Saskia Gabriele Hofmann

ESTABLISHMENT OF A PERFUSION CULTURE SYSTEM FOR BOVINE GRANULOSA CELLS TO INVESTIGATE THE RELATION BETWEEN NEGATIVE ENERGY BALANCE AND DECLINING FERTILITY IN DAIRY CATTLE

INAUGURAL-DISSERTATION zur Erlangung des Grades eines **Dr. med. vet.**
beim Fachbereich Veterinärmedizin der Justus-Liebig-Universität Gießen

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eingereicht von

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Tierärztin aus Mannheim (Baden-Württemberg)

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1 Introduction and Motivation

Cultivating animal cells and tissues *ex vivo* has been expediting the humanity's understanding of the organisms' functionality since the first experiments at the beginning of the 20th century. Nowadays, cell culture systems are not only the most widely used alternative for replacing animal experiments, but also essential in modern biomedical research.

Investigations on the level of the bovine granulosa cell (bGC) have been a topic in reproductive research for a longer period of time. However, to get information out of *in vitro* systems that are mirroring the *in vivo* situation and therefore being suitable for producing clinically relevant results, it is necessary to imitate *in vivo* conditions as exactly as possible in the applied systems. Therefore, the aim of this work was to establish a perfusion cell culture system for bovine granulosa cells.

After the successful establishment, the influence of metabolic imbalances on the cells' function will be elucidated. This topic is especially relevant in veterinary bovine health management, because the constant decline in the reproductive performance of dairy cows over the last years is linked to the deficient metabolic situation of those high yield animals. One approach to analyse this multifactorial syndrome of subfertility during high lactation is to investigate the interaction between metabolic disorders and mechanisms of the physiology of reproduction. Metabolic aberrations as the negative energy balance in high yielding dairy cows involve changes in plasma parameters, which influence the delicate balance in the ovary's endocrine system. Perturbed granulosa cell function has negative consequences for folliculogenesis, steroidogenesis and development of oocyte and embryo. Investigations of the interaction of metabolic disorders and granulosa cell function in cows have so far only been conducted using static cell culture systems.

New findings in this area will lead to the formulation of new recommendations for the management of high yielding dairy cows and contribute to the improvement of reproductive performance and animal health on dairy farms. In addition, reproductive failure in females has not only great relevance in veterinary, but also in human medicine. This study will provide the basis for cultivating other mammalian granulosa cells, including human cells, under these organotypic conditions.

2 Literature Survey

2.1 Bovine Granulosa Cells

2.1.1 Morphology

Granulosa cells (GCs) are cells of the female gonads, the ovaries. In mice, widely used as experimental model for mammalian development, their embryonic rise is not surely known. Though they appear to be epithelial cells, there is discussion if they have their origin in supporting cell precursors of the bipotential gonad, the ovarian surface epithelium or in the rete ovarii at the ovary-mesonephros border, or, in distinct populations, in both the supporting cell precursors as well as the surface epithelium instead [1,2]. In cows, however, for which mice were previously doubted as suitable embryonic models [3], the origin of the later GCs has been recently shown to be the surface epithelium exclusively using immunohistology for cytokeratines [4].

As part of the avascular compartment of the ovary the GCs are, together with the oocytes, separated from the other ovarian cells by a basement membrane [5]. The later granulosa cells first appear as a flattened layer of pregranulosa cells surrounding the oocyte, the follicular epithelium or follicular envelope, in which the cells are arrested in the G_0 phase of the cell cycle [6]. The structure comprising oocyte and follicular epithelium is called the primordial follicle (Figure 2-1, left side). If a primordial follicle is recruited in the course of a reproduction cycle by still not precisely known mechanisms [7], massive morphological and biochemical changes are taking place in order to prepare the conditions necessary for the generation of a fertilisable oocyte. Folliculogenesis from primordial to the mature, Graafian stage takes about 6 month [8]. In the beginning, the follicular epithelium becomes cuboidal to cylindric and the follicular structure is now referred to as primary follicle [9] (Figure 2-1, right side).

In the secondary follicle, the follicular epithelial cells start proliferating and form up to 10 layers. In these layers, the follicular cells are polyedric and have only few cytoplasma [10], so that this area appears very rich in nuclei, which leads to the name stratum granulosum. The follicular epithelial cells can now be referred to as granulosa cells (GCs). Among themselves and with the oocyte, GCs communicate via gap junctions and build a structural and functional syncytium [12–15].

