995; Rodríguez-Martínez, 2001), which is presumed to be under hormonal control (Hunter, 1991; Hunter, Petersen & Greve, 1999). Local progesterone concentration in the oviduct vessels may be up to 35% higher compared with the concentration in other vessels in the body (Stefanczyk-Krzymowska *et al*., 1994). This is due to the presence of a local counter-current transfer vascular system, which is thought to be a passive transfer of hormones

from venous ovarian blood to arterial ovarian blood (Krzymowski *et al.*, 1982; Einer-Jensen & Hunter, 2005). Since progesterone has been reported to play a role in sperm capacitation (Barboni, Mattioli & Seren, 1995), any disturbances in the hormonal patterns prior to fertilisation might affect sperm transportation as well as pre-fertilisation changes in the spermatozoa. An appropriate number of spermatozoa should be present at the site of fertilisation. If the spermatozoa are too few, the number of fertilised oocytes will be low; if the spermatozoa are too many, there is a higher risk of polyspermia, which will lead to early embryonic death (Hunter, 1991). There are few data on the impact of ACTH injections on sperm transport in the genital tract of the sow, especially with emphasis on the period around ovulation.

The oviduct

The oviduct is divided into three morphologically different segments: the isthmus (adjacent to the uterine horn), the ampulla and the infundibulum (adjacent to the ovary). The oviduct is provided with an epithelium consisting of ciliated and non-ciliated cells, and the oviduct wall has two layers of smooth muscle. The number of ciliated cells as well as the lumen of the oviduct increase from the isthmus to the infundibulum (Rodríguez-Martínez, 2001). The intraluminal environment of the oviduct undergoes changes around the time of ovulation (Buhi *et al.,* 1990; Mburu, Rodríguez-Martínez & Einarsson, 1997), possibly responding to the same signals that trigger the release of spermatozoa from the sperm reservoir. The amount of fluid present in the lumen of the pre-ovulatory oviduct diminishes rapidly after ovulation (Johansson, Tienthai & Rodríguez-Martínez, 2000), possibly facilitating sperm transport. Shortly after ovulation, the isthmic frequency of phasic pressure fluctuations is reduced (Mwanza *et al*., 2000d), thereby allowing the oocyte passage to the isthmus. The changes in the isthmus fluid content and environment are thought to be closely related to early embryo development (Bavister, 1988; Archibong, Petters & Johnson, 1989; Buhi, Alvarez & Kouba, 1997). The protein content in the oviductal fluid appears to be controlled by oestrogen and progesterone (Buhi *et al.*, 1990). The motility of the oviduct is also under the influence of prostaglandin (Rodríguez-Martínez & Einarsson, 1985), oestrogen and progesterone (El-Mowafi & Diamond, 1998): hormones that can all be affected by stress. The impact of ACTH injections during standing oestrus before ovulation on the intraluminal environment in the UTJ and isthmus has yet to be investigated.

Oocyte and embryo transport

It takes the oocytes 30–45 min to reach the AIJ after ovulation. After fertilisation, it takes approximately 46 h for the zygotes/embryos to reach the uterine horn (Oxenreider & Day, 1965; Hunter, 1974). The isthmic segment of the oviduct is thought to be necessary for normal embryo transport to the uterus (Rodríguez-Martínez, Larsson & Einarsson, 1985). Transport time has been reported to deviate after both progesterone injections (Day & Polge, 1968) and food deprivation (Razdan *et al.*, 2001). An increase or decrease in embryo transportation time can cause asynchrony between embryo development and the uterine environment, which could have a detrimental effect on the embryos (Pope,

1988). It is not known if the transport of embryos through the oviduct is affected by pre-ovulation stress.

Early embryo development

Most embryos have completed their first cleavage 17–19 h after ovulation. The 2-cell stage lasts for around 7 h, but the 4-cell stage is longer and lasts for around 22 h. At the passage between the isthmus and the uterine horn, most embryos will be in their 4-cell stage (Hunter, 1974). The activation of the embryonic genome, *i.e.* the replacement of maternal transcripts by transcripts from the embryo's own genome, takes place in the late 4-cell stage embryo in the pig (Tomanek, Kopecny & Kanka, 1989; Hyttel *et al*., 2000). This is believed to be a crucial developmental step and deficient rRNA gene activation has been associated with the 4-cell block (cleavage arrest) in *in vitro* culture. The rRNA activation and the associated nucleolus formation may be used as a marker of the activation of the embryonic genome (Kanka, 2003). Embryo development can be affected by many factors. However, the exact mechanisms and consequences of stress on embryo development and survival are not evident, and further research is needed.

Hormonal parameters around oestrus

Around the time of oestrus, the normal reproductive hormonal pattern includes a rise and fall of oestradiol and luteinising hormone (LH), and after ovulation, a gradual rise in progesterone. Inhibin is produced in the follicles and is thought to be the main regulator of follicle-stimulating hormone (FSH) release (Redmer *et al.*, 1986; Hasegawa *et al*., 1988). Several hormones are closely connected and controlled by feedback mechanisms that can act on several levels in the hormonal pathways. All these feedback mechanisms are not fully understood, but oestradiol, for instance, exerts a positive feedback effect on the release of LH from the pituitary gland, and also on the production of GnRH from the hypothalamus (Hafez, 1993).

Progesterone

Progesterone in the pig is mainly produced in the ovaries by the corpora lutea. The biosynthetic pathway for progesterone, as well as for other steroids such as oestradiol, begins with cholesterol. Progesterone is formed from all steroidogenic tissues as an intermediate in the production of other steroid hormones (Burris, 1998). Therefore progesterone is also released from the adrenal glands after ACTH stimulation (Bolaños, Molina & Forsberg, 1997; Tsuma *et al*., 1998). The pregnancy-promoting actions of progesterone include regulating the contractility of the oviduct and inducing endometrial differentiation (Burris, 1998). Concentrations of progesterone (and oestradiol) are greater in the arteries supplying the oviduct than in the jugular vein due to a counter-current transfer mechanism that retains these hormones locally (Krzymowski *et al*., 1982). If progesterone concentrations are high during the follicular phase there is a higher frequency of polyspermia and also a higher transport rate of oocytes/embryos through the oviduct (Day & Polge, 1968). Progesterone also has an inhibitory effect on LH (Harris *et al*., 1999) and might contribute to an impaired LH-surge.

Oestradiol

Whereas many tissues have the capacity to generate oestradiol, during the follicular phase it is produced mainly in the ovaries. Oestradiol-17β is the most potent circulating oestrogen (Smith, 1998). Oestrous symptoms are oestradiol-dependent and high cortisol concentrations *in vitro* have been shown to inhibit the follicular secretion of oestradiol-17β (Kawate, Inaba & Mori, 1993). A premature decrease in oestradiol concentrations might therefore cause disturbances in the signs of standing oestrus and also the duration of standing oestrus.

LH

LH is a member of the glycoprotein hormone family and is produced by and released from the anterior pituitary gland. LH induces ovulation and luteinisation, and together with FSH it stimulates the oestrogen production by the preovulatory follicles (Bousfield, 1998). The production and release of LH is stimulated by GnRH (gonadotropin-releasing hormone) from the hypothalamus. Gonadotropin-inhibitory hormone, a newly discovered hormone, can inhibit the release of GnRH and consequently also LH (Kriegsfeld *et al*., 2006). The pulse frequency and amplitude of GnRH release is important for stimulation of the LH surge and, subsequently, for ovulation. High concentrations of progesterone can block transmission of the oestradiol induced signal for LH surge generation (Harris *et al.*, 1999). After treatment with high doses of ACTH or hydrocortisone, the LH surge is blocked which leads to ovulation failure (Barb *et al*., 1982). The effect of ACTH on LH might at least in part be due to the increase in progesterone concentrations after ACTH treatment (Bolaños, Molina & Forsberg, 1997; Tsuma *et al*., 1998). However, the effect on LH seems to be dependent on the timing of the increase in progesterone concentration in relation to the oestrous cycle (Harris *et al.*, 1999). The exact mechanism behind this is not known and needs to be evaluated further.

Inhibin

Inhibin is a glycoprotein hormone group that consists of inhibin-A and inhibin-B. The inhibin molecules that suppress the FSH secretion are heterodimers that contain a subunit α and either a subunit βA or βB (determining if it is an inhibin -A or -B) (Schwall, 1998). The follicles are the primary source of inhibin. The subunit βA seems to originate mostly from larger, growing follicles. The subunit βB has been found mostly in pre-antral or small antral follices (Welt & Schneyer, 2001). However, inhibin is also expressed in other tissues (*e.g*. the pituitary and the adrenal glands) (Schwall, 1998). The level of expression of inhibin in the adrenal gland is stimulated by ACTH (Spencer *et al*., 1992; Schwall, 1998, Vänttinen *et al*., 2002). A change in FSH concentrations due to an altered concentration of inhibin might affect the development of growing follicles. Inhibin does not seem to have any effect on LH concentrations (Vale *et al*., 1988). However, both FSH and LH stimulate the production of the subunit βA from mature follicles (Welt *et al*., 2001). Little is known of the impact of ACTH injections in sows on the peripheral concentrations of inhibin in plasma.

Aims

The general aim of these studies was to investigate the effect on reproduction in the sow after ACTH injections, which were given to simulate the social stress seen after regrouping. The period of treatment lasted for around 48 h from the onset of standing oestrus, and the reproductive events compared between the ACTH treated group and the control group were:

- The duration of standing oestrus and time of ovulation.

