On Repeat Breeding in Dairy Heifers

With special focus on follicular dynamics, ovulation, and oocyte quality.

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Till Else

Det är visst möjligt att att kreaturen icke skötas bättre för det att husmodern går och ser till dem, men bestämdt blifva de alldeles icke sämre deraf, och det är ju rätt och godt att husmodern dagligen förvissar sig om kreaturens välbefinnande.

Alla korna skola hafva namn, eljest har man icke något intresse för hvar och en särskildt, utan blott för alla i klump, och det är långtifrån detsamma. Stjerna, Dockan, Hjertros, Gullros, Brunetta och dylikt skola de heta; först då värderas de som sig bör. Det är en stor skillnad på att blifva underrättad helt enkelt om att en ko kalfvat, eller att få veta att Majros fått en kalf, ”en så’n liten vacker kvigunge, krusig i huvfudet och hvitbrokig”.

Ur ”Husmodern”, Mathilda Langlet, 1892

Abstract

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Reproductive efficiency is essential for profitable dairy farming. Some heifers require several artificial inseminations (AIs) to conceive, and after repeated pregnancy failures, they are usually culled. The aim of this thesis is to contribute to the characterisation of repeat-breeder heifers (RBH), focusing on the oestrous period.

Oestrous behaviour, follicular dynamics, and hormone patterns were studied in RBH (≥3 AIs) and virgin heifers (VH, controls). RBH displayed endocrine aberrations, with progesterone concentrations of ≥0.5 nmol/L (suprabasal levels) at ovulation, whereas concentrations during the luteal phase were lower than normal. The preovulatory LH surge was delayed, standing oestrus was prolonged, and the preovulatory follicle persisted longer than in VH. In a field survey, the occurrence of suprabasal progesterone levels at insemination was confirmed and determined as a risk factor for non-pregnancy.

Conception rates declined with increasing numbers of AIs, indicating the involvement of intrinsic factors in RBH. Pharmacological stress was induced in ovariectomised heifers, resulting in similar adrenal progesterone production (exceeding the suprabasal progesterone concentrations) in all heifers but higher cortisol-producing capacity in RBH, indicating that stress may contribute to repeat breeding.

The question of whether in-built endocrine aberrations affected follicular and oocyte quality was studied. Results from light and electron microscopy (TEM) and the degree of apoptosis in the follicular wall (evaluated by the TUNEL technique) indicate that RBH enter standing oestrus with prerequisites similar to those of VH.

Ability for in vitro maturation (IVM) was studied in oocytes collected by ovum pick-up (OPU), and effects of OPU on RBH characteristics were evaluated. Immature RBH oocytes were scored to be of lower quality at retrieval but not at evaluation by confocal laser scanning microscopy or TEM. After IVM, spatial organelle reorganisation was, however, less advanced in RBH oocytes, revealing cytoplasmic maturation asynchrony. Performing AI subsequent to OPU resulted in pregnancy rates similar to field fertility in RBH and VH, thus apparently not impaired by OPU.

Multiple deviations (caused by intrinsic factors) occur along the course of events toward ovulation, which may affect oocyte developmental capacity and conditions for fertilisation and early embryonic development, thereby to some extent explaining the sub-fertility in RBH.

Keywords: Bovine, infertility, cattle breeding, reproductive performance, IVF, AI, endocrinology, morphology.

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Hurra for den lille Forskel!

Contents

Appendix

Papers I-V

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

I. Båge, R., Gustafsson, H., Larsson, B., Forsberg, M. & Rodríguez-Martínez, H. 2002. Repeat breeding in dairy heifers: follicular dynamics and oestrous cycle characteristics in relation to sexual hormone patterns.
Theriogenology 57, 2257-2269.

II. Båge, R. Conception rates after AI in Swedish red and white dairy heifers: Relationship with progesterone concentrations at AI.
Manuscript submitted for publication.

III. Båge, R., Forsberg, M., Gustafsson, H., Larsson, B. & Rodríguez-Martínez, H. 2000. Effect of ACTH-challenge on progesterone and cortisol levels in ovariectomised repeat breeder heifers.
Animal Reproduction Science 63, 65-76.

IV. Båge, R., Bosu, W.T.K. & Rodríguez-Martínez, H. 2001. Ovarian follicle apoptosis at the onset of standing estrus in virgin and repeat breeder dairy heifers.
Theriogenology 56, 699-712.

V. Båge, R., Petyim, S., Larsson, B., Hallap, T., Bergqvist, A.-S., Gustafsson, H. & Rodríguez-Martínez, H. Effects of ovum pick-up on follicular development, oocyte maturation and fertility in repeat-breeder heifers.
Manuscript submitted for publication.

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Abbreviations

ACTH	adrenocorticotropin
AI	artificial insemination
BSA	bovine serum albumin
COC	cumulus-oocyte-complex
CRH	corticotropin-releasing hormone
ECS	oestrous cow serum
FSH	follicle stimulating hormone
GnRH	gonadotropin-releasing hormone
IVF	in vitro fertilisation
IVM	in vitro maturation
IVP	in vitro production
LH	luteinising hormone
LM	light microscopy
$NaBH_4$	sodium borohydride
NS	non-significant statistical difference
OPU	ovum pick-up
PBS	phosphate-buffered saline solution
PI	propidium iodide
RBH	repeat-breeder heifer
RIA	radioimmunoassay
RT	room temperature
SD	standard deviation
SEM	scanning electron microscopy
SRB	Swedish red and white breed
TCM	tissue culture medium
TEM	transmission electron microscopy
TP-Hepes	modified Tyrode's-lactate solution
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling
VH	virgin heifer

Introduction

Background

Reproductive efficiency is essential in well-managed and profitable dairy farms (Nebel & Jobst, 1998). With the trend of decreasing profit in dairy farming reported worldwide, it is necessary to identify where efficiency improvements can be made. The production of replacement heifers and the herd-culling rate represent a large portion of the total cost of milk production (Tozer & Heinrichs, 2001). In Swedish dairy herds, replacement animals are traditionally brought up within the herd (Pettersson et al., 2001). The cost for rearing replacement heifers is substantial, and to minimise cost and optimise the production potential of each individual, it is recommended that heifers have a calving age of 24 months and thereafter produce new offspring every 12 months (Mjölkkor, 1997). According to a Swedish cost-benefit analysis, hundreds of millions of SEK could be saved if the national average calving age reached this recommended level (Nordgren, 1998). Some heifers require several artificial inseminations (AIs) to become pregnant, resulting in a higher age at calving. Every day of delayed breeding involves costs. Therefore, after a certain number of repeated AIs with pregnancy failure, heifers are usually culled and replaced. In Finnish farms with fertility problems such as low heat detection and conception rates, replacement costs have been identified as the major cause for the herd's economic loss (Rajala-Schultz et al., 2000).

In lactating dairy cows there is a strong unfavourable genetic relationship between milk production and fertility (Oltenacu et al., 1991), and in many countries, years of intensive breeding for high production have involved a decline in dairy cow fertility (see, e.g. Royal, 2000). Among the major problems associated with decreased fertility in dairy cows, repeat breeding with its practical consequences has the greatest impact on dairy herd economy. The current definition of repeat breeding includes pregnancy failures occurring after three or more AIs performed at oestruses with normal inter-oestrous intervals in the absence of detectable abnormalities (Zemjanis, 1980). In Sweden, about 10% of the dairy cows are culled annually because of reproductive failure, mainly because of repeat breeding (Gustafsson & Emanuelsson, 2002). Repeat breeding is diagnosed worldwide, although the definitions used are not unanimous. A short review of the repeat-breeding syndrome is contained in the next section.

Repeat breeding

The repeat-breeding syndrome

Although conception rates usually are high in the bovine, close to 90% in normal heifers (Roche et al., 1981), early embryonic deaths during the first weeks of pregnancy decrease the pregnancy rate to 60% (Diskin & Sreenan, 1980), and in Sweden only between 50% and 55% of inseminations result in the birth of a calf (Svensk Mjölk Årsredogörelse, 2000-2001). The total reproductive wastage in heifers and cows is about 40%-50%. It is composed of fertilisation failures to a lesser extent (10%) and embryonic deaths to a greater extent (25-30%), whereas foetal deaths and abortions only account for 5-10% (Roche et al., 1981; Maurer & Chenault, 1983; Sreenan & Diskin, 1985; Sreenan et al., 2001). In cases of repeat breeding, the cause for infertility is either fertilisation failure or early embryonic death. The aetiology may involve a combination of many factors, e.g. management and nutritiol disorders, physiological conditions, infections, disturbed hormonal interplay, and genetic factors (Laing, 1952; Casida, 1961; Hanly, 1961; Boyd, 1965; Ayalon, 1978, 1984; Hawk, 1979; Bruyas et al., 1993; Levine, 1999). The many risk factors presented have a more or less apparent effect on reproduction. Still, the impact of management on fertility is considerable and must never be ignored, with poor oestrus detection as an important factor greatly influencing reproductive performance (De Kruif, 1976; O'Farrell et al, 1983).