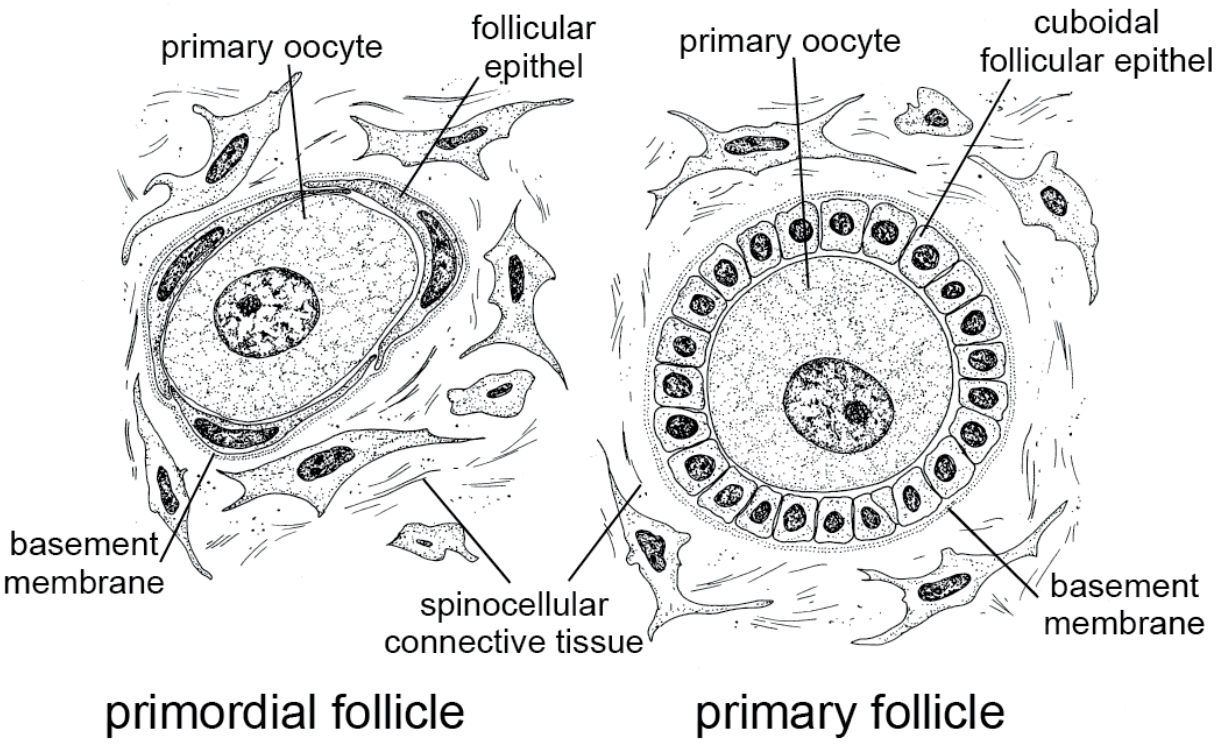

Figure 2-1 Primordial and primary follicle, modified after [11].

The GCs directly surrounding the oocyte are cylindrical and referred to as corona radiata [9]. The size of a bovine secondary follicle (also called multilaminar follicle (MUF)) is 150 - 300 μm (Figure 2-2, left side). With further proliferation of the GCs, which is mainly dependent on gonadotrophins [16,17], their cell shape is flattening out and a fluid-filled cavity is generated. This so called antrum is filled with follicular fluid secreted by the GCs, a modified trassudate from the peripheral plasma [18,19].
Its composition is essential for creating ideal preconditions for oocyte maturation. As soon as this antrum has formed, the follicle is referred to as being tertiary. In the tertiary follicle, the oocyte lies eccentric in a group of GCs called the cumulus oophorus [9](Figure 2-2, right side). Three different populations of granulosa cells have been suggested, based on the content of enzymes and the secretion of proteins, steroids and acids [20–22]. The mural population comprises the granulosa cells connected to the basement membrane, and the antral population comprises the cells closest to the antral cavity. The third population constitutes the cumulus oophorus, comprising the GCs population adjacent to the oocyte and the corona radiata cells, which directly surround the oocyte. As soon as the follicle reached its final dimensions and is ready for ovulation, it is called the Graafian follicle. A bovine Graafian follicle is approximately

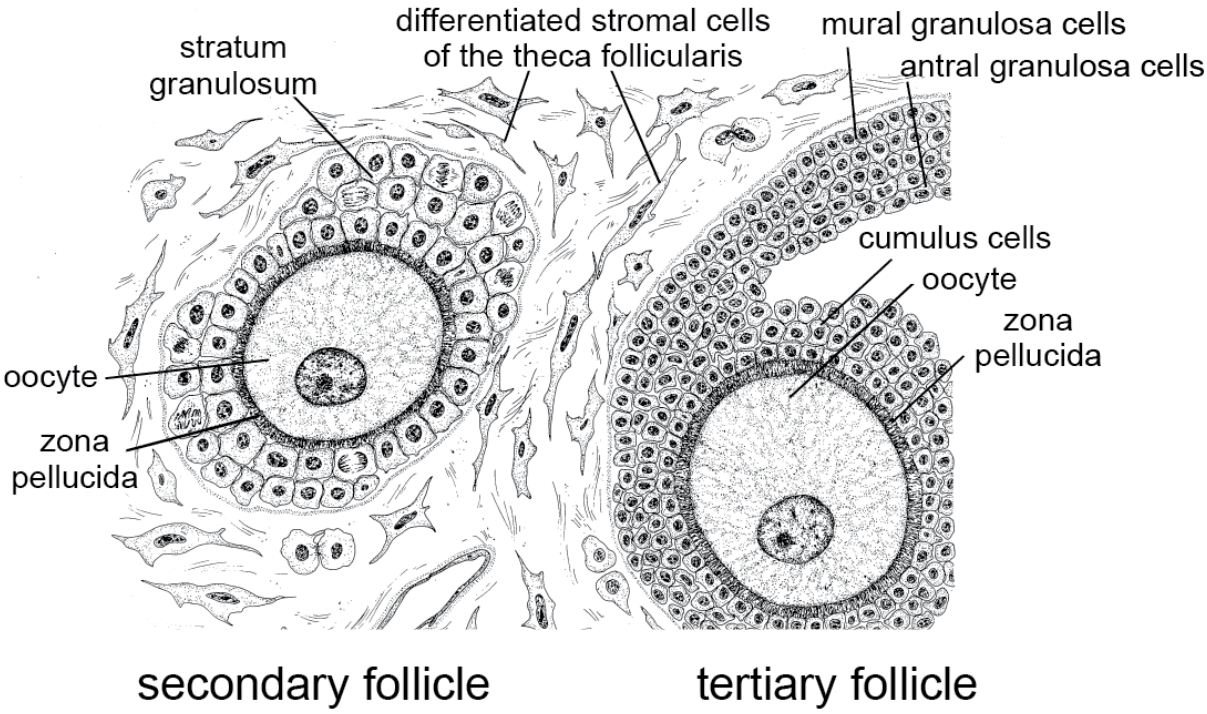

Figure 2-2 Secondary and tertiary follicle, modified after [11].

18-22 mm in diameter. Closer to ovulation, the connection between cumulus oophorus and the other GCs is loosened up, finally resulting in complete disconnection. Shortly before ovulation, granulosa cells penetrate the basal lamina and hence prepare the invasion of blood vessels and theca cells for the formation of the corpus luteum [19]. On one place the follicular wall thins and forms the stigma, the later place of ovulation. At ovulation, the oocyte and its surrounding cells flow out of the collapsing follicle in follicular fluid. The remaining follicular structure is filled with a later resorbed blood coagulum – forming the corpus haemorrhagicum – and changes under reorganisational processes like vascularisation and differentiation of bGC into granulosa lutein cells into the corpus luteum (CL) [9]. The granulosa lutein cells are two to three times larger than nonluteinised GCs [9]. In the cow, no theca-lutein cells occur [9]. If a pregnancy is established, the corpus luteum is maintained as corpus luteum graviditatis, otherwise, as a corpus cyclicum, its fate is fatty degeneration, which, after a while, only leaves scar tissue as corpus albicans on the ovarian surface [9]. GC proliferation and differentiation is regulated by gonadotrophins and different intraovarian factors from the oocyte and somatic surrounding cells [23–26].