- The hormonal patterns and concentrations of cortisol, progesterone, oestradiol-17β, LH and inhibin α in the jugular vein blood during standing oestrus.

- The transport of spermatozoa and their morphology in the UTJ and isthmus shortly after ovulation.

- The intraluminal and local endocrine environment in the UTJ and isthmus shortly after ovulation.

- The early oocyte/embryo transport in the oviduct.

- The early embryo development.

Methodological considerations

Materials and methods used in the present studies are presented in detail in the papers (I-IV) listed in the appendix above. A more generalised description of materials and methods is presented here. All procedures involving the use of animals were reviewed and approved by the Ethical Committee for Experimentation with Animals, Uppsala, Sweden.

Animals

A total of 29 crossbred sows (Swedish Landrace x Swedish Yorkshire) were used in these experiments: 14 sows for papers I and II, and 15 sows for papers III and IV. The sows were in their second to fourth parity and weighing between 153–253 kg. The sows were transported from a commercial farm to the Department of Clinical Sciences, SLU, on the day of weaning. They were kept on straw in individual pens with two adult boars in nearby pens. The sows were fed according to Swedish commercial pig production standards (Simonsson, 1994) twice per day (at 7 a.m. and 3 p.m.) and water was provided *ad libitum*. On the day after arrival, the sows were given a general health examination, with special emphasis on udder problems and lameness. The rectal temperature was recorded every morning to detect early symptoms of disease. The sows were studied during two consecutive oestruses, and the time of ovulation from the first oestrus was used to predict the timing of the second one (Mburu *et al*., 1995). The animals were divided randomly into two groups: a control group (C group) and an ACTH-treated group (ACTH group).

Detection of oestrus and ovulation

The sows were checked for prooestrus twice daily from day two post-weaning. From the onset of prooestrus, the sows were checked for oestrus every 4 h using the back-pressure test during which the sow had head to head contact with a boar. Ovaries were monitored for follicle growth using transrectal ultrasonography (annular array sector scanner [Scanner 250, Pie Medical b.v. Maastricht, Netherlands] with a 5 MHz multiple angle transducer as described by Mburu *et al*., 1995) once a day during prooestrus and every 4 h during oestrus. The onset of oestrus was defined as 2 h before the time when the sow first showed a standing reflex. The time of ovulation was defined as 2 h before the first time when no follicles were visible. This procedure was repeated during the second oestrus of the sows. Day one in the oestrous cycle was defined as the day when standing oestrus was first detected.

Venous catheterisation for blood collection

Between day 8 and day 17 (papers I and II) or day 8 and day 10 (papers III and IV) in the first oestrous cycle after weaning, all sows were catheterised in a jugular vein. The sows were sedated with azaperon (Stresnil®) and general anaesthesia was induced with a combination of romifidin (Sedivet®) and zolazepam and tiletamine (Zoletil®). The anaesthesia was maintained by open mask inhalation of

halothane (Fluothane®). A jugular vein catheter was surgically fitted according to Rodríguez & Kunavongkrit (1983). For prophylactic reasons, all sows were treated with penicillin (Novocillin®), 6.3 mg/kg every 12 h for 3 days post-surgery. The 15 sows used for papers III and IV were also given pain-relief by injections of the non-steroid anti-inflammatory drug, ketoprofen (Romefen®, 3 mg/kg), every 24 h for 3 days after surgery.

Blood sampling and treatment (papers I and II)

During the second oestrous cycle, blood was collected from the jugular catheter every 2 h from mid-prooestrus (estimated) until 6 h after ovulation. The first blood collected was discarded to prevent contamination and/or dilution of the sample. A blood sample was thereafter collected in a heparinised tube. The catheter was flushed after each collection with heparinised saline solution. The blood samples were centrifuged and the plasma was stored at -20 °C until analyses were performed.

In the C group sows (n = 7), saline solution, followed by heparinised saline solution, was injected into the catheters every 2 h, beginning 4 h after the onset of standing oestrus. The ACTH group sows (n = 7) were injected with synthetic ACTH (Synacthen Depot® 1 mg/ml) at a dose of 2.5 µg/kg into the catheters every 2 h, beginning 4 h after the onset of standing oestrus. Before injection, the catheter was flushed with saline solution, and thereafter the ACTH, diluted with saline, was injected, followed by saline solution. Finally, the catheter was flushed with heparinised saline solution. The sows received injections either until ovulation or for a maximum of 24 times.

Blood sampling and treatment (papers III and IV)

During the second oestrous cycle, blood was collected from the jugular catheter every 4 h from mid-prooestrus (estimated) until 60 h after the onset of standing oestrus. Extra blood samples were taken after onset of standing oestrus, 45 min after each injection.

In the C group sows (n = 8), saline solution, followed by heparinised saline solution, was injected into the catheters every 4 h, beginning immediately after the onset of standing oestrus. The ACTH group sows (n = 7) were injected with synthetic ACTH (Synacthen Depot®, 1 mg/ml), 5 µg/kg, via the catheters every 4 h, beginning 2 h after the onset of standing oestrus. Each sow received 12 injections.

The blood sample collection and the injection procedures were the same as those described for papers I and II.

Artificial insemination (AI)

The sows were inseminated once during their second oestrus after weaning, 16-18 h prior to the expected ovulation (as estimated from their first oestrus). For this we used 100 ml of fresh extended semen (extended with Beltsville Thawing Solution,

Pursel & Johnson 1976) pooled from two boars of proven fertility containing a total of 10 billion spermatozoa with motility above 70%.

Euthanasia and macroscopical examination of the reproductive organs

The sows were euthanised with an overdose of pentobarbital. For papers I and II the sows were euthanised during anaesthesia, 6 h after ovulation. For papers III and IV, the sows were euthanised 48 (n = 4) or 60 (n = 11) h after ovulation. The internal reproductive organs were then retrieved and macroscopically examined. The number of corpora lutea was counted.

Blood analyses

Cortisol

Plasma cortisol was determined by a radioimmunoassay (Coat-A-Count Cortisol, Diagnostic Products Corporation, Los Angeles, CA, USA), used according to the manufacturer's recommendations and after validation for porcine plasma by Mwanza *et al.* (2000b).

Progesterone

Plasma progesterone was measured by a solid phase radioimmunoassay (Coat-A-Count Progesterone, Diagnostic Products Corporation, Los Angeles, CA, USA), used according to the manufacturer's recommendations and evaluated for porcine plasma by Epelu-Opio & Madej (1988).

Oestradiol-17β (papers III and IV)

Plasma oestradiol-17β was determined by means of a radioimmunoassay (Double Antibody Estradiol, Diagnostic Products Corporation, Los Angeles, CA, USA), used according to the manufacturer's recommendations, but with modifications according to Mwanza *et al*. (2000b). Measurements of oestradiol were taken on every second plasma sample pair (*i.e.* every 8 h and on the sample taken 45 min after injection at this time).

LH (papers III and IV)

The concentration of luteinising hormone (LH) in plasma was determined using a radioimmunoassay developed and validated by Forsberg *et al*. (1993).

Inhibin α (papers III and IV)

Plasma inhibin α was determined by means of an enzyme immunoassay (Inhibin, α-subunit [l–32] Porcine EIA Kit, Phoenix Pharmaceuticals, Inc., Belmont, CA, USA), used according to the manufacturer's recommendations.

Sampling of uterine/UTJ-isthmus venous blood, spermatozoa and oviductal tissue (papers I and II)

The sows were anaesthetised 6 h after ovulation during their second oestrus after weaning. Anaesthesia was induced by pentobarbital (Thiopental®) and maintained with halothane. In the first part of the operation, blood samples were collected from the left side uterus in one vein draining the UTJ-isthmus, and one vein draining the uterine horn. The sampling was repeated every 10 min for 30 min, thereby resulting in 3–4 samples from each site. Parallel blood samples were simultaneously collected from the catheter in the jugular vein. The blood samples were centrifuged and frozen together after the operation, since the 1–40 min storage in room temperature was not expected to result in a drop in the concentration of progesterone (Oltner & Edqvist, 1982).

In the second part of the operation, the right side oviduct was fixed *in situ* by vascular perfusion through a branch of the uterine artery close to the UTJ. The artery was first flushed with saline solution and then cut out and immediately flushed through the prepared artery with approximately 300–500 ml of a 2.5% glutaraldehyde solution in Na-cacodylate buffer (0.067 M). The entire sample was stored in buffered glutaraldehyde solution at 4–8 °C for the morphological examinations.

Immediately post-mortem, the UTJ, isthmus and AIJ of the left side oviduct were removed and separated into 4 segments:

- UTJ, comprising 1 cm of the tip of the uterine horn and 1 cm of the isthmus.
- AIJ and ampulla, comprising approximately 1 cm of the isthmus and 15 cm of the ampulla.
- The remaining isthmus was divided into two equal segments, defined as isthmus 1 (I1) adjacent to the UTJ and isthmus 2 (I2) adjacent to the AIJ.

The UTJ and isthmic segments were intraluminally flushed twice with 0.5 ml of BTS and trained laboratory personnel determined the total numbers of spermatozoa in the flushing from each tubal segment.