It is known that genital infections may affect fertility in many different ways. Bovine viral diarrhoea virus, as an example of an infective agent, predominantly colonises the reproductive organs for its multiplication (Kirkland et al., 1991), with multiple effects on reproductive performance. The consequences might be manifested as affected follicular growth, impaired oocyte developmental capacity, disturbed ovulations, conception failures, and early embryonic deaths or, more obviously, as abortions, stillbirths, and foetal malformations (Fray et al., 2000a, b; Kafi et al, 2002). However, when infections induce embryonic deaths after the signal for maternal recognition of pregnancy and placentation has occurred, the inter-oestrous interval will be markedly prolonged beyond what is defined for repeated breeding. Furthermore, it is obvious that dystocia, retained foetal membranes, and other postpartum problems (including metabolic disturbances) may have a direct effect on fertility and cause repeat breeding (Boland et al., 2001; Gustafsson & Emanuelsson, 2002; Moss et al., 2002).

Ovarian disorders reduce fertility, with anovulatory conditions such as anoestrus and cystic ovary disease as common diagnoses (Wiltbank et al., 2002). Anoestrus is mainly diagnosed in prepubertal heifers, underfed cows and postpartum cows, and is usually not seen in connection with repeat breeding, because of the simple fact that these animals are seldom inseminated during the period of acyclicity. Ovarian cysts usually have a pathogenesis with too long a duration to be regarded as a factor in the repeat-breeding syndrome, whereas a similar but milder condition, namely persistent dominance of the preovulatory follicle (or large follicle anovulatory condition), definitely is of interest for further investigation from a repeat-breeding perspective.

The underlying physiology of anovulation seems to be a lack of a preovulatory LH surge in response to the high, oestrual concentrations of oestradiol (Wiltbank et al., 2002). One reason, besides a primary function failure in the hypothalamus, could be that the progesterone priming of the hypothalamus (which is necessary to gain increased hypothalamic responsiveness to oestradiol) has not occurred. Another reason may be the presence of suprabasal progesterone concentrations during oestrus, which has an inhibitory effect on the positive feedback of high oestradiol concentrations on the hypothalamus, resulting in high LH pulse frequency and effects on follicular growth (Stock & Fortune, 1993). Spontaneously occurring delayed ovulation or anovulatory conditions have been reported in connection with repeat breeding in postpartum cows (Bulman & Wood, 1980; Lamming & Darwash, 1998). Several studies have reported development of persistently dominant follicles subsequent to pharmacologically induced suprabasal progesterone concentrations, with inhibitory effect on oestrous signs and LH release in a dose-dependent manner (Sirois & Fortune, 1990; Savio et al., 1993b; Roche et al., 1999; Noble et al., 2000). In cases of anovulation, as evidence for an absent or deficient LH-surge trigger mechanism, GnRH treatment can stimulate ovulation and, in some cases, result in increased pregnancy rates (Stevenson et al., 1990; Tanabe et al., 1994). It thus appears that many anovulatory events are primarily due not to ovarian disorders but rather to deficient hypothalamic function.

Genetic factors may contribute to repeat breeding. Chromosome aberrations, visible by light microscope, have been detected in 5-10% of bovine embryos (King, 1990). In addition, aberrations may occur at the molecular level (e.g. mutations) and because of problems at genetic recombination of the parental genomes (Hyttel et al., 1996). The latter two aberrations are believed to occur at a very low frequency in cattle. In vitro-produced embryos, on the other hand, display chromosome aberrations at a significantly higher frequency, resulting in early embryonic death (Viuff et al., 2001). Inbreeding is another genetic aspect that is increasing dramatically (Hansen, 2000), with direct negative effects on reproductive traits in dairy cows (Hermas et al., 1987).

Stress has been addressed as a cause of impaired reproductive efficiency (Dobson et al., 2001), and the hormonal mechanism for effect on fertility is common irrespective of stressor involved. In a stressful situation, the function of the hypothalamus-pituitary-gonadal axis might be disrupted at each level (Dobson & Smith, 2000). LH pulse amplitude and frequency decrease, and the preovulatory LH surge might even be inhibited, with effects on ovarian activity and ovulation. In addition, the cortisol and progesterone produced by the adrenal glands in response to stress might, independently or together, have direct inhibitory effects on the preovulatory LH surge (Stoebel & Moberg, 1982). Many factors in modern dairy farming have been identified as potential stressors, e.g., high milk production, postpartum disorders and negative energy balance, inflammations and infections, lameness, social factors, transport, and heat stress. Moreover, because the propensity for stress varies amongst individuals, it can be hypothesised that certain animals become repeat breeders because of this, which is something that needs to be investigated.

If the impact of the above-mentioned conditions on fertility is apparent, it becomes more difficult to find the explanation for repeat breeding in cases in which the cows or heifers are well managed and seemingly normal in oestrous cyclicity and behaviour, without abnormalities in the genital tract (examined by rectal palpation or ultrasound examination). In those cases, the reasons for sub fertility are considered to lie within the individual animal as intrinsic factors. This theory is confirmed by the fact that pregnancy rates decline with increasing numbers of AIs.

In the effort to achieve improved reproductive efficiency, there is a need for either treatment or exclusion of repeat-breeder animals. Even though repeat-breeder cows or heifers may eventually become pregnant, there is a risk for the repeat breeding to occur again in following lactations (Gustafsson & Emanuelsson, 2002; Moss et al., 2002), which motivates the exclusion of those animals from further breeding. Heritability is not yet determined for repeat breeding, but this might be an aspect for consideration in future breeding programs. Fertility traits indirectly associated with repeat breeding (e.g., number of inseminations per service period) are already included in the total merit index for Swedish AI bulls. For early identification and diagnosis of repeat-breeder animals, it is necessary to determine certain characteristics defining those individuals. To study reproductive variables isolated from the many interfering factors present in lactating pregnant dairy cows, it is therefore suitable to perform the studies on sexually mature, non-pregnant heifers. Over the years, this has been done in Sweden in particular, and the overall aim of this thesis is to further contribute to the characterisation of the repeat-breeding syndrome in dairy heifers.

Repeat breeding in dairy heifers

A series of controlled studies have been performed for the characterisation of the repeat-breeding syndrome, both in spontaneous RBH (Linares, 1981a; Gustafsson, 1985a; Albihn, 1991a) and in normal heifers with induced hormonal aberrations similar to those recorded in RBH (Duchens, 1995). The compiled results from the above-mentioned theses provide a picture of RBH reproductive characteristics that, in short and in comparison to VH, can be described as follows:

During spontaneous oestrous cycles, the duration of standing oestrus was prolonged in RBH, as was the interval from onset of standing oestrus to the preovulatory LH peak and ovulation (Gustafsson et al., 1986; Albihn, 1991b; Albihn et al., 1991). In the two latter studies, in cycles when AI was performed, the same type of prolonged intervals (from onset of standing oestrus to LH peak and ovulation) tended to occur in connection with repeat breeding, irrespective of whether pregnancy failure occurred in a normal heifer or in a RBH.

In general, fertilisation rates after AI were good under the prevailing, controlled conditions of the studies, with early embryonic deaths being the dominant cause for the pregnancy failures (Linares et al., 1980a; Linares, 1981b; Gustafsson, 1985b; Gustafsson & Larsson, 1985). Embryos were non-surgically collected at different days after AI, and there was a gradual embryonic loss up to day 35 after AI both in RBH and VH, although it occurred at a higher frequency in RBH (Linares et al., 1980a; Linares, 1981b; Gustafsson & Plöen, 1985; Albihn et al., 1991). Moreover, the collected RBH embryos were smaller and morphologically deviated to a higher degree than embryos from VH. In the embryos collected from RBH, abnormalities were already present by 48 h after AI, during the period of transport through the uterine tube, but also at 7 and 17 days after AI (Linares et al., 1980b; Gustafsson & Plöen, 1985).