2.1.2 Function

The mammalian ovary has two major functions: the production of a fertilisable oocyte and the synthesis of steroid hormones [27]. For both, GCs play an indispensable role.

2.1.2.1 Hormone Production: 17β-estradiol and Progesterone

One of the major hormones produced by the bGCs is 17β-estradiol ((17β)-estra-1,3,5(10)-triene-3,17-diol, E2), and its principal functions are the promotion of sexual behaviour and the stimulation of secondary sex characteristics [19]. The production of this steroid hormone is triggered by hypophysal FSH, which binds to a G-protein associated receptor expressed on the surface of bGCs and basically activates the cAMP/protein kinase A intracellular second messenger pathway [28,29]. The so initiated cascade of signals ends in activating the expression of the enzyme cytochrome P450 aromatase (P450arom), which converts androgens into estrogens [30]. Both bovine theca cells (bTCs) and bGCs are needed for the synthesis of E2, because bTCs provide the androgens necessary as substrate for the GCs' E2 production [31]. This also refers to the two cell/two gonadotropin theory, established by Fortune and Quirk in 1988 [32]. After that theory, bGCs have FSH receptors (FSH-R) and bTCs have LH receptors (LHr) in early follicular stages, and both are capable of metabolising cholesterol ((3β)-cholest-5-en-3-ol) to pregnenolone (3β-hydroxypregn-5-en-20-one, P5) with the cytochrome P450 side-chain cleavage enzyme (P450scc). Since the so called Δ4 pathway is preferred in ruminants, P5 is further metabolised to 17α-hydroxypregnenolone (17OH-P5) and then to dehydroepiandrosterone (3β-hydroxy-androst-5-ene-17-one, DHEA) by the enzyme cytochrome P450 17α-hydroxylase/17-20 lyase (P450c17). P450scc is only expressed in bTCs, not in bGCs [33–35], and is stimulated by LH binding to its receptor on the bTCs [36,37]. DHEA is then metabolised to androstenedione (4-androstene-3,17-dione, A4) by the enzyme 3β-hydroxysteroid dehydrogenase/Δ-5-4 isomerase (3β-HSD), which is found in bTCs of all follicular stages as well as in late follicular stages in bGCs [38]. The instance that pieces of the follicle wall (containing theca interna together with attached bGCs) produce more A4 than only theca interna preparations alone, is due to the fact that bGCs supply bTCs with more metabolisable progestin precursors [39]. The supply with A4 was stated to be the limiting factor in the follicular E2 production [40–43].

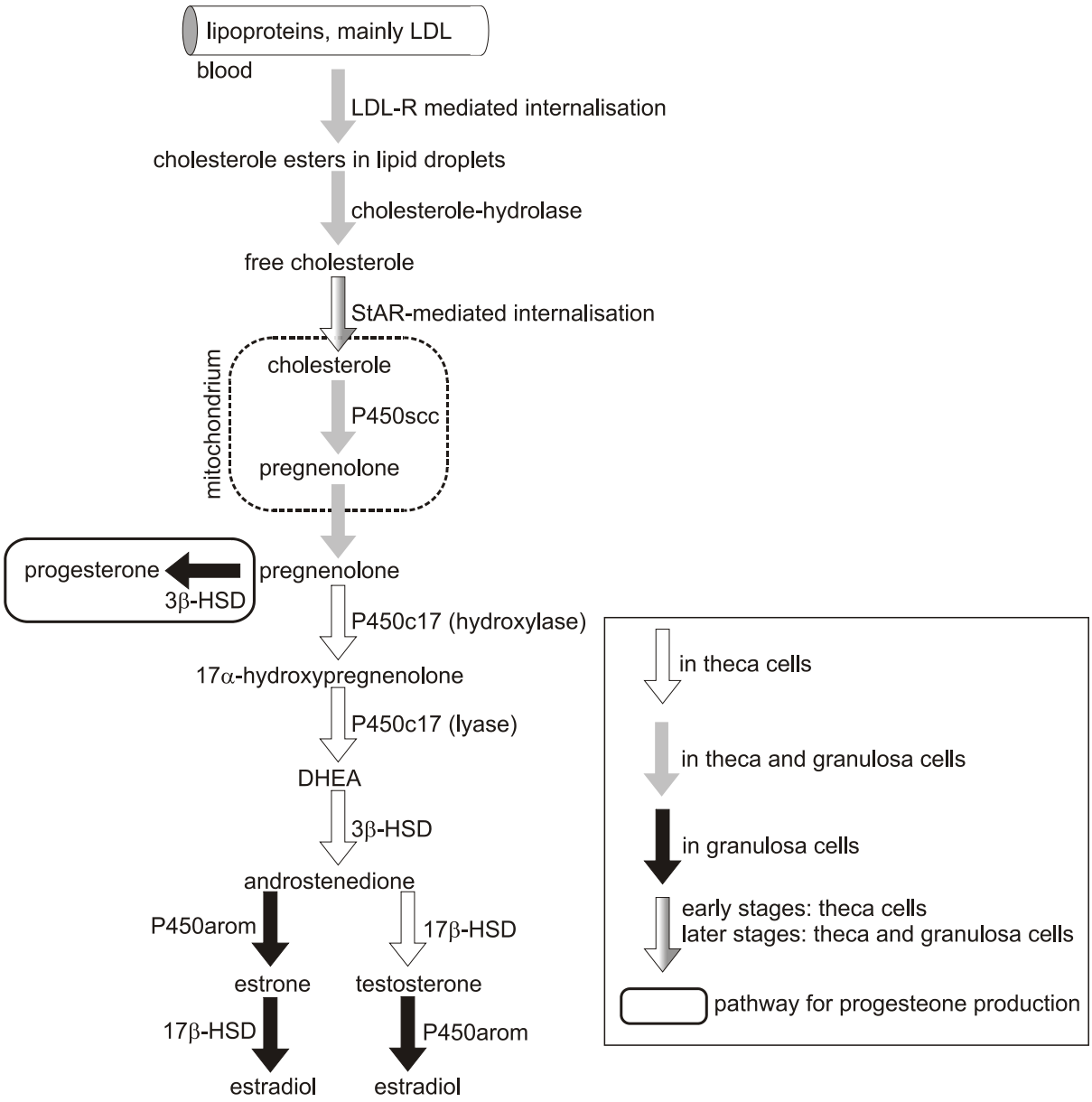

Figure 2-3 Production of E2 in the ruminant follicle (pathway for P4 production indicated).