Assessment of number of spermatozoa attached to the zona pellucida (ZP) (papers I and II)

The AIJ-ampulla segment was flushed for retrieval of ova. The ova were stored in 2.5% glutaraldehyde solution in Na-cacodylate buffer (0.067 M) at 4–8 °C and subsequently stained with propidium iodide (Sigma Chemical Company Ltd, St. Luis, MO, USA). The number of spermatozoa attached to the ZP was then determined in an epi-fluorescence microscope.

Retrieval of embryos (paper IV)

The oviduct and the adjoining first 10 cm of the uterine horn were flushed separately with 30 ml 0.15 M PBS pre-heated to 38 °C. The fluid was collected in

Petri dishes and brought under a stereo microscope where the embryos were collected to record preliminary cleavage rate.

Microscopy

Preparations of UTJ and isthmus for scanning electron microscopy (SEM) (paper II)

Samples for SEM were taken from the UTJ, I1, and I2. To expose the lumen of the UTJ and isthmus, the samples were split longitudinally down to the lumen using a sharp razor blade. The convex serosa-muscle layer was glued onto a piece of cardboard, and stretched radially to expose the epithelial lining. The glued tissue-blocks were routinely processed for SEM with 2% buffered osmium tetroxide solution (OsO_4), dehydrated, mounted on stubs and sputtered with platinum/palladium to attain a 4–6 nm thick conductive layer. To maximise the exposure of the epithelial lining and the deeper crypts, the specimens were split longitudinally in one to three places and again mounted on stubs and sputtered.

Preparations of embryos for confocal laser scanning microscopy (CLSM), light microscopy (LM), and transmission electron microscopy (TEM) (paper IV)

From the embryos retrieved under the stereo microscope, 50% were fixed with paraformaldehyde (2%) for CLSM. These embryos were stained for visualisation of actin with Alexa Fluor 488 Phalloidin and for tubulin with FITC-labeled antibody. The embryos were then mounted in anti-fade medium (Vectashield®) with propidium iodide to visualise the DNA.

The remaining embryos were fixed in glutaraldehyde (3%) for LM and TEM. The embryos were embedded in 4% agar, post-fixed in 1% OsO_4, and stained in 0.5% uranyle acetate. They were then prepared for LM and TEM according to routine procedures. The embryos were cut in 2 μm semi-thin sections and stained with 1% basic toluidine blue for continuous light microscopy evaluation. Embryos with visible nucleoli had ultra-thin sections prepared for TEM.

Microscopy evaluations (papers II and IV)

The spermatozoa and the surroundings in the UTJ and isthmus were examined in a JEOL 6320F SEM microscope (JEOL LTD, Japan) at 5 kv. The amount of mucus in the individual sows was graded by two independent persons. Due to problems during fixation for SEM, one sow from the C group had to be excluded from this part of the study.

The recordings for embryos examined in the CLSM (Zeiss LSM 510, Germany) included cleavage rate, the number of accessory spermatozoa and, to some extent, the aspect of the cytoskeleton and nuclei morphology.

The embryos that were not included for CLSM were prepared for LM and TEM. The cleavage rate and the number and status of nucleoli were determined,

whenever possible. The status of the nucleoli in the ultra-thin sections was judged by two independent observers and if their judgement (inactive/intermediate/active) differed, a mean for the individual embryo was calculated. From each animal in the group euthanased at 60 h after ovulation, at least three ultra-thin sections from at least one embryo were examined using TEM (JEOL JEM-1230 Electron Microscope, Japan).

Statistical analysis

The statistical analyses were carried out using the SAS programme (version 8; SAS Institute Inc Cary, NC, USA).

Hormonal data in blood sample plasma were analysed using analysis of variance (PROC MIXED) with the effect of individual sow regarded as a random factor. The statistical models included the fixed effects of time of sampling, treatment, and the interaction between time of sampling and treatment. For "local samples" (from veins draining the uterus and oviduct *vs*. the jugular vein) the statistical model also included the effect of location and the interaction between treatment and location. Least squares means were estimated and pair-wise tests of significance were performed for the differences between the estimated means.

Peaks of oestradiol and LH (papers III and IV) were identified by using moving averages, based on three recordings. Student's t-tests were performed to test the differences between treatment groups for length of standing oestrus and time of ovulation (calculated from the onset of standing oestrus) in both first and second oestrus, as well as the difference in cleavage rate and in percentage of embryos recovered in relation to number of corpora lutea. Student's t-tests were also performed on the intervals between the onset of standing oestrus and oestradiol peak and LH-peak respectively, as well as the interval between insemination and ovulation. Paired t-test, within groups of sows, was performed for parameters recorded both in the first and second oestrus.

Since sperm number was distributed far from normally, logarithmic transformation (log10) of sperm numbers was applied in paper I. The statistical analyses were carried out using analysis of variance (PROC MIXED), and pairwise t-tests between least squares means were performed. The effects of sperm location (three segments), group of sows (C or ACTH) and the interaction between group and location were analysed. The random effect of sow within group was also included in the statistical model. Spearman rank correlations were calculated between sperm number in the different segments. Statistical analysis of differences between groups in sperm attachment to ZP of the oocytes was carried out using Fisher's exact test. In paper IV, analyses of number of accessory spermatozoa in the ZP of the embryos were performed using analysis of variance (PROC GLM). The statistical model included the fixed effects of treatment and time of euthanasia (48 or 60 h after ovulation), and the interaction between treatment and time.

Probability values equal to or less than 0.05 were considered to be statistically significant.

Results

Oestrus and ovulation

All sows displayed normal standing oestrus within 6 days of weaning. Ovulation occurred at approximately the same time calculated from onset of oestrus in both groups and also in the first and the second oestrus after weaning. However, there was a significant difference in the duration of the second oestrus after weaning (papers III and IV) between the C group and the ACTH group (66 h *vs*. 52 h, $p < 0.05$). Apparently the ACTH group stopped displaying signs of standing oestrus soon after the occurrence of ovulation in their second oestrus (during/after ACTH-treatment).

Hormone concentrations in serum

Cortisol

Cortisol concentrations in jugular plasma were significantly higher in the ACTH group sows than in the C group sows during the treatment period ($p < 0.05$). A marked drop in cortisol values was noted in the ACTH group after treatment withdrawal. The cortisol concentration pattern was different between the sows where ACTH-injections were given every 2 h *vs*. every 4 h. When the ACTH-injections were given every 2 h, the cortisol concentrations reached more or less steady elevated state after the first two injections. When the ACTH injections were given every 4 h, cortisol concentrations were elevated significantly in samples taken 45 min after each injection in the ACTH group compared to the C group ($p < 0.001$), but at 4 h after injection the concentration had returned to basal levels and there was no difference between the groups ($p = 0.16$). A similar pattern could be detected after each ACTH-injection throughout the treatment period.

Progesterone (Figures 1 and 2)

Progesterone concentrations in jugular plasma were significantly higher in the ACTH group sows than in the C group sows during the treatment period ($p < 0.001$). In the first blood sample after the first ACTH-injection the progesterone concentrations were notably elevated. The progesterone concentrations in the C group remained at a steady basal level until ovulation, when it started to rise. Among the ACTH group sows, the progesterone pattern was similar to that of cortisol, with a marked difference between the sows injected every 2 h and those injected every 4 h. At 4–8 hours after the last treatment, progesterone concentration in the ACTH group had begun to follow the concentration pattern displayed by the C group.

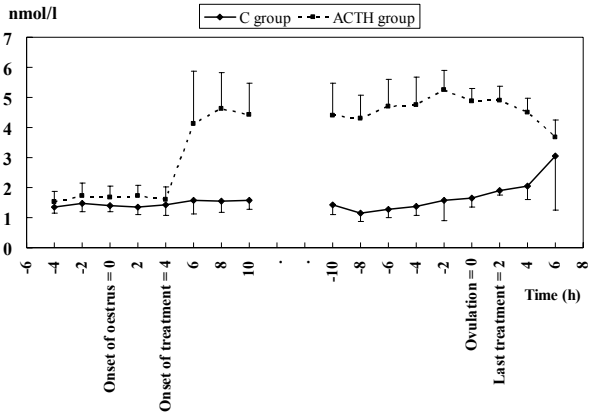

Fig.1. Progesterone concentrations (mean ± SD) during ACTH injections every 2 h.

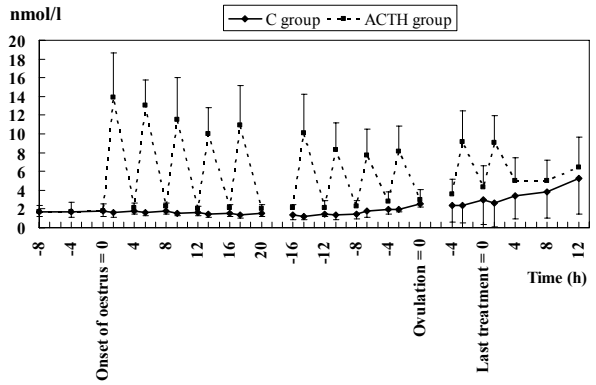

Fig 2. Progesterone concentrations (mean ± SD) during ACTH injections every 4 h.

Oestradiol-17β (papers III and IV)

Oestradiol had already peaked in the majority of sows (13 of 15) by the time treatment started. There was no overall significant difference in oestradiol concentrations between the C group and the ACTH group ($p = 0.11$) during treatment, nor was there any significant difference between the samples taken 45 min after injection and 4 h after injection. However, at 8 and 16 h after the onset of treatment, the ACTH group had significantly lower ($p < 0.05$) oestradiol concentrations than the C group.