Several hypotheses were suggested to explain the higher rates of morphologically deviating embryos in RBH. It was proposed that the early deviations developed as a consequence of an inherent low quality of the oocyte. Furthermore, after passage to the uterus, the deviations could be a result either of low developmental capacity of the embryo (due to the initial low oocyte quality) or of the inability of the uterus to support early embryonic development. The latter possibility was investigated further, and after reciprocal transfers of embryos between RBH and VH, it was confirmed that the uterine environment was sub-optimal in RBH (Gustafsson & Larsson, 1985). This became even more evident after the transfer of identical demi-embryos to the uteri of RBH and VH (Albihn et al., 1989). Because the RBH embryos were less capable of inducing a proper signal for maternal recognition of pregnancy and prolonging the inter-oestrous interval (after removal of the embryo on day 17 after AI), it was again suggested that, besides the presence of a sub-optimal uterine environment in RBH, the embryos from RBH were of initial lower quality compared to embryos from VH (Albihn et al., 1991). Hormonal aberrations occurring at oestrus were suggested as a probable cause of the impaired uterine environment and the asynchrony between embryo and uterus, and it was discussed, but, however, not investigated, that there might be similar negative effects on uterine tube function. Whether the reason for sub-fertility lies within the oocyte, and whether it is imprinted during follicular growth or after the preovulatory LH surge, during final maturation, are questions of utmost importance that require further elucidation. The period of final maturation should preferably be evaluated under standardised in vitro conditions, provided that oocytes can be collected without interference with RBH characteristics.

The hormonal interplay during oestrus tended to deviate in RBH, with numerically elevated (suprabasal) progesterone concentrations in standing oestrus (Gustafsson et al., 1986; Albihn 1991b; Albihn et al., 1991b) and significantly higher concentrations on cycle day 1 (Gustafsson et al., 1986). The source of the suprabasal progesterone was, however, never determined. In the different studies, increasing progesterone concentrations during early pregnancy (up to day 7) were either normal or numerically alternatively significantly lower in RBH (Linares et al., 1982; Gustafsson et al, 1986; Albihn 1991b; Albihn et al., 1991b). The effect of lower luteal progesterone concentrations was reinforced by the lower concentration of endometrial progesterone receptors in RBH on cycle day 15 after oestrus, something that was found to correlate with degree of embryonic development (Stanchev et al., 1991). The preovulatory LH peak was delayed in RBH in relation to onset of standing oestrus, and the amplitude of the peak, i.e. the total LH release, was significantly smaller in RBH compared to VH (Gustafsson et al., 1986). Oestradiol-17β was not analysed in any of these studies, and concerning progesterone, the sampling procedures differed between the studies. The monitored endocrine trends must be confirmed in well-defined RBH under controlled conditions and during consecutive oestrous cycles, as well as under field conditions, in a larger population of heifers.

The significance and consequence of suprabasal progesterone concentrations at oestrus was determined in a series of experiments (Duchens, 1995). By experimental induction of suprabasal progesterone concentrations (1.0 ± 0.3 nmol/L) in normal heifers, the hormonal conditions recorded in RBH were mimicked (Duchens et al., 1994; Duchens et al., 1995a, b). These heifers displayed prolonged growth of the preovulatory follicle before ovulation and a prolonged duration of standing oestrus. Oestradiol-17β release was prolonged and increased, and the LH peak was delayed. Altogether, the effects on fertility were negative, despite the fact that repeated inseminations were performed until ovulation was detected ultrasonographically. The induced hormonal asynchronies, linked to delayed ovulation, were associated with mild degenerative cell changes in the follicular wall (Duchens et al., 1996). This was, in turn, suggested as a possible cause for impaired developmental capacity of the oocyte and, thereby, the reduced fertility in those heifers. Before inferences are made concerning the situation in RBH, follicular wall and oocyte quality must be investigated in spontaneous RBH.

Aims

The overall aim of the present study was to further characterise the repeat-breeding syndrome in Swedish red and white (SRB) dairy heifers, with special focus on the oestrous period and reproductive events involved in the processes of (final) oocyte maturation and ovulation. The specific aims were to:

- Describe, under controlled study conditions, clinical reproductive variables in repeat-breeder heifers (RBH) such as oestrous behaviour, follicular dynamics and sexual hormone patterns over consecutive oestrous cycles,

- study, under field conditions, the conception rate of first-service dairy heifers and RBH, its relationship with progesterone concentrations at AI, and its congruence with the current repeat-breeder definition,

- determine the capacity of the adrenal gland to produce progesterone in RBH and the potential influence of stress in the repeat-breeding syndrome,

- assess the quality of the pre-ovulatory follicle wall as an indirect measure of oocyte quality before ovulation in RBH, and

- evaluate the ability of RBH oocytes, retrieved by an optimised OPU procedure, to mature and fertilise in vitro, as well as the fertility of heifers after OPU, with controlled AI.

Methodological considerations

Materials and methods used in the present study are described in detail in the specific papers listed above (I-V), whereas general comments of a conceptual nature are provided herein.

Animals

In all experiments, comparisons were made between groups of repeat-breeder heifers (RBH) and virgin heifers (VH, controls), focusing on the group and not on the individual animal to characterise them as two categories of heifer. According to the current repeat-breeder definition, besides the requirement for ≥3 AIs on apparently normal oestruses, the returns to oestrus should be within normal inter-oestrous intervals, excluding cases of late embryonic deaths. Furthermore, there should not be any palpable pathologies in the genital tract explaining the infertility or abnormal findings present at ultrasound examination per rectum.

A total of 26 animals were used in the controlled studies (Papers I, III, IV and V), 12 RBH and 14 VH. Of the 12 heifers in paper I, 5 RBH and 2 VH were included in the study in paper III and all of the RBH and 2 VH were included in the study in paper IV. The field study (paper II) comprised 211 heifers, 91 first-service heifers, 60 third-service heifers and 60 well-defined RBH that had received four to eight AIs. Heifers diagnosed as pregnant after the first AI were considered as controls.

In all studies, the heifers were SRB, one of the major cattle breeds (48% of the dairy cattle) in the country, along with the Swedish Holstein breed (47%) (Svensk Mjölk Årsredogörelse, 2000-2001). To perform the experiments on as homogeneous animal material as possible, besides restricting the studies to one breed, further criteria were used when heifers were purchased. Only dairy farms with good fertility rates were of interest, with the entire herds declared free from bovine viral diarrhoea virus and bovine leucosis virus infections.

Oestrus detection

Oestrous intensity was scored according to the same protocol, a five-grade scale, in all experiments including the field study. The term “strong oestrus” used in paper I equals the term “standing oestrus” used in papers II, IV and V. The same person performed the oestrus detections in the controlled studies (Papers I, III, IV and V).

Ovarian examination

Development and regression of ovarian follicles and corpora lutea (CL) was monitored over several consecutive oestrous cycles by transrectal ultrasonography (Papers I, IV and V). All sessions were recorded on videotape, which made it possible to retrospectively determine the pattern of follicular waves and in particular, study selection and development of the preovulatory follicle as well as ovulation.

Hormone determinations

Plasma progesterone concentrations were analysed with two different methods, a modified luminescence immunoassay technique (Forsberg et al., 1993; Duchens et al., 1995b) (Papers I, III, IV and parts of the samples in Paper II) and a radio-immunoassay (RIA) technique (Paper V and parts of the samples in Paper II). Parallel samples were tested with the two methods, and the results were in full agreement (M.-A. Carlsson, Dept of Clinical Chemistry, SLU, Uppsala, Sweden, personal communication). Progesterone and oestradiol-17β concentrations in follicular fluid were analysed by enhanced luminescence immunoassay techniques (Paper IV), and the oestradiol to progesterone ratio was calculated to indirectly determine whether the preovulatory LH surge had occurred (Ireland and Roche, 1982; Dieleman et al., 1983). Blood plasma concentrations of oestradiol-17β and LH (Paper I) and cortisol (Paper III) were analysed by RIA techniques (Paper I).

For evaluation of the pituitary-adrenal response to stress and the steroid-producing capacity of the adrenal glands, an ACTH challenge was performed in ovariectomised heifers (Paper III). A synthetic ACTH analogue was administered in a suitable dose for a physiological adrenocortical response, and cortisol and progesterone concentrations in the peripheral circulation were analysed as described above.

Morphology studies

The quality of ovarian follicles and oocytes was studied morphologically. Assessment of follicle wall quality (Paper IV) was done as an indirect measure of the oocyte's quality before final maturation and ovulation. For this, the combined results from light microscopy (LM), apoptosis detection with the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) technique, and transmission electron microscopy (TEM) were evaluated. Histological appearance was correlated to degree of apoptosis, and TEM was done to confirm apoptotic changes on cellular level. For this study, ovaries were surgically resected after the onset of standing oestrus from pre-defined RBH (ovaries, n=12) and VH (ovaries, n=5). Only five cumulus-oocyte-complexes (COC) were retrieved, and to study the ultrastructure of these COC, TEM was used.