A4 can be directly provided to bGCs as substrate for P450arom for further conversion into estrone (3-hydroxyestra-1,3,5(10)-triene-17-one, E1). Estrone is then metabolised into E2 by the enzyme 17β-hydroxysteroiddehydrogenase (17β-HSD), the preferred pathway in ruminants [44]. Alternatively, A4 can be first converted into testosterone (T4) by 17β-HSD and then be provided to bGCs for further metabolisation into E2 by P450arom [44] (Figure 2-3).

E2 is the major steroid produced by GCs before their luteinisation under the influence of luteinising hormone (LH) [45] and paracrine and autocrine factors [46]. The LH

surge, which leads to the ovulation of the follicle and the differentiation of follicular cells into luteal cells, is triggered by a positive feedback mechanism of E2 on LH secretion [47,48]. During luteinisation, GCs switch from a highly proliferating to a non-proliferative, terminally differentiated stage [15] and acquire more and more LHr [6]. It was also shown for rats, that in later stages of follicular development, E2 and FSH promote the expression of LHr on GCs, so that there is a positive feedback of E2 on its own secretion [49]. LH and FSH act via the same post-receptor second messenger system on GC function [34,50,51]. In ruminants, LH and growth hormone are the primary luteotrophic hormones [48]. For reaching preovulatory competence, a high pulse frequency of LH seems to be crucial: it has to stimulate TCs to produce higher amounts of androgene substrates for further metabolisation to E2 in GCs and to recoup the declining FSH levels in stimulating P450arom activity for this metabolisation [52]. In preovulatory follicles, the LH surge induces the expression of genes essential for ovulation [53].

The process of luteinisation starts shortly before ovulation of the dominant follicle. After ovulation, great morphological and functional changes lead to the formation of the corpus luteum (CL), which is composed of modified GCs, TCs and supporting cells and is highly vasculated. In the CL, the major product of luteinised GCs is progesterone (P4). P4 has a key function in the regulation of the length of the reproductive cycle and for the implantation of the blastocyst [54]. P4 is synthesised by the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD), which uses P5 as substrate. The rate-limiting step in the production of P4 is the transport from cholesterol into the mitochondria by the secretory acute regulatory protein (StAR), where it is further metabolised to pregnenolone by P450scc [55].

P4 is the key hormone for successful embryo development and maintenance of pregnancy [56,57], and only 18 hours after the LH surge, 90 % of the follicular steroid content is represented by P4 [58].

The estrogen to progesterone ratio is used to estimate the estrogenic activity and the health status of follicles and high estrogen production is the criterion of an intact dominant follicle [59].

2.1.2.2 Importance for Bovine Oocytal Development

GCs are responsible for creating optimal conditions for oogenesis and ovulation, and precursors for fertilisation and implantation [60]. They are the somatic cells with the

closest association to the developing oocyte. The growths of the oocyte as well as reaching its developmental and meiotic competence depend on adequate communication with the surrounding GCs [27] (Figure 2-4).

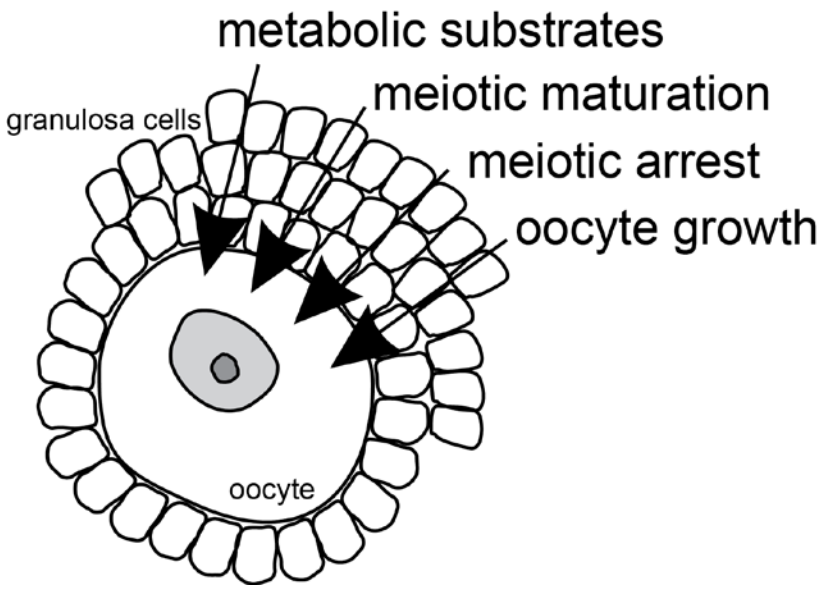

Figure 2-4 Influence of GCs on oocyte development and maturation, modified after [27].

FSH and intrafollicular factors that support oocytal development act on the oocyte via the GCs [27]. GCs influence the oocyte's transcriptional activity [61] as well as their protein expression [62]. This happens mainly over gap junctions between the GCs and the oocyte [63,64], but there is also ligand-receptor mediated communication [27]. GCs need to change from their predominantly estrogenic to a major progesterogenic environment under the influence of the LH surge to trigger meiosis in the oocyte [65].
In ewes, the preovulatory rise in P4 secretion by GCs results in downregulation of gap junctions between these cells, so that the cumulus oocyte complex is isolated and the E2 concentration in the oocyte is reduced below the threshold to maintain meiotic arrest [66]. Downs presumed the cumulus cells to act via paracrine factors on the oocyte to induce meiotic resumption [67]. There is also some evidence that P4 is involved in the polyadenylation of maternal mRNA and thereby regulating the expression of oocytal genes important for development [66].
Communication between GCs and oocytes is bidirectional.