LH (papers III and IV)

The overall LH concentrations in the ACTH group were significantly higher during the treatment period than they were in the C group ($p = 0.05$), although the concentrations in both groups returned to basal levels during the treatment period. However, LH values in the ACTH group were nearly significantly higher than in the C group before onset of treatment, a fact taken into consideration when interpreting the results. The ACTH group displayed notably higher LH

concentrations during the surge (the samples taken 4 and 8 h after the onset of treatment, $p < 0.001$). There was no significant difference in LH concentration between the samples taken 45 min *vs*. 4 h after injection.

Inhibin α (papers III and IV)

During the treatment period, the ACTH-group displayed a significantly higher concentration of inhibin α ($p < 0.05$). There was no significant difference in inhibin α concentration between the samples taken 45 min *vs*. 4 h after injection.

Correlations between hormones and intervals between oestradiol peak, LH peak, onset of oestrus, and ovulation (papers III and IV)

Spearman's correlation coefficients calculated for hormone concentrations revealed a positive correlation between cortisol and progesterone levels, and also between cortisol and inhibin α levels, measured 45 min after each injection in the ACTH group. There was a positive correlation between LH and oestradiol levels after the onset of treatment (onset of standing oestrus) in both groups. There was no correlation between the concentrations of inhibin α and LH. In the samples taken before the onset of treatment, positive correlations were evident between the progesterone and cortisol levels, progesterone and oestradiol, and progesterone and inhibin α. There was also a positive correlation between cortisol and inhibin α levels measured before treatment.

No significant differences were found between the experimental groups of sows in terms of the intervals between oestradiol peak, LH peak, onset of oestrus, and ovulation. The mean interval between oestradiol peak and onset of oestrus was 6 h in the C group and 0.5 h in the ACTH group ($p = 0.13$). The mean interval between oestradiol peak and ovulation was 44 h both in the C group and in the ACTH group ($p = 0.96$). The mean interval between LH peak and onset of oestrus was 17 h in the C group and 12 h in the ACTH group ($p = 0.30$). The mean values of the interval between LH peak and ovulation were 32 h both in the C group and in the ACTH group ($p = 0.93$). The mean interval between oestradiol peak and LH peak was 12 h in the C group and 11 h in the ACTH group ($p = 0.97$).

Progesterone and cortisol in plasma from veins draining the oviduct and uterus (paper I)

In the blood samples taken from the area draining the uterus-oviduct 6 h after ovulation and 4 h after the last treatment, there were no significant differences in the plasma concentrations of cortisol or progesterone between the two groups of sows. However, the C group, but not the ACTH group, displayed significantly higher ($p < 0.01$) concentrations of progesterone in the UTJ-isthmus plasma compared with the values in plasma taken in parallel from the jugular vein. There were no significant differences in cortisol concentrations between the sites of sampling (jugular vein, vein draining the uterus and vein draining the UTJ-isthmic region).

Sperm distribution and morphology (papers I, II and IV)

The spermatozoa were commonly found in the deeper crypts of the *in toto* vascularly fixed UTJ in both the ACTH and C group. The majority of the spermatozoa seemed to have intact membranes. The tails of most spermatozoa found both on the epithelial surface and protruding from deeper crypts were in curved or undulating positions. Not all spermatozoa were found in direct contact with the cilia or microvilli of the epithelial cells; rather, some were in positions above the epithelium and in some cases clearly attached to other spermatozoa. The morphology of these spermatozoa did not differ from that of the spermatozoa attached to the epithelial cells, and they were regarded as potentially viable. Occasionally, spermatozoa were found forming large clusters, in which the spermatozoa were entangled in each other and to some extent also in the intraluminal masses. These spermatozoa usually displayed damaged membranes and were considered to be morphologically deviant or dead.

The UTJ generally contained larger populations of spermatozoa than did the isthmus-segments, where only occasional spermatozoa were found. Regarding the distribution of spermatozoa in the different flushed segments, it was evident that there were significant differences between the various segments within both groups, except for the I1-segment and I2-segment in the ACTH group. However, there were no significant differences between the two groups either for total number of spermatozoa or for number of spermatozoa in different oviductal segments. Nevertheless, in the ACTH group there were tendencies for generally higher number of spermatozoa, and possibly a shift of spermatozoa from the I1-segment to the I2-segment, compared to the C group.

Mean numbers of spermatozoa attached to the ZP were not statistically different between the C group and the ACTH group, nor between the embryos/oocytes collected at 48 or 60 h after ovulation. However, in the sows euthanased six hours after ovulation, there was a tendency for the C group to have fewer spermatozoa attached to the ZP than the ACTH group.

UTJ and isthmus (papers I and II)

The epithelium consisted of ciliated and non-ciliated cells. In the isthmic segments, the epithelium consisted predominantly of ciliated cells, whereas the region of the UTJ consisted of approximately equal parts of ciliated and non-ciliated cells. Intraluminal mucus was found to different extents in all samples, ranging from well-defined secretory droplets of various sizes to an amorphous material partially covering the epithelial structures. More sows in the ACTH group (4 of 7 sows) than in the C group (2 of 6 sows) displayed large amounts of intraluminal mucus, covering both the lining epithelium and the spermatozoa. It seemed that in sows with great amounts of mucus, the spermatozoa were generally not located under, but rather partially embedded in the mucus.

Embryos and oocytes (paper IV)

Retrieval of embryos and oocytes (Table 1)

A total of 195 embryos/oocytes were retrieved from the sows euthanased 48 or 60 h after ovulation. In the C group, 81% of the embryos/oocytes were retrieved (calculated from the total number of corpora lutea). In the ACTH group only 51% of the embryos/oocytes were retrieved. This difference was statistically significant ($p < 0.05$). In the sows euthanased six hours after ovulation, a total of 78 embryos were retrieved (from the left side oviduct only). Related to the number of corpora lutea on the left side ovary, 71% of the oocytes were retrieved in the C group, compared to 44% in the ACTH group ($p = 0.09$). The total loss of embryos in the ACTH group (≥6 h after ovulation) was significantly higher than in the C group ($p < 0.01$).

Table 1
Number of embryos/oocytes retrieved

	No. of embryos/ oocytes retrieved	No. of CL
C group 6 h after ovulation	48	66
ACTH group 6 h after ovulation	30	69
C group > 48 h after ovulation	121	149
ACTH group > 48 h after ovulation	74	145

Transport of embryos and oocytes

The majority of the embryos/oocytes in the C group were found in the oviducts at 48 h after ovulation, while in the ACTH group the majority were found in the uterus. At 60 h after ovulation, the majority of the embryos/oocytes were found in the uterus in both groups, but in the ACTH group all embryos/oocytes were in the uterus. Thus, the transportation of the embryos/oocytes in the ACTH group might have been faster than in the C group.

Embryo development

The cleavage rate did not differ between the C group and the ACTH group. At 48 h after ovulation, all embryos were in the 4-cell stage, but at 60 h after ovulation about 25% of the embryos had evolved beyond the 4-cell stage (19% in the C group, 3 of 5 sows; and 32% in the ACTH group, 2 of 5 sows). No embryo had more than eight blastomeres. A total of 75 embryos were examined in CLSM. No differences were noted between the actin/tubulin patterns or nuclei morphology between the C group and the ACTH group. A total of 12 embryos were examined in TEM. No difference was noted in number of inactive/active nucleoli between the groups.

General discussion

Animals and experimental model

In the present studies, only multiparous sows were used. Gilts and primiparous sows are more sensitive to stress and other challenges and using them for the present studies might therefore have given more evident results. However, the advantages of using multiparous sows must also be taken into account. Multiparous sows have a documented successful reproductive record of at least two litters and, compared with primiparous sows, an enhanced ability to return to an anabolic state after weaning. The second oestrus after weaning was chosen to allow the sows to reach a more anabolic metabolism, and also to detect any incongruities in the first oestrus after weaning that would make the sows unsuitable for the experiment. The recorded time of ovulation from the first oestrus after weaning was also used to calculate the appropriate time of insemination during the subsequent oestrus after weaning (Mburu *et al*., 1995). The use of multiparous sows and the second oestrus after weaning will therefore to some extent ensure an experimental group that has normal reproductive functions, and whose members are in a similar metabolic state. Since the number of animals used in these studies was low, the need for a more homogenous group of experimental animals increased. Another reason for choosing multiparous sows was the use of transrectal ultrasonography to detect ovulation. The pelvic cavity might not be wide enough for this procedure in gilts or primiparous sows. In the present studies, the daily dose of synthetic ACTH did not differ between the ACTH groups, whereas the injection interval was changed from 2 h to 4 h due to practical reasons. The effects on reproductive events were assumed to be similar regardless of the change in injection interval. Minor deviations due to this cannot wholly be ruled out, but in this thesis I will mainly attempt to discuss the effect of the ACTH treatment, irrespective of injection interval.