For further evaluation of oocyte quality (Paper V), oocytes were collected by transvaginal ultrasound-guided ovum pick-up and studied by confocal laser scanning microscopy and TEM. Nuclear status and cytoplasmic arrangement of organelles (especially mitochondria and cortical granules) were studied in immature oocytes (RBH oocytes, n=10; VH oocytes, n=10) and after 24 h of in vitro maturation (IVM; RBH oocytes, n=10; VH oocytes, n=10).

OPU and in vitro fertility

The process of in vitro maturation and the ability of the oocytes to mature and fertilise in vitro were studied (Paper V). For this study, oocytes were repeatedly collected according to an optimised twice-weekly OPU schedule, restricting follicle punctures to the first 12 cycle days and thereby allowing the emergence of a third follicle wave and physiological selection and ovulation of a preovulatory follicle and a subsequent CL formation (Båge et al., 2001; Takenouchi et al., 2001; Petyim et al., 2002). After an average of 14 (range 11 - 19) OPU sessions performed per heifer during three to six consecutive oestrous cycles, the effects of OPU on repeat-breeder reproductive characteristics were evaluated. Retrieved oocytes were quality scored and cultured in a conventional in vitro system, and cleavage rates were recorded. Some of the oocytes were subjected to morphological studies as described above, either as immature oocytes or after IVM.

In vivo fertility

Conception rates after AI were studied in a field population of 211 heifers (first-service heifers, *n*=91; RBH, *n*=120) subjected to routine AI performed by AI technicians (Paper II). Pregnancy results, based on pregnancy diagnosis by rectal palpation 6-8 weeks after AI, were obtained from the records of the Swedish Dairy Association. Pregnancy results were studied in relation to progesterone concentrations at AI.

In vivo fertility was also studied in a group of experimental heifers, five RBH and five VH, subsequent to completed OPU sessions (Paper V). The heifers were subjected to AI according to the routine a.m.-p.m. scheme under controlled conditions (frequent oestrus detections and ultrasound examinations) for optimal timing in relation to ovulation. Pregnancy was diagnosed 30 days later by ultrasound examination and by analysis of progesterone concentrations in the peripheral circulation.

Statistical analyses

Data were in general compared group-wise for RBH and VH, with either Minitab software (Minitab® Release 12.21, Minitab Inc., State College, PA, 1998) or the Statistical Analysis Systems package (SAS Institute Inc., Cary, NC, 1996). When data were not normally distributed, they were either transformed to logarithms to achieve normality or compared with non-parametric methods. Adjustments were made for the repeated retrieval of data from individuals over consecutive cycles. For determination of variables characteristic for RBH, a discriminant function analysis was used and a classification model was designed for prediction of animal category.

Results

Oestrous behaviour

Oestrous intensity and inter-oestrous intervals were similar (NS) in RBH (*n*=7) and VH (*n*=5) studied under controlled conditions (Paper I). The total duration of oestrus was comparable in RBH and VH (*P*=0.12). Prooestrus lasted equally long (NS) in RBH and VH, but standing oestrus was longer (*P*<0.05) and postoestrus tended to be longer (*P*=0.07) in RBH. There was no difference (NS) in duration from onset of strong oestrus to ovulation, even though it was numerically longer in RBH. When the effect of manipulation of the oestrous cycle by OPU (Paper V) was evaluated, oestrous intervals and oestrous intensity during spontaneous cycles before the OPU period and during OPU cycles were similar in RBH (*n*=5) and VH (*n*=5). In the field study (Paper II), behavioural oestrus was good in all heifers and was unaffected (NS) by the occasional presence of suprabasal progesterone concentrations. In the field population of RBH heifers, strong oestrous signs were registered to a greater extent (*P*<0.05) than in first-service heifers, irrespective of subsequent pregnancy results.

Follicular dynamics

During consecutive spontaneous oestrous cycles, the interval from emergence of the preovulatory follicle to selection (deviation) was similar (*P*=0.11) in RBH and VH (Paper I). The interval from deviation to ovulation was clearly longer (*P*<0.05) in RBH, resulting in a longer (*P*<0.01) total interval from emergence to ovulation in RBH. Selection of the preovulatory follicle occurred at a significantly larger diameter in RBH (*P*<0.01), and maximum diameter preceding ovulation was greater than in VH, although this was not statistically significant.

The effect of OPU on follicular development was studied in RBH and VH (Paper V). The numbers of follicles punctured in the very first session were similar (*P*<0.01) as to those in the first sessions of the following three to six oestrous cycles, implying that there was no depletion of follicles over time in either heifer category. The median diameter of the pre-ovulatory follicle before AI was greater (*P*<0.05) in RBH than in VH controls.

Hormone patterns

Progesterone

Total progesterone production during spontaneous cycles (Paper I) was 167.3 ± 41.3 nmol/L per cycle in RBH compared with 200.2 ± 49.8 nmol/L in VH (NS). Focusing on the peri-ovulatory period (i.e., 1 day before and after ovulation), there was a strong tendency (*P*=0.06) for higher mean basal plasma progesterone concentrations, so-called suprabasal progesterone levels, in RBH (0.64 ± 0.43 nmol/L) than in VH (0.33 ± 0.16 nmol/L), which were recorded during consecutive spontaneous oestrous cycles (Paper I). Moreover, the RBH had a late postovulatory rise in progesterone, reflected by a numerically lower quantity (*P*=0.13) of hormone produced by Day 7: 20.8 ± 5.6 compared to 26.0 ± 8.4 nmol/L in VH (Paper I).

Correspondingly, in the field study (Paper II), mean plasma progesterone concentrations at AI were 0.6 ± 0.6 nmol/L in heifers (RBH and first-service heifers) subsequently diagnosed as non-pregnant (*n*=97), and 0.4 ± 0.3 nmol/L in all heifers subsequently becoming pregnant (*n*=109). Moreover, there was a significantly higher (*P*>0.05) occurrence of suprabasal progesterone concentrations (>0.5 nmol/L) in those failing to conceive.

In correspondence with progesterone patterns in spontaneous cycles (Fig. 1), luteal phase plasma progesterone concentrations were lower (*P*<0.001) in RBH than in VH during OPU cycles (Paper V). Furthermore, during the OPU periods, significantly higher (*P*<0.05), suprabasal progesterone concentrations were present at standing oestrus in RBH, 0.6 nmol/l (range 0.2-0.8 nmol/l) compared to 0.2 nmol/l (range 0.2-1.1 nmol/l) in VH controls.

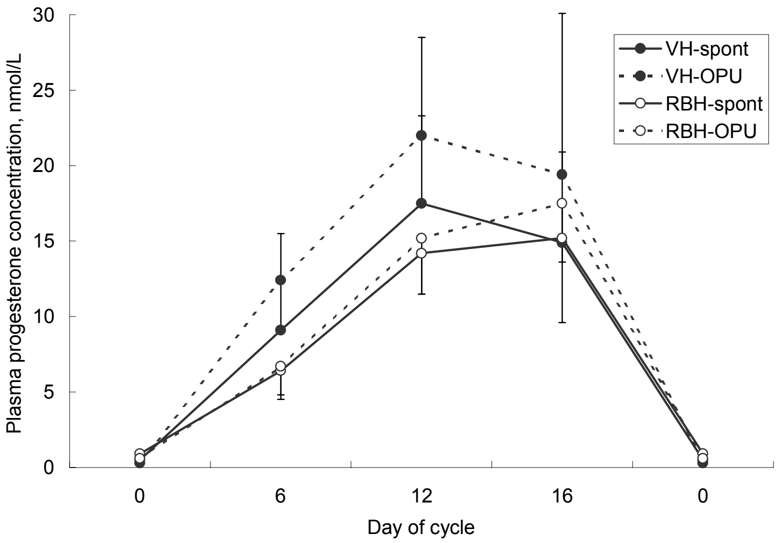

Figure 1. Mean plasma progesterone concentrations (±SD) during spontaneous oestrous cycles (solid lines) and during oestrous cycles with OPU (dotted lines) in RBH (white) and VH (black).

Oestradiol

The rise in oestradiol-17β before and during oestrus resulted in similar average production (71.6 ± 19.0 pmol/L) in the two categories of heifers (Paper I).

Luteinising hormone (LH)

A preovulatory rise in LH, indicating the occurrence of a peak, was detected in 44 of 68 (65%) examined oestruses, although blood sampling was only performed three times a day (Paper I). In VH, a majority of the detected LH peaks coincided with onset of oestrus, whereas the interval from onset of standing oestrus to the LH rise was significantly longer ($P<0.05$) in RBH (4.6 ± 6.0 h vs. 0.4 ± 1.0 h in VH).