2.2 Perfusion Culture

2.2.1 Perfusion Culture for Granulosa Cells

Investigations on the level of the granulosa cell, which is significantly involved in follicular and oocytal development and maturation [12], have been a topic in reproductive research for a longer period of time. As soon as cell biological methods had been introduced in endocrinological research, the *in vitro* investigation of granulosa cells began in the mid-1970s (porcine [68], equine [69,70], human [71], bovine [72,73]). Studies with static GC cultures elucidated many fundamental mechanisms of the physiology of these cells.

Because the focus of this work lies on dynamic, perfused cultures for GCs, the following overview will concentrate on publications referring to the perfusion technique.

Excursion: Etymologic Elucidation of Terms Used for Perfusion Cultures

In the literature, different terms are used to describe the same act of pumping medium over cell cultures that are held back in some kind of container by being attached to a substrate and/or with using a mesh at the out- or out- and inflow site. At first, the term ‘perfusion’ and its alternatives will be etymologically elucidated.

To **perfuse** means

1: to suffuse or permeate (a liquid, colour, etc.) through or over (something),

2: *surgery* to pass (a fluid) through organ tissue to ensure adequate exchange of oxygen and carbon monoxide [74]

or

1: **suffuse**,

2a: to cause to flow or spread: **diffuse**,

2b: to force a fluid through (an organ or tissue) especially by way of blood vessels,

whereby **suffuse** means

to flush or spread over or through in the manner of a fluid and especially blood

and **diffuse** means

1: to subject (as a light beam) to diffusion,

2: to break up and distribute (incident light) by reflection (as from a rough surface), *vi*: to undergo diffusion [75] .

It has its origin in the Latin verb ’perfundere’, which means ’to drench, flood’.

Perfusion is

> an act or instance of perfusing, *specific*: the pumping of a fluid through an organ or tissue.

When searching the verb to **superfuse** one finds

> *obsolete*: to pour or be poured so as to cover something

and **superfusion** referred to as the accordant noun [74].

It has its origin in the Latin verb ’superfundere’, which is composed of the word super, meaning above, beyond, in addition, to an especially high degree, and fundere, meaning to pour.

The verb to **perifuse** is defined as

> to flush a fresh supply of bathing fluid around all of the outside surfaces of a small piece of tissue immersed in it,

with the noun **perifusion** being

> the act of perifusing [76].

In the literature cited here, to perfuse, to perifuse and to superfuse, respectively perfusion, perifusion and superfusion are all used to describe the same technique. In the following overview, the term originally used in a cited publication was further kept up with, whereby the verb to perfuse and perfusion is preferred by the author of this work, as it was by the developers of the here applied system [77].

The first perfusion cell culture models were developed in the 1970s and were used for adrenal tissues [78]. Superfusion systems for elucidating the function of female reproductive tissue using whole organs, respectively organ slices, have been first reported for rat ovarys in the 1980s [79,80]. Concerning cattle, bovine follicles were first examined for their P4 and T4 production in a short term superfusion system by Zimmermann et al. in 1985 [81].

An early publication concerning superfusion cultures of isolated ovarian cells, as it will be performed in this work, is the survey of Rodway and co-workers from 1976, where the steroid secretion of porcine ovarian cells was examined. Isolated porcine luteal cells and follicular TCs and GCs were cultured using a modified chromatography column with in- and outflow blocked by filters as superfusion chamber. Isolated granulosa cells secreted P4 and E2, and both were enhanced by addition of LH or hCG. Combined culture of TCs and GCs produced slightly more E2 and P4 as the single culture, but the response to LH or hCG was similar [82].

1985, Woody and La Barbera also described the perifusion of porcine GCs in abstract form and found the cells to maintain their responsiveness to FSH better if stimulated in pulses and not continuously [83].

Rolfes-Curl and co-workers published an abstract about perifused rat GCs 2 years later. They tested the influence of pulsatile in contrast to continuous stimulation with FSH and could not find any difference in P4 production between the two protocols [84].

In 1988, Johanson and Johanson developed a superfusion system for isolated rat GCs to investigate the dynamics of cAMP production and steroidogenesis in response to gonadotrophins. Therefore, cells were allowed to attach to Cytodex^{TM} microcarrier beads and a modified syringe was used as superfusion chamber. The stimulation with FSH showed a concentration dependent transient rise in cAMP and P4, but not in E2 or testosterone production [85].

In the same year, Kadota and co-workers also superfused Cytodex^{TM}-bound immature rat GCs in a perfusion chamber made of a modified syringe. They detected a self-priming effect of FSH, shown in a biphasic reaction in P4 production during continuous stimulation, which could not be mimicked by continuous stimulation using a cAMP analogon [86].

A survey concerning the refractoriness to FSH in rat GCs was done by Johanson and co-workers one year later, using the same syringe and Cytodex^{TM} superfusion system described before. The cells were stimulated with one or two pulses of FSH, whereby the height of the first pulse was changed and the second was kept constant. P4 and cAMP were following the second pulse the higher the lower the first pulse was, whereby also the total amount of P4 and cAMP released during both pulses was lower when the first pulse was higher [87].

In the same year, Woody and La Barbera used a dynamic flow perifusion system to elucidate the FSH-responsiveness and adenylyl cyclase capacity of immature porcine GCs. They found that short FSH-pulses with FSH-free intervals were most effective and stated a refractoriness due to changes in the interaction between FSH receptor and a regulatory component of the cyclase [88].

Also 1989, Weiss and co-workers perifused human GCs on Cytodex^{TM}-3 beads and stimulated them with different pulse frequencies and amplitudes of LH, which stimulated P4 production in form of an initial rise followed by a decline according to decreasing LH concentration. Over a period of 10 hours, pulsatile stimulation was

more efficient for P4 production than continuous stimulation. No P4 production could be activated using hCG [89].