Oestrus and ovulation

All sows in the present studies displayed normal oestrous behaviour during both the first and second oestrus after weaning. There were no differences in the time of ovulation (calculated from the onset of standing oestrus) in either the first or the second oestrus, nor between the groups. The duration of standing oestrus could only be determined in the second experimental group since the first experimental group was euthanased 6 h after ovulation when they were still in standing oestrus. The duration of the treated oestrus was significantly shorter for the ACTH group, but since there was no effect on ovulation times, this might be of little practical consequence in relation to time of insemination. The disruption in oestrous behaviour after ACTH treatment might be due to a premature decline in oestradiol concentrations, perhaps in combination with increased progesterone concentrations. Oestrous behaviour is dependent on oestradiol and on the relation between oestradiol and progesterone (Etgen, 1998), and the signs of oestrus can be inhibited by treatment with ACTH before the onset of standing oestrus (Gee, Geissinger & Liptrap, 1991). All sows displayed normal oestrous symptoms and

ovulated within the normal time span in the present study, and no cystic ovaries were found after the ACTH injections. Earlier studies where sows and gilts were treated with ACTH showed high frequencies of cystic ovaries resulting from this treatment (Liptrap, 1970; Scholten & Liptrap, 1978; Barb *et al.*, 1982; Gee, Geissinger & Liptrap, 1991). However, in the instances where ACTH injections resulted in ovarian cysts, ACTH was given during a longer period than 48 h, and also well in advance of the onset of standing oestrus (during the entire follicular phase). No cystic ovaries were seen when the onset of ACTH injections began only one day before the expected onset of oestrus (Liptrap, 1970). Whether ACTH injections, with subsequently increased concentrations of progesterone, are given before or after the onset of standing oestrus might thus be an important factor in the effects seen (Hennessy & Williamson, 1983; Harris *et al.*, 1999). Since both the rise in oestradiol and the LH-surge commence before, or close to, the onset of standing oestrus, the sow might be less sensitive to disturbances regarding time of ovulation when ACTH injections are given from the onset of standing oestrus.

Endocrinology

Cortisol

It is well known that ACTH injections result in an increase in cortisol concentrations. ACTH stimulates both the immediate release and the production of cortisol in the adrenal glands. When ACTH was administered every 2 h, cortisol concentrations were significantly elevated throughout the treatment period, whereas when ACTH was administered every 4 h, cortisol concentrations were significantly higher 45 min after ACTH injection but had receded to pre-treatment concentrations 4 h after injection. Cortisol concentrations measured 2 h after each ACTH injection were well correlated with cortisol concentrations seen during regrouping of sows (Pedersen *et al*., 1993; Tsuma *et al.*, 1996), whereas cortisol concentrations 45 min after ACTH injection were moderately higher. It is possible that there is a peak concentration of cortisol around 60 min after intravenous ACTH stimulation. This was supported by a study where blood samples were taken every 20 min, and where the peak concentrations of cortisol were found around 1 h after ACTH injection (Madej *et al*., 2005b). Between 4 and 8 h after the last ACTH injection there was a marked decline in cortisol concentrations, which resulted in concentrations below those seen during pre-treatment. It is possible that this was the result of negative feedback of cortisol on ACTH (Kemppainen & Behrend, 1997), which could not be seen during treatment due to the iterations of exogenous ACTH. Cortisol concentration in the follicles decreases at a slower rate than in the blood and the effect of ACTH-injections might therefore linger there, with detrimental effects on follicular development (Montgomery *et al*., 1997). High concentrations of cortisol present in follicles have also been shown to have a negative effect on fertilisation *in vitro* (Jimena *et al*., 1992), but fertilisation rate in the present studies did not differ between the two groups. During pregnancy, high concentrations of maternal cortisol might affect the embryo directly since the maternal concentrations are reflected in the embryo (Klemcke, McGuire & Christenson, 1999), the survival of the offspring (Kanitz *et al*., 2003) and also in the stress-axis of the offspring later in life

(Haussmann *et al*., 2000). However, in the present study only the early embryos were examined, and no differences could be found between the embryos from the C group and those from the ACTH group.

Progesterone

The hormonal pattern of progesterone was positively correlated with that of cortisol. The increase of progesterone concentrations seen after ACTH injections during the follicular phase is believed to originate mainly from a release from the adrenal glands (Bolaños, Molina & Forsberg, 1997; Tsuma *et al*., 1998). ACTH stimulates the production of enzymes and proteins involved in the steroid production from the adrenal glands, a process during which progesterone is formed as an intermediate (Burris, 1998). A decline in progesterone concentration, similar to that of cortisol, was seen after the last ACTH injection in the ACTH group where the injections stopped when ovulation was detected. From the results from this group, which was euthanased 6 h after ovulation, it was not possible to determine if the decline in progesterone concentration would have continued despite the onset of ovarian progesterone production. The ACTH group euthanased at a later stage displayed a rise in progesterone around 8 h after ovulation that did not differ significantly from the rise in the C group. It is therefore possible to conclude that the ACTH treatment during oestrus did probably not affect the progesterone production from the corpora lutea in the ovaries. However, the premature rise in progesterone might have affected the responsiveness to progesterone in reproductive tissues after ovulation, since the number of progesterone receptors are reported to decrease after exposure to progesterone (Funk & DeMayo, 1998). Elevated concentrations of progesterone might have had an impact on the functions of the uterus, oviduct (Day & Polge, 1968; Hunter, 1981; Gawronska, Stepien & Ziecik, 2000), ovary (Close & Liptrap, 1975) and possibly also on the acrosome reaction of spermatozoa (Sueldo *et al*., 1993; Melendrez, Meizel & Berger, 1994). In the present study there was a possible disturbance of the intraluminal environment of the UTJ and isthmus, and a tendency towards increased transport rate of both spermatozoa and embryos, whereas there was no effect on the ovaries or on the spermatozoa themselves.

Oestradiol-17β

There were no significant differences overall in oestradiol concentrations between the C group and the ACTH group. However, immediately after the onset of treatment, there were significantly lower oestradiol concentrations in the ACTH group, which persisted for the first 16 h after the onset of treatment. The effect of ACTH treatment on oestradiol might be limited since the concentrations of oestradiol were already declining in most sows when treatment started. Detrimental effects on oestradiol concentrations due to ACTH treatment were observed in one sow during a previous experiment (Lang *et al*., 2004b), and lower concentrations of oestradiol and/or altered pattern of oestradiol secretion have been reported after treatment with dexamethasone or ACTH commencing before the onset of standing oestrus in sows (Frautschy & Liptrap, 1988; Gee, Geissinger & Liptrap, 1991). A significant decrease of oestradiol concomitant with an elevation of cortisol was also seen during fasting in pregnant sows (Tsuma *et al*.,

1996). High cortisol concentrations are reported to inhibit oestradiol secretion from the follicles *in vitro* (Kawate, Inaba & Mori, 1993), and this might have contributed to the decline in oestradiol concentration in the present study. The early decrease in oestradiol concentration might also be connected with the reduced duration of standing oestrus seen in the ACTH group.

LH

There was a near-significant difference between the groups before treatment that could help account for the significantly higher concentrations of LH seen in the ACTH group compared to the C group during ACTH injections. However, during the LH surge, the LH concentrations were notably augmented in the ACTH group in comparison with the C group. The findings in the present study concur with results seen in an earlier study at the department where the concentrations in the pre-ovulatory LH surges tended to be higher in the ACTH treated sows than in the control sows (Lang *et al*., 2004a). An increase in basal LH concentrations has also been found after short-term injections of cortisol in gilts (Pearce, Paterson & Hughes, 1988), and *in vitro,* where pituitary cells were incubated with cortisol (Li, 1987). Other earlier studies using other stressors and ACTH doses have shown that stress and ACTH injections result in a reduced LH release and in some cases even a complete absence of the LH surge (Barb *et al*., 1982). The effect on LH concentration might be dependent on the type of stressor, the way stress is simulated, the duration of treatment or the dose of ACTH used. The increase in LH seen during the present study might also be connected with the fact that the ACTH injections co-occurred with a period when oestradiol concentrations were elevated (Brann & Mahesh, 1991). In oestrogen-primed rats, progesterone is thought to induce the release of LH (Brann, Putnam & Mahesh, 1991). Since progesterone was elevated significantly in the ACTH group, this might have contributed to the increase in LH concentrations. Furthermore, at the time of the onset of treatment, an increase in LH had already begun in several sows and the effect on LH seems to be dependent on the timing of the increase in progesterone concentration in relation to the oestrous cycle (Harris *et al*., 1999). When interpreting the consequences of high LH concentrations during the pre-ovulatory LH surge, one must also consider that the frequency of LH pulses were not measured in the present study. A high frequency of GnRH/LH pulses is believed to be an important positive feedback factor for follicular growth and oestradiol production (Dobson & Smith, 2000). In the present study, all sows ovulated and neither ovulation rates nor the interval between the onset of standing oestrus and ovulation differed significantly between the groups. Thus, the increase in LH concentrations seen in the ACTH group did not seem to affect the number of ovulations, nor the time thereof.