Adrenal steroid production

Pre-treatment, baseline concentrations of progesterone were equal (NS) in ovariectomised RBH ($n=5$) and VH ($n=5$), whereas cortisol baseline concentrations were significantly higher ($P<0.01$) in RBH (mean 10.1 ± 2.3 nmol/L) than in VH (2.6 ± 0.2 nmol/L). After ACTH-treatment, all animals had a significant and highly correlated ($r=0.8$) rise in both cortisol and progesterone. RBH had a significantly greater ($P<0.001$) cortisol response than VH, most prominent ($P=0.06$) in the peak values within an hour after the treatment. Progesterone concentrations after treatment were higher than the spontaneous suprabasal concentrations occasionally present at ovulation, although there was no difference (NS) between peak concentrations in RBH (2.2 ± 0.8 nmol/L) and VH (3.5 ± 1.9 nmol/L). The duration of significantly elevated progesterone concentrations after treatment was similar (NS) in RBH and VH. There was a tendency ($P=0.08$) for longer duration of elevated cortisol concentrations in RBH (mean 276 min) compared to VH (mean 240 min).

Relations between oestrous events

Suprabasal progesterone levels at the time of ovulation were seen in correlation with prolonged life span of the preovulatory follicle ($P<0.05$) and with short intervals from the LH rise to ovulation ($P<0.01$) (Paper I). Suprabasal progesterone of similar concentrations implied a 58% relative risk of pregnancy failure and a 42% chance of pregnancy in a field population of heifers (Paper II). As already mentioned, the duration from onset of standing oestrus to a LH rise was considerably longer ($P<0.05$) in RBH than in VH, for which the rise in LH coincided with onset of strong oestrus in 95% of the oestruses (Paper I). Duration from the LH rise to ovulation was shorter ($P<0.05$) in RBH than in VH. The late postovulatory rise in plasma progesterone was the variable best categorising the heifers as RBH, but plasma progesterone level at ovulation, duration of strong oestrus and life span of the preovulatory follicle were also characteristic. Using the three latter variables together, the simulation model predicted 81% of VH and 79% of RBH correctly.

Reproductive data from the pregnant first-service heifers ($n=53$) in the field study (Paper II) were considered as suitable control material for VH in general. Their mean plasma progesterone concentration at AI was 0.4 ± 0.3 nmol/L. Mean oestrous intensity was scored as “strong” (4 on the 5-graded scale). They were predominantly (74%) inseminated the same day that oestrus was first recorded. Corresponding figures for former RBH finally becoming pregnant were exactly the same (NS) concerning progesterone concentrations (0.4 ± 0.3 nmol/L) and oestrous intensity (score 4).

Morphology studies

Follicular wall quality

As judged by the oestradiol to progesterone ratio in follicular fluid, ovaries were resected before the occurrence of the preovulatory LH surge in 11 of the 12 heifers (RBH, n=7; VH, n=5) included in the study (Paper IV). Apoptotic, TUNEL-labelled cells were present in the granulosa (G) and theca interna (TI) layer with a mean G-to-TI ratio of 50:9 (P<0.001). The relative degree of apoptosis in the G and TI in preovulatory and subordinate follicles was similar in RBH and VH (NS), but the relative mean numbers of apoptotic cells differed significantly (P<0.001) between the preovulatory follicles (2.5 ± 1.8) and subordinate follicles (21.5 ± 16.8) in both animal categories. All preovulatory follicles had very low relative numbers (0.5-5.7) of apoptotic cells and were histologically classified as either healthy or primary atretic (NS). The largest subordinate follicles (6- to 7-mm diameter, n=7) had comparatively high density of apoptosis (relative mean numbers 35.7 ± 12.1) in both VH and RBH (NS), whereas small subordinate follicles (2 to 5 mm diameter, n=40) had a more moderate apoptosis density (relative mean numbers 19.1 ± 16.4) in all animals (NS). This was compatible with the degree of atresia observed histologically.

Oocyte quality

Most cumulus-oocyte-complexes (RBH, n=3; VH, n=1), recovered from surgically resected ovaries (Paper IV), had a homogeneous ultrastructure and a healthy appearance. Only one oocyte, deriving from the single heifer (a RBH) that had the ovaries resected after the occurrence of the LH surge, showed signs of maturation with dispersed cumulus investment, disrupted contacts between cumulus cells and oocyte and spatial reorganisation of organelles.

Maturational changes in oocytes

A majority of the OPU-retrieved, immature oocytes (10 oocytes each from RBH and VH) had the expected morphological appearance (Paper V). The chromatin was not condensed, and mitochondria, many in hooded, immature shape, were located in small clusters at the very periphery of the ooplasm. Golgi complexes were well developed, and a large number of vesicles were present and cortical granules appeared in groups. After IVM, 7 of 10 VH control oocytes and 6 of 10 RBH oocytes had reached the expected maturational stage, i.e., the metaphase of the second meiotic division. Nuclear status was similar in oocytes from RBH and VH, but the cytoplasmic appearance deviated in oocytes from RBH. Mitochondria were still located in peripheral clusters, as in the immature oocytes, with some showing immature, hooded shape. Cortical granules were still present in peripheral clusters instead of being reorganised as solitary granules along the oolemma. In VH, mitochondria were to a higher degree (6 of 10) evenly distributed in the ooplasm.

In vitro fertility

Oocyte recovery rate after OPU was lower (*P*=0.05) in RBH (55%) compared to VH (62%). Fewer oocytes from RBH (42%) than from VH (55%) were scored as high-quality oocytes (grade 1 or 2) at retrieval after OPU (*P*<0.05). The median cleavage rates after IVF were equal (NS), about 34%, in RBH and VH and in the slaughterhouse-derived oocytes that were used as controls for laboratory efficiency at the time of the experiment.

In vivo fertility

In a survey of field conception rates (Paper II), 60% of the first-service heifers and 47% of RBH (P=0.07) were diagnosed as pregnant 6 to 8 weeks after AI. As many as 61% of the heifers conceived after the third AI, but with increasing numbers of AIs, the pregnancy rates declined gradually. There was a tendency (*P*=0.08) for significant differences between pregnancy rates when comparing first (60%) and third (61%) AI to fourth (41%) and fifth (36%) AI, respectively. The group of heifers inseminated six to eight times had significantly lower (19%, *P*<0.01) pregnancy rates than those inseminated once or three times.

After completed OPU sessions (Paper V), AI performed under controlled conditions resulted in pregnancy in four VH control (80%) and two RBH (40%). The non-pregnant VH control had clinical signs of endometritis at AI. Of the three non-pregnant RBH, one presented a perfectly normal progesterone profile of a non-pregnant oestrous cycle, in the second RBH the preovulatory follicle became persistently dominant and ovulation (of a newly developed follicle) occurred a week later and in the third RBH, early embryonic death occurred after implantation, between cycle days 21 and 23.

General discussion

The repeat-breeder heifer definition

Throughout this thesis, repeat breeding is approached and discussed as a primary reproductive disorder. Other causes for infertility are disregarded, such as infections, postpartum disorders, and negative energy balance or other metabolic disturbances. The experiments were carried out on heifers to better distinguish between intrinsic reproductive disorders and fertility problems secondary to some of the aforementioned conditions. The use of VH as controls in the experiments was not without risk, because a proportion of these could be potential repeat breeders. Instead of actually testing their fertility by subjecting them to AI and pregnancy diagnosis and thereafter inducing abortion – a treatment that might entail unwanted side effects – the decision was made to regard them as presumed normal. Inseminations may cause endometritis, albeit rare, and some heifers may come into anoestrus after prostaglandin-induced abortion. Such animals are not desirable as controls, leaving VH as the best possible control animals. All possible precautions were made to ensure the normality of the VH. Their genital tracts were examined by palpation and ultrasound examination per rectum, and they were checked for regular cyclicity, normal oestrous behaviour and sexual hormone patterns before any experiments were carried out. An important aspect was the selection of bovine viral diarrhoea virus-free heifers because this infection can cause repeat breeding, e.g. reflected in lower conception rates (Fray et al., 2000b).

To be diagnosed as a repeat breeder, the current repeat-breeder definition used in Sweden requires that a heifer should have received at least three AIs without conceiving but appear normal in all other aspects. From the results obtained in this thesis (Paper II), it is suggested that this definition should be retained, considering the high fertility after the third AI and the low fertility after the fourth and subsequent AIs in the field study. Swedish field surveys of dairy cow fertility were performed in 1968 (Hewett, 1968) and 1991 (Gustafsson & Emanuelsson, 2002), and a 10% incidence of repeat breeding was reported in both studies, although they were performed more than 20 years apart. Corresponding figures for heifer fertility are not available, and they are hard to determine, because the reproductive data records for heifers are not complete. In the field study of the present thesis, 60% of the first-service heifers successfully conceived and can, therefore, be regarded as a reference group for normal heifers. As many as 81% in the group of heifers that had received six to eight repeated AIs were diagnosed as non-pregnant after an additional AI. These results should not be considered as general heifer fertility data because the heifers included were a selected sub-population of heifers presented for AI during that particular period of time. Furthermore, because pregnancy was not diagnosed until 6-8 weeks after AI, this sub-population not only includes well-defined repeat breeders (who are supposed to return to oestrus within normal intervals) but also represents (although never diagnosed) cases of embryonic and foetal deaths causing prolonged inter-oestrous intervals. An interesting observation, though, is that pregnancy rates declined markedly after the fourth AI and onward, implying the existence of intrinsic factors in the aetiology of repeat breeding. If a certain proportion of pregnancies should fail solely depending on chance (Hyttel et al., 1996), there would not have been this decrease but rather similar pregnancy rates after each AI in a series performed in a group of heifers.