In 1992, Wang and co-workers perifused rat GCs with calcium-free medium to study the mechanism of the LH-releasing hormone. They concluded that the increase in intracellular calcium after stimulation with LH-releasing hormone results from a release of intracellular calcium without involving voltage-sensitive or sodium-dependent calcium channels [90].

Human GCs were cultured in a superfusion system in 1993 by Bódis and co-workers. Using a superfused cell column and Sephadex^{TM} beads, they showed that serotonin induces P4 release in human GCs and this could be blocked using mianserin, but not propanolol [91].

Benoit and Veldhuis cultured porcine GCs on Cytodex^{TM}-3 micro carrier beads in perifusion columns in 1995. They found the upregulation of P450scc mRNA to be dependent on frequency and concentration of FSH stimulation [92].

Rabe and co-workers cultured human GCs on Cytodex^{TM} beads in superfused columns and found vasopression, calcium ions and inositol trisphosphate to stimulate P4 secretion of these cells [93].

2000, Török and co-workers superfused human GCs on Sephadex^{TM} G10 and found out, that the basal P4 production of these cells is pulsatile and that LH stimulation results in a rapid increase of P4 production, which is mainly due to de novo synthesis [94].

Bodis and co-workers superfused human GCs on Sephadex^{TM} G10 again in 2010 to investigate the effect of catecholamines, acetylcholine and histamine on progesterone release. Adrenaline, noradrenaline and histamine had no influence on P4 production, whereby acetylcholine stimulated P4 production and this stimulation could be blocked using atropine [95].

In 2004, Koppan and co-workers examined the influence of serotonine on gonadotropin-induced steroid secretion of human GCs in a superfusion as well as in a static culture system. In the static system, serotonin had no influence on gonadotropin-induced P4 secretion and augmented E2 production. In the superfusion system, serotonin had a negative influence on gonadotropin-induced P4 secretion, but the general hormone response was prolonged [96].

This overview of publications shows that a perfused culture can be used to get a better insight into the dynamics of the GCs' hormone secretion and their dynamic reactions to different stimuli. Until now, bGCs have not been cultured in a perfusion system. Also, no publication directly compared the static to a perfused culture using cells from the same cell pool. So far, all publications concerning dynamic systems for granulosa cell cultures have in common, that they depend on self-made constructions of the perfusion apparatus. The repeatability and comparability of these culture systems is difficult and a higher degree of reproducibility is desirable.

Therefore, this study is aimed to fill these gaps by establishing a perfusion culture for bGCs and comparing it to a static culture from the same cell pool, using a reproducible, since commercially available, cell culture system. This bGC perfusion cell culture system will then be used to elucidate the context between negative energy balance and GC function in cattle.

2.2.2 The Perfusion Apparatus

During *in vitro* investigations, it is important that cells maintain their physiological biochemical properties to get relevant results [97]. But a great problem when using traditional cell culture methods is the proceeding dedifferentiation of the cells under static conditions, comprising loss of cell specific morphology as well as physiological and biochemical processes [98,99]. Also, the *in vivo* environment of cells with its optimal cell adhesion, nutrition supplement and discard of metabolic products is difficult to reproduce in conventional culture dishes like Petri dishes or cell culture flasks [98]. In a perfusion cell culture system, the conditions for the cultured cells can be kept stable. With the continuous exchange of culture medium, accumulation of metabolites and cell secretion products is prevented. The cells always have contact with unconsumed medium and are optimally supplied with nutrients, hormones and growth factors [100].

Minuth and co-workers from Regensburg University, Germany, developed the perfusion cell culture system used in this survey. The so called Tissue Factory^{TM} was constructed as a modular system and developed to provide optimal perfusion culture conditions for the generation of artificial tissues. In the tissue culture containers optimal *in vivo* like conditions can be provided for each individual tissue by a precisely tuneable calibration [101]. The Tissue Factory^{TM} methodically combines the advantages of static cell culture as widespread and approved tool, advanced tissue culture as requested in

modern biomedicine, and micro reactor technology [77]. Adhesion of cells can be optimised by determining an individual biomaterial for every cell type [77]. Most of the components of the culture system can be used more than once and therefore be sterilised using an autoclave [77]. The Tissue Factory™ used in this experiment is commercially available (minucells and minutissue Vertriebs GmbH, Bad Abbach, Germany), which provides the basis for a high reproducibility of the experiments. The perfusion cell culture apparatus used in this study has already been established for the culture of various other mammalian cells, where a high level of differentiation could be initialised and maintained over a longer period of time (for example epithelia, connective, muscular and nervous tissue, summarised in [77]). A detailed description of the Tissue Factory™ is given in chapter 3.1.2.4.

2.3 Declining Fertility in Dairy Cows and the Link to Granulosa Cell Function

2.3.1 The Influence of Negative Energy Balance on Fertility

Fertility in high yielding dairy cows is declining year after year over the last decades (expressed in terms of pregnancy rates 0.45 % in the USA [102,103], expressed in terms of conception rate to first service 0.75 % in Ireland [104] and 1 % in the UK [105,106], and expressed in terms of pregnancy rates 0.5 % per year between 1991 and 2000 in Spain [107]). Reduced fertility has an influence on average daily milk production, average days in milk, the number of calves per year, the generation interval and consequently on the income of the farmer [106,108,109]. The decline in fertility has multiple causes and involves genetic components, inadequate nutrition, poor reproductive management, an increase in diseases and overall poor cow welfare [110]. One important fact is that the reproductive performance of a dairy cow is linked to its energy reserves and metabolic responses to nutrition [111] and one major risk factor for impaired reproductive performance is negative energy balance (NEB) [103,112]. Already Aristotle claimed nutrition to be the most important factor influencing conception [113].

Cows in NEB, which occurs regularly in the post partum period and is triggered by the massively increasing milk yield over the last decades, need more energy for their milk

production than they can acquire through feed. There is an obvious antagonism between the increase in milk yield and the reproductive performance in dairy cattle [110,114–117].