Inhibin α

Inhibin α was significantly higher in the ACTH group during treatment, which is in agreement with results reported by Madej *et al*. (2005a). The origin of the increment of inhibin α after ACTH treatment is not clear. The main source of inhibin α during the follicular phase is the follicles, but inhibin α is also reported to be released from the adrenal glands after stimulation of ACTH (Spencer *et al*.,

1992; Schwall, 1998, Vänttinen *et al*., 2002). However, increased concentrations of LH are thought to stimulate the production of inhibin from the mature follicles (Welt *et al*., 2001). There was a positive correlation between cortisol and inhibin α concentrations, possibly indicating the simultaneous release from the same source after ACTH stimulation, whereas there was no correlation between the concentrations of inhibin α and LH before or during ACTH injections. Nevertheless, this connection cannot be ruled out since the effect of high LH concentrations on the production of inhibin α from the follicles might be delayed (Vale *et al*., 1988). It is possible that the increase is a result of the combined stimulatory actions of ACTH and LH. An increase in inhibin α concentrations may result in impaired follicle growth due to the negative feedback on FSH. The concentration of FSH was not measured in the present study, but no differences in follicular growth between the groups were noted according to the transrectal ultrasonography. Since significantly fewer oocytes and embryos were retrieved in the ACTH group, it can be speculated that the increase in inhibin α was a consequence of a modification in the granulosa cell function, connected with an impaired follicle development and oocyte maturation that led to a high number of failures of the ovum pick-up by the oviduct. However, when comparing the embryos retrieved, there were no differences in embryo development between the groups.

Intervals between oestradiol peak, LH peak, onset of oestrus, and ovulation

Injections of ACTH in the present study, where the interval between injections was 4 h had no significant effect on the interval between LH peak, oestradiol peak, and onset of standing oestrus or ovulation. In an earlier study by Lang *et al*. (2004a) where injections were repeated every 2 h, the ACTH-treated sows experienced a prolonged interval both from the LH peak to ovulation, and from the oestradiol peak to ovulation. It is possible that the mode of ACTH-injections or the individual variation can influence these parameters.

Progesterone and cortisol in veins draining the oviduct and uterus

In the present study, blood samples were only taken from the veins draining the oviduct 6 h after ovulation and 4 h after the last ACTH injection. It is therefore not possible to draw any conclusions regarding the concentrations of progesterone and cortisol in these veins during treatment. There were no significant differences between the ACTH group and the C group as regards the concentrations of progesterone and cortisol taken from veins draining the UTJ 6 h after ovulation. At this time the progesterone concentrations in the ACTH group were stable or decreasing, whereas the progesterone concentrations in the C group were increasing. In the C group, but not in the ACTH group, there was a significant difference between the concentrations of progesterone in the vein draining the UTJ and the corresponding concentrations in the jugular vein. This supports the presence of a counter-current transfer mechanism that can recover progesterone, and possibly oestradiol (not measured in the present study), in the local circulation (Krzymowski *et al.*, 1982; Einer-Jensen & Hunter, 2005). No increase in the concentrations of cortisol could be found in parallel to the increase of

progesterone. It is possible that the counter-current transfer does not include all steroid hormones, but only those directly related to the functions of the oviduct and ovary. The ACTH injections resulted in an increase in progesterone before ovulation, possibly affecting the counter-current transfer through an alteration in the ratio between progesterone in arterial *vs*. venous blood. A local alteration of progesterone concentration or progesterone:oestradiol ratio could cause an equally local disturbance in the area, possibly related to the milieu of the oviduct.

Spermatozoa

Transport

To determine the number of spermatozoa in the UTJ and isthmus, the segments of the oviduct were flushed two times, since, according to Mburu *et al*. (1996), one flushing is not enough to retrieve a satisfactory representation of the numbers present. No attempt was made to calculate the exact number of spermatozoa found during *in situ* observation in SEM, since the already limited area that was investigated contained numerous crypts inaccessible by SEM. The flushing technique is also reported to be a better assessment of distribution and number of tubal spermatozoa than *in situ* observation with SEM (Mburu *et al.*, 1996).

After treatment, there was no significant difference between the numbers of spermatozoa retrieved between the two groups. As have been reported in earlier studies (Viring, 1980; Viring & Einarsson, 1981; Mburu *et al.*, 1996) the number of spermatozoa decreased significantly in each segment closer to the AIJ. However, there was a slight tendency towards higher numbers of spermatozoa in the portion of the isthmus adjacent to the AIJ in the ACTH group, and there was no significant difference between the two isthmic segments in this group. This finding might indicate an augmented transport of spermatozoa in this group, either through the entire uterus and oviduct, or only in the isthmus. It could also reflect an increased release rate from the sperm reservoir. Irrespective of the extent of this increased transport, it might give rise to an excess of spermatozoa at the site of fertilisation (the AIJ), which could result in polyspermic fertilisation (Hunter, 1991). However, even though pig oocytes are extremely sensitive to polyspermia *in vitro*, they are thought to be more resistant *in vivo* (Hunter, 1991). The resistance to polyspermia *in vivo* in the pig was supported by the fact that only one possible case of polyspermia was found among the embryos flushed out 48 and 60 h after ovulation.

The reason behind this facilitated transport or release from the sperm reservoir is not apparent, but might be connected with the high concentrations of progesterone in the ACTH group during treatment. An increase in the number of polyspermic oocytes after injections of progesterone was found in a study by Day & Polge (1968). Progesterone reduces the oedema in the UTJ and oviduct (Hunter, 1981) and also causes a relaxation of the porcine oviduct (Gawronska, Stepien & Ziecik, 2000). The functional closure of the oviduct is also thought to be controlled by high concentrations of oestradiol in the absence of progesterone (Jansen 1995). Under the influence of progesterone, the oviduct might therefore have been less of

a mechanical barrier to the spermatozoa. The oviduct also shows changes in its contractility during the oestrous cycle, with strong peristaltic waves during the fourth day of the oestrous cycle when progesterone concentrations have risen notably after ovulation (Rodríguez-Martínez, Einarsson & Larsson, 1982). It is possible that the increase in progesterone concentrations in the ACTH group had an effect on sperm (and embryo/oocyte) transport due to increased peristaltic movement of the oviduct. An injection of ACTH is followed by a $PGF_{2\alpha}$-metabolite peak (Razdan *et al.*, 2002; Lang *et al*., unpublished results) and administration of $PGF_{2\alpha}$ also results in an increased frequency and amplitude of contractions of both the uterus and oviduct (Edqvist *et al*., 1975; Rodríguez-Martínez & Einarsson, 1985; Pettersson, Einarsson & Kindahl, 1993; Mwanza *et al*., 2002a), which might facilitate sperm transport.

The tendency towards higher numbers of spermatozoa found in the ACTH group might also be connected with the high amount of mucus present in most of the sows in this group. It is possible that the mucus retained spermatozoa that would otherwise have been transported on to the ampulla and abdominal cavity. Some spermatozoa may have been hidden from detection by SEM or been trapped in the mucus and not retrieved in the flushing, factors which might contribute to discrepancies seen between the numbers of spermatozoa in flushings compared to the amount of spermatozoa observed when using SEM.

Accessory spermatozoa in the zona pellucida (ZP)

Accessory spermatozoa in the ZP can be used to obtain information on the number of spermatozoa in the sperm reservoir and isthmus (Alanko, 1974). The number of accessory spermatozoa in the ZP tended to be higher in the ACTH group than in the C group 6 h after ovulation. This tendency was not confirmed when accessory spermatozoa were counted on embryos retrieved 48 or 60 h after ovulation. The number of spermatozoa attached to the ZP at 6 h after ovulation might reflect the number of spermatozoa present at the site of fertilisation (the AIJ), whereas the number of spermatozoa in the ZP from 48 h after ovulation most probably is a measurement of the number of spermatozoa the embryo has encountered during the first two days after fertilisation, when moving down the isthmus. Since there was no difference between the number of spermatozoa in the ZP in the groups euthanased at 48 or 60 h after ovulation, it is reasonable to assume that most spermatozoa attach to the ZP within the first 48 h after ovulation, in consistency with the time when the embryos will reach the uterus (Hunter, 1974), where they are unlikely to encounter any more viable spermatozoa.

Morphology

In the present study, most spermatozoa observed by SEM in both the C group and the ACTH group had intact membranes and curved or undulating tails, indicating that the spermatozoa were viable and in an active state. This corresponds to SEM observations during the peri-ovulatory period made by Mburu, Rodríguez-Martínez & Einarsson (1997). The significance of these seemingly viable spermatozoa in the UTJ and isthmus 6 h after ovulation is not clear, but they might

be an indication of a continuous release of spermatozoa from the sperm reservoir after ovulation (Mburu *et al*., 1996; Rodríguez-Martínez *et al*., 2005).

The UTJ and isthmus

Earlier studies on the morphology of the epithelial lining of the UTJ and oviduct have often used immersion fixation methods where the lumen of the specimen has to be opened to ensure fixation, and sometimes even rinsed with fixation media. In the present study, the UTJ and the isthmus were perfusion fixed and the lumen was not opened until the specimens were mounted for SEM. This procedure might therefore have ensured a better representation of the true intraluminal environment.