Characteristics of RBH

Deviating oestrous events

The duration of standing oestrus was significantly prolonged in RBH, and the interval between onset of standing oestrus and ovulation, a period of both physiological and practical importance (e.g., affecting timing of AI) was numerically longer in RBH, although not statistically different from the situation in VH. Interestingly, the period from the LH surge to ovulation was, on the other hand, clearly shorter in RBH than in VH. The occasional normal values presented by RBH caused a wide distribution in the data set, consistently making it difficult to statistically verify the biological differences between RBH and the VH controls.

Results from the repeatedly monitored oestrous cycles can be compiled and recapitulated in chronological order for description of a typical RBH oestrus: First there was a period of prolonged oocyte captivity in the dominant follicle microenvironment of physiologically increasing oestradiol concentrations, followed by a delayed LH surge and by a remarkably short but exceedingly progesterone-dominated period from the occurrence of a delayed LH surge until ovulation. This latter period, initiated by peak concentrations of LH, is crucial for the final maturation of the oocyte, when developmental capacity is achieved (Dieleman et al., 2002; Rizos et al., 2002). Although the oestrous events and hormone patterns in RBH only marginally deviated from normal, the biological effect should not be underestimated, and it is suggested that the total effect on the oocyte is detrimental.

The occurrence of suprabasal progesterone levels in the controlled study was confirmed in a larger group of heifers by analyses of blood samples collected at the time of field AI. Suprabasal progesterone concentrations were, to a high extent, found in heifers that subsequently were diagnosed as not pregnant after AI and were connected to a 58% risk of non-pregnancy. The significance of suprabasal progesterone concentrations during oestrus has already been confirmed by induction of suprabasal progesterone levels (1.0 ± 0.3 nmol/L) in normal heifers (Duchens et al., 1994; 1995a, b). In these animals, asynchronies similar to those observed in RBH were provoked, e.g., prolonged and increased oestradiol release, delayed LH surge, and prolonged growth of the preovulatory follicle, altogether resulting in an ultimate effect on fertility. Concerning the late postovulatory rise in progesterone in RBH, this has been seen in connection with poor embryo development and with an asynchrony between the embryo and the uterine environment, resulting in failed inhibition of the luteolytic PGF2α release and early embryonic death (Mann & Lamming, 2001). Total progesterone production during the luteal phase, which is crucial for the maintenance of pregnancy, was numerically lower in RBH. Low luteal progesterone concentrations (Lucy et al., 1998) as well as abnormal luteal phases (Opsomer, et al., 1998) have been observed in high-merit and high-yielding cows. It would be misleading to interpolate this to the heifer situation, but in future studies, the genetic merit should be included to elucidate if a similar relationship exists in heifers.

The growth pattern of the preovulatory follicles in RBH resembled that of persistently dominant follicles, although as a milder and transient form of persistence. These transient or definite anovulatory conditions have shown to aggravate in a progesterone dose-dependent manner, including ovarian cysts as the most serious manifestation of the common pathogenesis (Noble et al., 2000). Interestingly, there are similar tendencies in the normal physiological situation, in oestrous cycles with two instead of three follicular waves. In two-wave cycles, the follicles are older and larger at ovulation and fertility is lower than in cycles with three waves (Townson et al., 2002). In the present study, the follicular growth phase was longer in RBH, both from emergence to ovulation and from deviation (or selection) to ovulation. In addition, deviation occurred at a greater diameter than in VH and than what has been reported by others as normal deviation diameter (Ginther, 2000a). This delayed selection could be interpreted as an impaired ability of the largest follicle to attain dominance. It could be caused either by an insufficient oestradiol production for suppression of FSH production, which is necessary for the induction of atresia in the competitors of the follicle cohort (Ginther et al., 1999, 2000b), or by a deficient response in the feedback system of the hypothalamus to the increasing oestradiol concentrations. Moreover, there is most likely involvement of a differential intrafollicular activity and regulation of growth factors in the complex process of dominant follicle selection (Mihm & Austin, 2002). It might also be that the LH pulsatility is not optimal for sustaining continued growth and oestradiol synthesis in the dominant follicle (Savio et al., 1993a). These are all interesting hypotheses, but they are outside the scope of the experiments of this thesis.

Because of the extended growing phase of the preovulatory follicle in RBH, there was a prolonged exposure of the oocytes to the microenvironment of the preovulatory follicle, a microenvironment with importance for oocyte maturation (Moor et al., 1998). A process characterised as “prematuration” or “pseudomaturation” occurring independently of an LH surge has been noted in oocytes from degenerating, subordinate follicles (Assey et al., 1994). This process has also been shown to improve the in vitro competence of oocytes derived from slightly atretic follicles of 3-7 mm (Hendriksen et al., 2000). Experiments with induced persistent dominant follicles report similar observations: treatment with subluteal concentrations of progesterone resulted in increased LH-pulse frequency, which in turn initiated maturational changes in the oocyte (Revah & Butler, 1996) and luteinisation of the follicles (Bigelow & Fortune, 1998). Mihm et al. (1999) found that oocytes recovered from persistently dominant follicles had initiated final maturation and progressed to metaphase I before the occurrence of a preovulatory LH surge. One explanation for the reduced pregnancy rates seen in connection with this was that the prolonged interval between onset of maturation and fertilisation was detrimental to the developmental capacity of the oocyte. It has been demonstrated that oocytes from persistently dominant follicles may be competent enough to be fertilised, but their ability to develop further is compromised (Ahmad et al., 1995). This is in accordance with the general opinion of previous studies of RBH, claiming that fertilisation rates were good and that early embryonic deaths accounted for most of the pregnancy failures (Linares et al., 1980a; Linares, 1981b; Gustafsson, 1985b; Gustafsson & Larsson, 1985). The similar cleavage rates after in vitro fertilisation of oocytes derived from RBH, VH, and offal ovaries in the present study support the theory that fertilisation might be achieved and that the cause of pregnancy failures probably lies within the period of early embryonic development. Before this can be ascertained, further studies should be performed with a general laboratory performance better than the one during the current experimental period.

Consistency in deviating events

The results from this thesis are unique in that they prove that the deviating oestrous events are characteristic and can be used as exclusion criteria for RBH. The picture is, however, complicated by the fact that RBH are not sterile but rather sub-fertile animals that occasionally present normal hormonal interplay and manage to establish pregnancy. Practical implications are further complicated by the necessity of performing repeated ovarian examinations, preferably with ultrasonography, and repeated blood analyses to diagnose the characteristic symptoms. A great part of the economic loss caused by repeat breeding is already established when a heifer has received repeated unsuccessful inseminations, but it might still be beneficial to exclude such an animal. Even if a RBH eventually would become pregnant, there is a high risk that the repeat-breeding problems will recur with the following lactations (Gustafsson & Emanuelsson, 2002; Moss et al., 2002).

Propensity for stress

It is well known that stress can interfere with reproductive events in the bovine as well as in other species, and propensity for stress can be regarded as one intrinsic factor in the repeat-breeding syndrome. The ovariectomised heifers of the present study had significantly higher cortisol production from the adrenal glands, both concerning baseline concentrations and after stress induced pharmacologically by ACTH treatment. Although adrenal progesterone production did not differ between RBH and VH, it can still be hypothesised that the adrenals might be the source of the suprabasal progesterone monitored during oestrus in RBH, because of a higher propensity for stress in those animals. In both RBH and VH, the levels of progesterone produced by the adrenals after ACTH treatment (in a suitable dose for a physiological-like response) resulted in progesterone concentrations far higher than the suprabasal concentrations observed in RBH during oestrus. In modern dairy farming, the cows encounter many stressful situations, both acute and chronic, and those individuals that have high stress sensitivity are in risk of becoming repeat breeders. Stressful situations in connection to oestrus are of specific importance because LH pulsatility and, hence, preovulatory follicle growth and ovulation will be affected (Dobson & Smith, 2000).