The selection for high milk yield leads to an intensivation of the ‘nutrient highway’, where all energy, either from nutrition or mobilised from body reserves, goes directly to the udder [118]. Also, it is from a biological viewpoint more sensible for the cow to favour milk production (to save current offspring) over fertility (to invest in unsure future offspring, ‘nutrient prioritisation’) [119]. To prioritise the milk production over body functions is a universal strategy in mammals to safe the offspring’s nutrition independent of the mother’s nutritional state [120]. But in comparison to the need of energy for milk production, the energy needed for the ovarian cycle resulting in the ovulation of a fertilisable oocyte, the formation of a CL and the maintenance of early pregnancy, is negligible. Therefore, not an insufficient energy supply, but the endocrine and biochemical changes associated with NEB seem to have a negative influence on fertility [121].

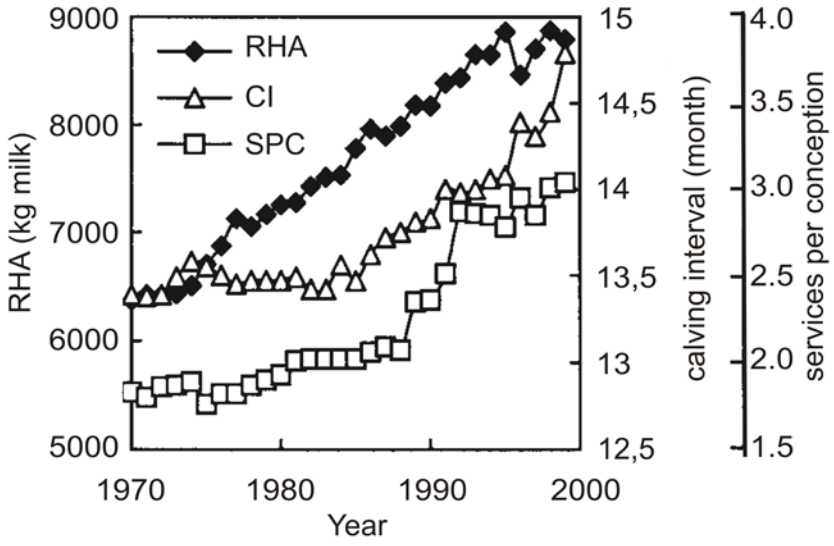

Figure 2-5 Rolling herd average (RHA, kg milk per lactation), calving interval (CI), and services per conception (SPC) for 143 dairy herds continuously enrolled in the Raleigh DHIA record system from 1970 to 1999 [110].

One major factor recognised for reproductive failure in connection with NEB is disturbed endocrine signalling on the hypothalamus-pituitary-ovary-uterus axis, which leads to a later resumption of ovarian cyclicity post partum [111,122,123]. However,

the focus changed recently towards ubiquitous low conception rates (Figure 2-5) [110,111,124] and high early embryonic loss [56,125] as a major problem of high yielding dairy cows [118]. There is evidence that one major factor contributing to this problem is reduced oocyte and embryo quality [118].

Endocrine and biochemical changes in association with NEB reach the follicle and so the microenvironment of the developing oocytes and can influence their growth and maturation (Figure 2-6) [118].

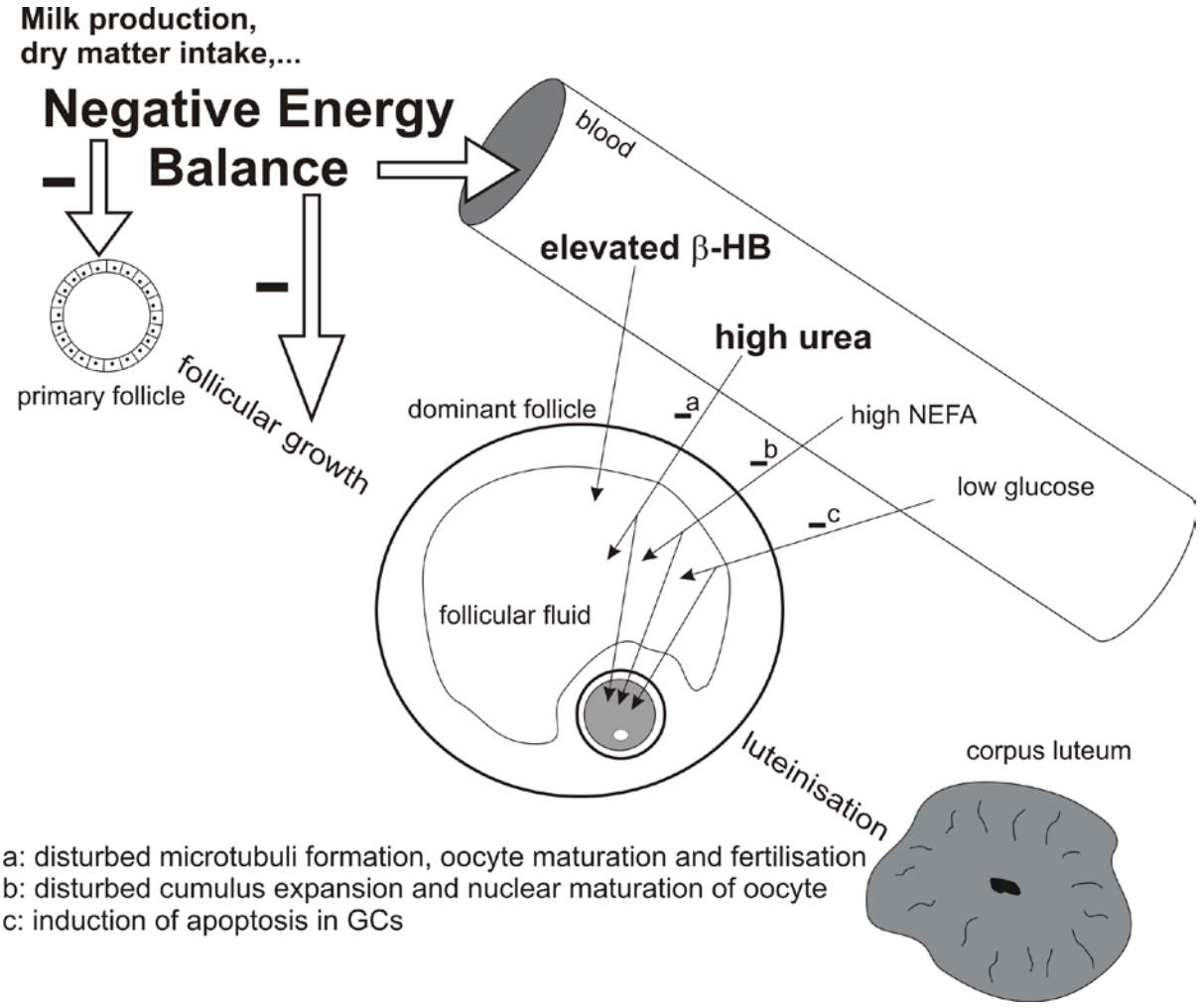

Figure 2-6 Influence of negative energy balance on follicular and oocytal development, modified after [118].