The epithelium consisted of ciliated and non-ciliated cells with microvilli (secretory cells). In consistence with earlier studies (Stalheim, Gallagher & Deyoe, 1975; Hunter, Fléchon & Fléchon, 1987; Mburu, Rodríguez-Martínez & Einarsson, 1997), the proportion of ciliated cells increased in the isthmus compared to the UTJ and dominated the epithelium completely close to the AIJ. The surface of the epithelium of the individual sows was covered with various amounts of mucus. The structure of the mucus ranged from well-defined droplets to an amorphous mass, which wholly or partially embedded epithelial structures and spermatozoa. The number of sows with high amount of mucus was higher in the ACTH group than in the C group. The main components of epithelial mucus in the oviduct is reported to be acidic glycoproteins (Jansen, 1995) and the presence of mucus in the UTJ and oviduct is apparent during the pre-ovulatory period (Jansen, 1978; Mburu, Rodríguez-Martínez & Einarsson, 1997; Johansson, Tienthai & Rodríguez-Martínez, 2000) and decrease after ovulation (Hunter, Fléchon & Fléchon, 1987; Johansson, Tienthai & Rodríguez-Martínez, 2000). The high amount of mucus seen after ovulation in several sows might be due to an increased mucus production or an impaired cleansing of mucus. The reason for this accumulation of mucus is not implicit, but might be connected with the altered concentrations of progesterone or the relationship between progesterone and oestradiol concentrations (Jansen, 1978; Leese *et al*., 2001). The effects of an altered environment in the UTJ and oviduct might include altered transport and survival of both spermatozoa (see above) and embryos. However, when sows were euthanased 48 or 60 h after ovulation, there were only tendencies towards an altered transport rate of embryos in the ACTH group, and no effects were seen on embryo development (see below).

Embryos and oocytes

Transport

Compared to the number of corpora lutea, the number of embryos retrieved at 48 and 60 h after ovulation was significantly lower in the ACTH group than in the C group. A tendency towards a lower percentage of retrieved oocytes was also noted in the ACTH group that was euthanased 6 h after ovulation. Retrieval rates of embryos and oocytes in the C group were similar to those reported after flushing of oviducts and uterine horns in earlier studies (Oxenreider & Day, 1965; Mwanza

et al., 2000ac; Razdan *et al*., 2001; Mwanza *et al*., 2002b). The reason for the low number of embryos and oocytes retrieved in the ACTH group might be failure of the oviduct to retrieve the oocytes at ovulation, either due to an unsuccessful expulsion of the oocyte or a failure of the oviduct pick-up procedures of the oocyte. The oocyte pick-up is a delicate process that can be influenced negatively if the immediate environment is not suitable or if the oocyte is not mature enough (Talbot, Geiske & Knoll, 1999; Talbot, Shur & Myles, 2003). Since there seemed to be a disturbance in the oviductal environment 6 h after ovulation, this might be a factor contributing to the loss of oocytes and embryos. Injections of ACTH before and during standing oestrus in sows have resulted in necrotic changes in the follicles as well as degenerative changes in the oocytes (Gee, Geissinger & Liptrap, 1991). However, the embryos that were retrieved 48 or 60 h after ovulation seemed to have developed normally (see below) and no evidence was found concerning the possible immaturity of the oocytes at ovulation.

The transport rate of embryos through the oviduct tended to be augmented in the ACTH treated sows. The reason for this might be linked to the same factors as the possible augmented transport of spermatozoa. The exact mechanism(s) is/are not clear, but high progesterone concentrations can, for example, cause a relaxation of the porcine oviduct (Gawronska, Stepien & Ziecik, 2000) and might therefore contribute to a facilitated embryo transport to the uterus. The peristaltic movement of the oviduct is increased during the early rise in progesterone concentrations after ovulation (Rodríguez-Martínez, Einarsson & Larsson, 1982). The early rise in, or the lingering effects of, the progesterone concentration in the ACTH group might also have contributed to the increased transport rate of oocytes/embryos. The development of the embryos is dependent on the environment in the oviduct and uterus (Gandolfi & Moor, 1987). If the embryos reach the uterus before the environment is prepared for their arrival, the asynchrony between embryo development and uterine environment might have detrimental effects on the further development of the embryos (Polge, 1982). However, the slight deviation in transport time seen in the ACTH group compared to the C group might not be enough to cause an asynchronous situation, since pig embryos are normally found in the uterus at 46–60 h after ovulation (Hunter, 1974). In a study by Mwanza *et al*. (2000c), ACTH was injected repeatedly for 48 h after ovulation, and no effect was seen on transport rate of the embryos through the oviduct. However, when ACTH is administered after ovulation, the increase in progesterone after each ACTH injection is likely to have little effect, since progesterone concentrations are already increasing from the ovaries.

Development

In the present study, embryo development was assessed by cleavage rate, morphology and the activation of nucleoli. No differences were found between the ACTH group and the C group on any of the factors investigated in this study. These results stand in contrast to those in a study by Razdan *et al.* (2002) where the sows were repeatedly injected with ACTH for 48 h after ovulation and the cleavage rate of the embryos in the ACTH treated group tended to be lower. The follicle function has also been reported to be altered (fewer viable granulosa cells

per follicle) after repeated ACTH injections in mid-cycle (Viveiros & Liptrap, 1995). The timing of stress or ACTH injections seems to be an important factor for effects on many reproductive events (Hennessy & Williamson, 1983; Harris *et al*., 1999) and it is likely that injections of ACTH after ovulation have other effects than injections given before ovulation. It is possible that ACTH treatment during the first 48 h of standing oestrus had no impact on embryo development or that the parameters examined were not affected by the ACTH treatment.

Concluding remarks

Among all living beings the basic requirements of survival are closely followed by the requirement to reproduce. Through years of evolutionary and man-driven selection, pigs have developed an enormous potential for overcoming stressful situations that could cause disturbances in the reproductive functions. In the present studies, the clear effect of ACTH injections seems to be moderate. The possible disturbances observed in oestrous behaviour, sperm and embryo transport, hormonal patterns and the oviduct did not have any effects on the quality parameters evaluated in the early embryos. However, the significant loss of oocytes (or embryos) along the way from ovulation to the uterus could be a significant threat to the reproductive performance. The exact mechanism of this cannot be determined from these studies and further investigations are needed.

Even though the daily dose of ACTH was the same in all ACTH group sows in the present study, the mode of injection (2 *vs*. 4 h) might have affected the outcome of the studies. The hormonal responses were similar after both injection intervals and doses, but the pattern differed, at least concerning cortisol and progesterone. In other reports where a moderately higher ACTH dose was used, the rise in cortisol concentrations was not higher than in the present studies and cortisol concentrations returned to pre-treatment concentrations within 4 h (Razdan, 2003; Madej *et al*., 2005b). The reason for this might be the metabolic half-life of cortisol in the body, which is reported to be between around 36–164 min in pigs (Montgomery *et al*., 1997; Hari & Pliska, 2005).

In studies where high doses of ACTH have been administered to gilts and sows during more than 48 h (Liptrap, 1970; Scholten & Liptrap, 1978; Barb *et al*., 1982), the negative effects on reproduction seem enhanced compared to more frequent injections of low doses of ACTH, such as the present study. However, it is questionable whether the sow will ever be exposed to such extremely high concentrations of endogenous ACTH, *e.g.* during regrouping. This is not to say that the stress during regrouping is less severe than simulated stress. The period needed to create a stable group of pigs after a regrouping event might well last for up to several weeks for certain individuals (Moore, Gonyou & Ghent, 1993; Stookey & Gonyou, 1994) and the models for stress cannot recreate and control all pathways and factors involved in the reaction to a stressful situation. *In vitro* studies have also indicated that synthetic ACTH ($ACTH^{1-24}$) might not have the exact same effects as entire ACTH ($ACTH^{1-39}$) (Phogat, Smith & Dobson, 1997).

Progesterone is the factor that time and again appears throughout the discussion of possible mediators for the observed differences between the ACTH group and the C group. Even though it is clear that the effects seen after ACTH injections are likely to originate from several sources, it is possible that progesterone plays an important role. It might also account for some of the differences seen between studies where stressors were applied during different periods of the oestrous cycle. The increase in progesterone concentration after ACTH injections seems to be less obvious during the luteal phase of the cycle and during pregnancy when progesterone concentrations are rising or are already high (Razdan *et al*., 2002), and might during this phase not affect reproduction to the same extent.

An important factor that should not be neglected is the fact that even though chronic stress has documented negative effects on reproduction, the effects of short-term stress (acute or repeated acute stress) are not always negative (Rivier & Rivest, 1991). Transportation-stress can, for instance, induce oestrus and ovulation in anoestrous sows (Rojanasthien, 1989). Since, in all likelihood, most sows, domestic or wild, will experience stressful situations on a daily basis, reproduction needs not be adversely affected by repeated acute stress.

The conclusions drawn from the present studies are not clear enough to be implemented directly in the pig reproduction industry today. Injections of ACTH from the onset of standing oestrus seem to have negative effects on *e.g.* the number of oocytes/embryos present in the uterus and oviduct compared to the number of corpora lutea. However, injections of synthetic ACTH cannot be considered equivalent to the stress seen during regrouping of sows, and the number of animals used in the studies is relatively low. Even though the results indicate detrimental effects of stress during standing oestrus, this must be further investigated in more extensive studies.

Future prospects

It is difficult to investigate all consequences of the complex stress which arises from regrouping of sows. The stressors involved may range from feed restriction, recurring confrontations with other sows, and pain from injuries, possible in connection with inflammatory processes. Were all stressors concomitant in the same individual, the effects would most likely be severe, whereas an individual could probably cope to some extent with one stressor at a time. The wide range of stressors and individual responses make controlled field studies of stress during regrouping virtually impossible. However, the effects of the different stressors can be assessed during experimental settings where well-defined stressors can be applied and monitored both in general and locally. With increased knowledge of the exact effects of the stressors, it might be possible to piece together the whole "stress-picture". However, the stressor type is not the only factor determining the response. The timing and frequency with which the stressor is applied, as well as the duration the application, will also affect the outcome. Furthermore, the effects might not be evident instantaneously, but emerge during later stages during gestation or in the offspring.