Effects on oocyte quality and fertility

In the previous studies of RBH performed at our clinic (Linares, 1981a; Gustafsson, 1985a; Albihn, 1991a), one question was repeatedly raised in the search for an explanation for the low fertility in RBH: Could the low developmental capacity in embryos from RBH be already imprinted in the oocyte during follicular growth, before ovulation? According to the results of the present thesis, it seems that RBH enter standing oestrus with normal morphological prerequisites concerning follicular wall quality, as judged by the degree of apoptosis in granulosa and theca cells in correlation with morphological changes. This was further confirmed by the morphological appearance of the few oocytes that were retrieved from the surgically resected ovaries of the same study. The monitored hormone deviations in RBH, i.e., suprabasal progesterone concentrations and delayed LH surge, had been observed at a later stage of standing oestrus. Because it is the preovulatory LH surge that induces the steroidogenic shift in the follicle (Ireland & Roche, 1982; Dieleman et al., 1983) and initiates the final maturation of the oocyte (Kruip et al., 1983), it was suspected that negative influence on this period might have decisive effects on oocyte quality, fertilisation and early embryonic development.

The period of final oocyte maturation was therefore studied under in vitro conditions. The method used for retrieval of oocytes (OPU restricted to the first 12 cycle days and allowing spontaneous ovulation) affected neither cyclicity nor the reproductive variables investigated and was thus considered a suitable experimental method. Although the conditions were standardised for oocytes retrieved from RBH and VH, the degree of maturation differed, implying that their developmental capacity was not equal. There was an asynchrony between nuclear and cytoplasmic maturation in RBH, visualised by delayed spatial reorganisation of organelles. Mitochondria were still located in peripheral clusters, as in immature oocytes, and some of them were showing an immature, hooded shape. Cortical granules were present in peripheral clusters instead of being reorganised as solitary granules along the oolemma. Reorganisation of mitochondria has been associated with metabolic activity and correlates well with developmental capacity of the oocyte (Bavister & Squirrel, 2000), whereas migration of cortical granules is important for prevention of polyspermy in the oocyte (Austin, 1956; Wang et al., 1997). As mentioned above, the cleavage rates after in vitro fertilisation were similar in RBH and VH, whereas the capacity to develop to the blastocyst stage and further remains to be investigated. It can be speculated that the negative effects will manifest as a lower blastocyst rate, because it has been shown that asynchrony between nuclear and cytoplasmic maturation may result in impaired developmental capacity (Mermillod et al., 1999). As a further evidence for this, it has been proven possible to treat low-competence oocytes and thereby increase the blastocyst rate (Mermillod et al., 2000; Lonergan et al., 2000).

In vivo fertility after the completed OPU period was comparable to field fertility in VH and even better than field fertility in RBH, and it was concluded that the OPU scheme used had no carry-over effects on fertility. There were, however, only five heifers in each group, and they were only inseminated once at an optimal time point, which is why it is hard to draw any general conclusions. To really ensure that AI is performed with optimal timing in relation to ovulation and that spermatozoa are present and capacitated at the site of fertilisation when the oocyte arrives, a more frequent AI schedule than the routine a.m.-p.m scheme should be experimentally applied.

Concluding remarks

Causes for sub-fertility in RBH have been found at different segments of the genital tract and the ovaries. As for the latter, unfavourable conditions associate with hormonal aberrations in RBH. If those occur during the final maturation of the oocyte, this might result in an ovulated oocyte with impaired developmental capacity. The hormonal aberrations might also affect the functionality of the uterine tube, with additional negative consequences for gamete transport, fertilisation and early embryonic development. The high frequency of abnormal embryos collected from the uterine tubes of RBH confirms that the tubal conditions in RBH are detrimental for the early embryo (Linares et al., 1980b), and there are indications for a deviating morphology in the epithelium of the uterine tubes of RBH, both before and after the pre-ovulatory LH surge (Båge et al., 2002b). Furthermore, a hormonal asynchrony might also modify the uterine environment and, in turn, result in a fatal asynchrony between the conceptus and the uterus (Albihn et al., 1991a). Morphologically deviating embryos have been collected from uteri of RBH, indicating that the organ was apparently not supportive enough for the elongation of the early embryo (Linares, 1982; Gustafsson & Plöen, 1985). This hypothesis was further confirmed after the transfer of demi-embryos to two different uterine environments, normal and sub-normal, with elongation occurring in the first but being restrained in the second (Albihn et al., 1989). Thus there are several explanations available for the sub-fertility in RBH, and the multifactorial causality of the repeat-breeder syndrome is apparent.

Future prospects

The characterisation of RBH is far from complete. What remains to be done as an extension of the work of this and previous theses is, e.g. a more rigorous determination of the LH pattern, especially concerning frequency and amplitude of the basal LH pulsatility, starting at the time of selection of the preovulatory follicle and onward (including monitoring of the LH surge) until ovulation. Oestradiol-17β production is also not fully determined during this period in spontaneous RBH. Concerning progesterone, peripheral concentrations are not always correlated to the local progesterone levels because of the counter-current mechanism of exchange of progesterone between the ovarian vein and artery (Einer-Jensen & McCracken, 1981). Still, concentrations in the systemic circulation affect the gonadotrophin secretion and thereby the ovarian activity. It should be studied further how the observed endocrine aberrations in systemic circulation of RBH affect hormone levels in the target organs, if possible monitored in the ovarian vein and in follicular fluid. More information about endocrine interplay and its regulation in RBH is needed to elucidate whether hormonal deviations occur because of deficiencies on a central or a gonadal level.

As already mentioned in the general discussion, an experiment should be performed with frequently repeated AIs from the onset of standing oestrus until ovulation is observed. It would thereby be possible to eliminate any suspicions about poorly timed AI or lack of fertile spermatozoa at the critical site and time point as the cause for infertility.

The developmental capacity of oocytes deriving from RBH should be further evaluated, concerning both in vitro blastocyst rate and, subsequently, embryonic development in uteri, in parallel to what has been performed in previous studies with in vivo-derived embryos (e.g., Gustafsson & Larsson, 1985; Albihn et al., 1989). The experiments in the present thesis have focused on the preovulatory follicle. In future studies, the subordinate follicles in the cohort should be given more attention. Interesting comparative aspects of ovarian activity can be extrapolated to the situation observed in obese women suffering from polycystic ovary syndrome (Crosignani et al., 2002).

The effects of endocrine aberrations described here on the functionality of the uterine tubes should be further studied in RBH. Before the final establishment of pregnancy in the uterus, a series of critical events must take place under particular conditions in the uterine tube (Ellington, 1991). These events, such as gamete transport, sperm capacitation, final oocyte maturation, fertilisation and early embryo development, are all occurring in direct contact with the lining epithelium and its secretions, being mainly regulated by the steroid hormones oestradiol and progesterone (Hunter, 1988). These functions are at apparent risk when subjected to the deviating hormone patterns in RBH, which may emphasise the negative effects on fertility. Morphological differences in tubal epithelium have been observed in RBH, together with tendencies for differing steroid receptor distribution, particularly in the isthmic region (Båge et al., 2002). Moreover, it has been shown that cows with persistent dominant follicles have differences in the pattern of oviductal secretory proteins during oestrus (Binelli et al., 1999).

One important topic not covered by this thesis is the effect of nutrition or metabolic state on reproductive events. Dairy heifers are genetically programmed for effective energy conversion and high milk production, but without energy-consuming pregnancy and lactation, they rapidly gain weight. There are reports available on both positive and negative effects of high body condition, but most research focuses on detrimental effects on fertility by negative energy balance in dairy cows (Butler, 2000). The situation in heifers is not fully clarified, although it is highly plausible that the obesity that often develops in RBH somehow will affect fertility. For dairy heifers, it has been shown that an excessive energy diet will result in a lower number of transferable embryos produced after superovulation (Humblot et al., 1998), and that following a period with overfeeding, dietary intake restriction may increase the blastocyst rate (Freret et al., 2002). High dietary intake results in higher liver weight and increased progesterone clearance, and progesterone (being a fat-soluble steroid) may be stored and released from adipose tissue depending on the metabolic condition (O'Callaghan et al., 2001). In cows, changes in the peripheral circulation of, e.g., insulin, glucose, non-esterified fatty acids, and insulin-like growth factor-1 concentrations can be used as markers of metabolic status directly or indirectly influencing reproduction (Wathes et al., 2001). Insulin-like growth factors and leptin have been shown to have direct effects on follicular cells (Lucy, 2000; Driancourt, 2001; Smith et al., 2002). The question is, however, whether the described markers can be directly applied to heifers. Reports on metabolic effects on heifer fertility have so far been contradictory. Because heifers are still growing, genes different from those in lactating dairy cows probably regulate their metabolism and their reproduction. This theory is supported by the fact that heifer fertility has remained stable during the last 20 years, while high-yielding cow fertility has decreased considerably (Butler & Smith, 1989). Moreover, genetic correlations between fertility traits have shown to be lower in heifers and first-parity cows than in cows of higher parities (Roxström et al., 2001).

Conclusions

- RBH exhibited several reproductive perturbations over the course of consecutive oestrous cycles. Among the most prominent perturbations were prolonged duration of oestrus, delayed LH peak, prolonged life span of the preovulatory follicle, and a late postovulatory rise in progesterone concentrations. Furthermore, RBH showed a strong tendency for suprabasal progesterone concentrations at ovulation.

- Suprabasal progesterone concentrations occurred at AI, involving a risk for pregnancy failure. Pregnancy rates were as good after the third AI as after the first, but declined markedly after the fourth AI. Thus, the current definition of RBH, $\geq$3 AIs, is justified and should be retained.

- There was a high propensity for stress in RBH, as judged from the great cortisol-producing capacity from the adrenal glands in response to induced stress. The amounts of adrenal progesterone produced were more than sufficient to cause suprabasal progesterone concentrations. Stress may be regarded as a potential factor in the repeat-breeding syndrome.

- The quality of the preovulatory follicle wall, as an indirect measure of the oocyte quality, was similar in RBH and control heifers in early standing oestrus (before the occurrence of a preovulatory LH surge), implying that RBH entered oestrus with the same prerequisites as normal heifers concerning follicular morphology.

- The use of an optimised OPU procedure for collection of oocytes did not influence the characteristics of either RBH or control heifers, or the in vivo fertility after controlled AI performed subsequent to completed OPU.

- After IVM, oocytes from RBH presented a deviating spatial reorganisation of organelles in the cytoplasm, whereas nuclear maturation appeared normal as expected. Cleavage rates after in vitro fertilisation were similar in RBH and controls.

- RBH, in contrast to VH, present a heterogeneity of symptoms and deviations with values widely distributed from occasionally normal to pathological, still characteristic for the syndrome. This demonstrates the multifactorial aetiology for the sub-fertility that constitutes the syndrome.

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Anders and Else, my private universe.

Populärvetenskaplig sammanfattning

För att en ko ska producera mjölk måste hon först bli dräktig och föda en kalv. Mjölkproduktionen pågår sedan i ca 9 månader, och därefter följer en 2 till 3 månader lång vila, den sk sinperioden. Efter det krävs en ny förlossning för att kon ska sätta igång att mjölka igen, och i praktiken innebär det att hon måste bli dräktig så snart som möjligt efter förlossningen. God fruktsamhet bland kor och kvigor är därför mycket viktigt för att få lönsamhet i mjölkproduktionen. En modern mjölkgård behöver ha så hög produktion som möjligt till så låg kostnad som möjligt. Utslagning av dåliga kor och rekrytering av ersättare för dessa är en av de största utgifterna, och den vanligaste orsaken till utslagning är just fruktsamhetsproblem.

Lönsamhetsberäkningar har visat att en kviga bör kalva in (få sin första kalv) när hon är 24 månader gammal och därefter får en ny kalv med 12 månaders intervall. En del kvigor blir dock inte dräktiga vid första försöket, utan ibland krävs det upprepade inseminationer under flera brunstcykler. En brunstcykel är 21 dagar, och om en kviga inte blir dräktig kommer hon i ny brunst igen efter 21 dagar, d.v.s. hon ”löper om”. Sålunda innebär en misslyckad insemination direkt tre veckors försening där varje dags försening kostar pengar. Vanligtvis slaktas kvigan efter ett visst antal misslyckade inseminationer. En del av dessa kvigor med fruktsamhetsproblem är till synes normala utan tecken på sjukdom eller fel i könsorganen, och kallas därför ”symptomlösa omlöpare”. För att få diagnosen omlöpare krävs det att de inseminerats under minst tre brunstcykler utan att bli dräktiga. I Sverige förekommer symptomlös omlöpning hos ungefär 10% av korna och kvigorna, och internationellt sett är siffrorna liknande eller högre. Målet med denna avhandling var att finna de särdrag som kännetecknar omlöparkvigor, framför allt under brunsten och vid tiden för ägglossning, för att hitta en förklaring till deras låga fruktsamhet. Vi vet sedan tidigare att det kan förekomma hormonella störningar under brunsten och den tidiga dräktigheten hos omlöparkvigor.

Omlöparkvigor av SRB-ras köptes in till institutionen för obstetrik och gynekologi, SLU, och undersöktes under flera brunstcykler med avseende på brunstsymptom, äggstocksaktivitet och könshormoner i blodet. Som jämförelsematerial användes normala SRB-kvigor som aldrig tidigare inseminerats. Omlöparkvigorna visade sig ha en del hormonstörningar, framför allt rörande gulkroppshormonet progesteron, både runt ägglossningen och i perioden mellan två ägglossningar. Hormonspelet inför ägglossningen förlöper normalt sett enligt ett komplicerat schema, och detta schema måste noggrant följas för att ett befruktningsdugligt ägg ska kunna avlossas och dessutom avlossas vid rätt tidpunkt. Hos omlöparkvigorna upptäcktes små förskjutningar i detta hormonspel och i brunstsymptomen, och utvecklingen av äggblåsorna i äggstockarna skilde sig från de normala kvigornas. Högbrunsten varade längre hos omlöparna, och äggcellen fick tillbringa längre tid i äggblåsan. Den sista perioden före ägglossning var dock kortare, den känsliga period då ägget ska erhålla den slutgiltiga mognad som krävs för att bli befruktat och utvecklas till ett embryo.

En fältstudie genomfördes parallellt för att bekräfta förekomsten av de specifika hormonstörningarna i en större grupp av kvigor. De kvigor som inte blev dräktiga uppvisade mycket riktigt hormonstörningar i högre grad. Dessutom sjönk dräktighetsprocenten i takt med ökat antal inseminationer, vilket tolkas som att utebliven dräktighet inte beror på slumpen utan att det måste finnas inre faktorer hos kvigorna som orsakar fruktsamhetsproblemet.

Det är sedan tidigare känt att stress påverkar fruktsamheten negativt. För att undersöka om omlöparna var mer stresskänsliga än normala kvigor, och om de avvikande progesteronnivåerna som uppmätts under brunst kunde bero på en ökad progesteronproduktion från binjurarna, behandlades kvigorna med ett hormon (ACTH) som framkallar en stressliknande situation i kroppen. Vid stress frisätts nämligen progesteron parallellt med stresshormonet kortisol från binjurarna. Som svar på den stressframkallande behandlingen producerade både omlöparna och de normala kvigorna progesteron från binjurarna i koncentrationer som överskred de förhöjda progesteronkoncentrationer som tidigare uppmätts under brunst hos omlöparkvigor. Vidare var produktionen av kortisol mycket högre hos omlöparna. Utifrån försökets resultat kan man misstänka att stress är en faktor i omlöparsyndromet.

I en annan studie opererades äggstockarna ut från omlöparkvigor i ett tidigt skede i brunsten. Avsikten var att undersöka om hormonstörningarna under brunsten påverkat kvaliteten på den miljön där äggcellen vistas fram till ägglossning. Bedömningen blev att kvaliteten på vävnaden var god, och att omlöparkvigorna inträder i brunst med samma förutsättningar som normala kvigor i detta avseende.

För att studera äggcellens kvalitet under den senare delen av brunsten, under den känsliga mognadsfas som föregår själva ägglossningen, samlades omogna äggceller från levande kvigors äggstockar med en metod liknande den som används på kvinnor inför s.k. provrörsbefruktning. Den sista mognaden fick ske i mognadsmedium under laboratorieförhållanden (in vitro), och kvaliteten hos de mogna äggcellerna jämfördes mellan omlöpare och normala kvigor. De omogna äggcellernas kvalitet var lika i de båda grupperna, men efter ett dygns mognad in vitro visade omlöparkvigornas äggceller tecken på försämrad kvalitet p.g.a. obalans i mognad mellan cellkärnan och cellens övriga beståndsdelar.

Sammanfattningsvis visar resultaten från denna avhandling att symptomlösa omlöparkvigor har vissa karakteristiska drag som kan urskiljas vid noggranna och upprepade undersökningar av deras brunstsymptom, äggstocksaktivitet och hormonspel under brunstcykeln. Resultaten visar att det räcker med mycket små störningar i det normala hormonspelet vid brunst för att brunstförloppet ska rubbas och förhållandena för den känsliga äggcellen påverkas negativt. En sådan äggcell kan ha viss möjlighet att bli befruktad, men har dåliga förutsättningar att utvecklas till ett livsdugligt embryo.