Conclusions

- Injections of ACTH from the onset of standing oestrus shortened the duration of standing oestrus, whereas the time of ovulation was unaffected.

- Injections of ACTH from the onset of standing oestrus resulted in increased concentrations of cortisol, progesterone and inhibin α, and possibly LH.

- Injections of ACTH from the onset of standing oestrus disturbed the hormonal patterns of progesterone and possibly also the hormonal patterns of oestradiol and LH.

- The transport of spermatozoa and embryos within the genital tract of sows might have been augmented due to injections of ACTH during standing oestrus.

- In both the C group and the ACTH group, there were seemingly viable spermatozoa present in the sperm reservoir at 6 h after ovulation.

- The intraluminal and local endocrine environment of the oviduct might have been altered due to ACTH injections. Fixation by vascular perfusion can be a useful method when investigating the intraluminal environment of the oviduct.

- The retrieval of oocytes and embryos from the oviducts and uterus was negatively affected by repeated injections of ACTH for around 48 h from the onset of standing oestrus.

- The early embryo development seemed unaffected by injections of ACTH during oestrus.

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Populärvetenskaplig sammanfattning

På bara några decennier har grishållningen gått från ett småskaligt till ett storskaligt intensivt system. Parallellt med detta har avelsarbetet och grishållningen tillsammans resulterat i större kullstorlek och högre tillväxt hos slaktsvinen. Trots de yttre skillnaderna mellan vildsvinet och tamgrisen så är de basala behoven och instinkterna fortfarande mycket lika. Tamgrisar som förvildas uppvisar i stor utsträckning samma beteende som vildsvin. Hur klarar då våra mer eller mindre vilda tamgrisar av livet i de nya djurhållningssystemen? I de mest intensiva systemen hålls gyltor och suggor fixerade på mycket begränsade utrymmen, där de har få möjligheter att uttrycka något naturligt beteende. I dessa system ser man ofta olika stereotypier, som av många anses vara tecken på att djuren försöker hantera en stressfylld situation. Sedan några år har flera länder förbjudit dessa system främst av etiska skäl. Istället går inriktningen idag mot olika grupphållningssystem, där suggorna får röra sig fritt under sinperioden. Vanligtvis hålls suggorna i separata boxar så länge de har smågrisar, men efter avvänjningen placeras suggorna tillsammans i stora boxar. De första dagarna efter grupperingen präglas av stridigheter, där rangordningen fastställs mellan suggorna. Denna stress, då den nya gruppen skapas resulterar bland annat i förhöjda kortisolkoncentrationer i blodet under åtminstone 48 timmar. Dessa förhöjda kortisolnivåer sammanfaller i stor utsträckning med tidsperioden då suggorna efter avvänjningen ska komma i brunst, insemineras och bli dräktiga. Brunstsymptom, ägglossning och fler andra viktiga skeenden i reproduktionscykeln regleras av bland annat könshormoner. Störningar i hormonbalansen runt brunsten kan därför ge upphov till nedsatt reproduktionsförmåga.

För att undersöka effekterna av stress hos suggor under brunsten genomfördes en serie försök med injektion av syntetiskt adrenokortikotropt hormon (Synacthen Depot®, ACTH), som frisätter kortisol från binjurarna. I det första försöket ingick 14 suggor och i det andra försöket ingick 15 suggor. Under suggornas första brunst efter avvänjningen genomfördes rektal ultraljudsundersökning för att fastställa den exakta ägglossningstidpunkten. Venkatetrar opererades in på samtliga suggor så att blodprovstagning och injektioner skulle kunna utföras utan att störa djuren. Den andra brunsten efter avvänjningen var behandlingsbrunst, där hälften av djuren injicerades med ACTH (ACTH-gruppen) och hälften med fysiologisk koksaltlösning (C-gruppen). Injektionerna upprepades varannan till var fjärde timme under *ca*. 48 timmar för att efterlikna (simulera) den stress, som ses hos suggor då en ny grupp har bildats. Blodprover samlades kontinuerligt under behandlingen. Alla suggor inseminerades före ägglossningen. I det första försöket sövdes och avlivades suggorna sex timmar efter ägglossningen och blodprover togs från vener som dränerar äggledare respektive livmoder. Delar av äggledaren tillvaratogs. Spermieantalet bestämdes i den s.k. spermiereservoaren (övergången mellan livmodern och äggledaren, där spermierna kan överleva i väntan på ägglossningen) och i olika avsnitt av äggledaren (i spolvätska). Äggledarens insida undersöktes med svepelektronmikroskop (SEM). I det andra försöket avlivades suggorna 48 eller 60 timmar efter ägglossningen och de tidiga embryona spolades

ut och undersöktes med konfokallasermikroskop (KLM) och transmissions-elektronmikroskop (TEM).

Brunsten var kortare hos de ACTH-behandlade suggorna, men det förelåg ingen skillnad i tid från brunstens början till ägglossningen mellan grupperna. ACTH-behandlingen bör därför inte ha påverkat förutsättningarna för dräktighet i detta hänseende.

Kortisolkoncentrationen i blodet hos de ACTH-behandlade suggorna var signifikant högre än hos kontrollsuggorna. Även progesteronkoncentrationen (dräktighetsbevarande hormon som normalt inte stiger förrän vid ägglossningen) var signifikant högre i ACTH-gruppen. LH (luteiniseringshormon som ger signal för ägglossning) var högre under behandlingsperioden i ACTH-gruppen, men var även högre hos denna grupp före behandlingen. Den exakta effekten av ACTH-injektionerna på LH-koncentrationen är därför oklar. Östradiol tenderade att sjunka snabbare hos ACTH-gruppen och eftersom brunstsymptomen är östradiolberoende kan detta ha bidragit till förändringen i brunstlängd hos denna grupp av suggor. Hormonet inhibin som frisätts från bland annat äggblåsorna i äggstockarna före ägglossningen, var förhöjt hos ACTH-gruppen, något som kan ha negativt påverkat frisättningen av det hormon som stimulerar äggblåsornas tillväxt (FSH). Kortisol- och progesteronkoncentrationerna mättes även i blod som samlats från vener som dränerar äggledare och livmoder; det var inga skillnader mellan grupperna förutom att kontrollsuggorna, till skillnad från de ACTH-behandlade suggorna, hade högre progesteronnivåer i venerna från äggledarna än i halsvenerna.

Antalet spermier i äggledare och spermiereservoar sex timmar efter ägglossningen tenderade att vara högre i den ACTH-behandlade gruppen. Det är möjligt att spermietransporten var snabbare eller mer effektiv i denna grupp, kanske tack vare de högre progesteronnivåerna, som ger en vidgning av äggledarna. Om det finns för många spermier på befruktningsplatsen ökar risken för polyspermi, d.v.s. att ägg befruktas av flera spermier, vilket leder till tidig embryodöd. Svepelektronmikroskopisk undersökning av äggledarna och spermie-reservoaren visade att flertalet av de ACTH-behandlade suggorna hade stor mängd sekret, som helt eller delvis täckte ytan av äggledarnas insida och ibland bäddade in spermierna. Denna förändring i äggledarnas miljö hos de ACTH-behandlade suggorna kan ha uppkommit som följd av de hormonella förändringarna. Miljön i äggledaren är mycket viktig för både spermiernas överlevnad och så småningom embryonas utveckling, men den exakta betydelsen av den ökade mängden sekret är inte klarlagd.

I det andra försöket, där embryona samlades upp 48 eller 60 timmar efter ägglossningen, återfanns signifikant färre embryon i ACTH-gruppen (i relation till antalet gulkroppar, d.v.s. antalet äggblåsor som haft ägglossning). Orsaken är inte känd, men äggledarnas förmåga att fånga upp äggen från äggblåsorna vid ägglossningen kan ha varit nedsatt hos suggorna i den ACTH-behandlade gruppen. En annan tänkbar förklaring är att ACTH-behandlingen påverkat äggen eller äggblåsornas mognad vid ägglossningen. Transporten av embryona genom

äggledarna verkade vara snabbare i ACTH-gruppen, kanske av samma orsak som gav upphov till den ökade spermietransporten i denna grupp. De tidiga embryonas utveckling undersöktes med hjälp av KLM och TEM men inga skillnader sågs mellan grupperna. Förändringarna i äggledarmiljön och i hormonspelet synes sålunda inte ha påverkat embryona.

Sammanfattningsvis synes suggor i god kondition kunna klara av 48 timmar med höga kortisolvärden under brunsten utan synbar negativ inverkan på embryonas kvalitet; trots ett förändrat hormonspel och en miljöförändring i äggledarna. Inte heller tendenserna till ett ökat antal spermier under befruktningen och en ökad transporthastighet av embryon ner till livmodern synes ha påverkat embryonas kvalitet. Däremot var antalet embryon signifikant lägre hos ACTH-suggorna jämfört med kontrollsuggorna. Om denna effekt också uppstår vid den stress som en omgruppering medför leder den till en allvarlig reproduktions-störning i form av färre födda smågrisar. Antalet suggor i de genomförda studierna är förhållandevis lågt och ytterligare studier bör därför göras för att klargöra om det föreligger ett reellt samband mellan stress under brunsten och färre födda smågrisar.