Follicles, and so GCs, that are exposed to an microenvironment affected by NEB may be negatively influenced in their ability to produce adequate amounts of E2 and P4 [126–128]. Even if a competent oocyte is fertilised, its survival can be endangered by inadequate CL function, which leads to an inadequate uterine microenvironment and results in early embryonic loss [118]. In cows during NEB, the CL secrets lower

amounts of P4 than in cows not in NEB [129], whereas a positive correlation between blood P4 levels and establishment of pregnancy was found [130]. After fertilisation, the microenvironment of the oviduct and uterus has a great impact on embryo quality [131,132]. Pregnancy rates after embryo transfer were lower in recipients with a low body condition score than in recipients with high body condition score [133].

In conclusion, ovarian tissue shows direct reactions to metabolic factors with consequences for folliculogenesis, steroidogenesis and development of oocyte and embryo [134]. Knowledge concerning the oocyte's microenvironment, which is greatly influenced by GCs, is still limited, and research should aim to fill this gap [121].

2.3.2 The Influence of Negative Energy Balance-Related Metabolites on Granulosa Cell Function

The physiological ovarian dynamics are affected by NEB and lactation, e.g. the postpartum number of large follicles and blood E2 concentrations are different in lactating and non-lactating cows [41]. There is also plenty of evidence that the ovary can react independent of gonadotrophic stimulation directly to metabolic inputs, especially those which occur during high yielding phases post partum in the modern dairy cow [134].

It is proposed that alterations in ovarian function in consequence of metabolic disorders are mediated through metabolic hormones like insulin-like growth factor I, growth hormone and leptin [135]. For example, Armstrong and co-workers supported the thesis that blood insulin and IFG-I levels directly affect the steroidogenic potential of bGCs [136]. But also metabolites accumulating during negative energy balance, like non esterified fatty acids, urea and ketone bodies (Figure 2-7), can have a negative influence on GCs and so follicular and ovarian function. This is also due to the fact, that, in correspondence, circulating gonadotrophin levels do not change [135]. Previous studies in the Clinic of Obstetrics, Gynaecology and Andrology of Large and Small Animals with Ambulatory Service of the Justus-Liebig-University Giessen showed, that in cows blood metabolite concentrations are reflected in their follicular appearance and that significant changes happen in the composition of the follicular fluid in the final phase of follicular maturation [137,138]. This is in accordance with data from other surveys [139–142]. The integrity of ovarian cells can be adversely influenced by metabolic factors with consequences for follicle and oocyte [134,139]. For example, Vanholder and co-workers found, that the three main non esterified fatty

acids in the serum of cows in NEB significantly inhibit the proliferation rate and the survival of granulosa cells *in vitro* [143]. The focus in this work will be on the influence of urea and β-hydroxybutyric acid, both metabolites accumulating during NEB, on GC function.

2.3.2.1 Urea

During NEB in dairy cows post partum, not only a massive lipid mobilisation, but also a protein catabolism takes place. The deamination and detoxification of proteins result in elevated systemic urea concentrations [118]. Also, the ruminal flora is not adapted to the sudden increase in food rations appropriate for early lactation, resulting in an imbalance between energy and protein in the rumen, which can also lead to increased blood urea levels [140]. Feeding high dietary protein also results in high concentrations of urea in plasma and milk, and both have been associated with impaired fertility in dairy cattle [144–146], but the knowledge about the linking mechanisms is limited [147]. High blood urea concentrations can also be detected in the follicular fluid [139,147,148], as the concentration of urea in plasma, follicular and uterine fluid have a very good correlation in high yielding dairy cows in the postpartum period [139,149].

Urea was long suspected to have a negative influence on follicular and oocytal development and maintenance of pregnancy [150], before a negative influence of urea on oocyte maturation and fertilisation was confirmed [151]. Fahey suggested a negative influence of elevated urea levels on embryo quality in sheep via changes in the follicle or the oviducts [152]. To our knowledge, no studies have previously investigated the influence of urea on bGC function.

2.3.2.2 β-Hydroxybutyric Acid

It was stated, that in the periparturient time, nearly all cows suffer from a kind of subclinical ketosis [153]. Ketone bodies, such as acetone, acetoacetate and the here investigated β-hydroxybutyric acid (β-HB), accumulate due to a misbalance of C2 and C3 compounds during NEB. Is the ability of the liver to oxidise non esterified fatty acids and to store triacylglycerol exceeded, the blood concentration of ketones is rising [154]. Additionally, lipogenic feed supplements increase blood β-HB concentrations [155]. Ketones have been shown to have a direct influence on ovarian cells [119]. High β-HB concentrations are mirrored in the composition of the follicular fluid and influence the micro-environment around the maturing oocyte [139], and high plasma β-HB

concentrations were shown to have a negative effect on reproductive performance [156].

Vanholder and co-workers tested the influence of β-HB in concentrations of 0, 0.5, 1 and 1.5 mM on bGCs in medium with low glucose content. No negative effect of β-HB on bTCs was detected. In bGCs, β-HB significantly decreased the production of E2 and P4 per cell, but not per well. Accordingly, it had a positive effect on the number of cells. When medium with normal glucose concentrations was used, β-HB had no influence on the number of bGCs [157]. A reason for these findings might be, that ketones can be used as energy source by metabolisation in the Krebs cycle, but not in the pentose phosphate pathway [158], which yields the coenzyme NADPH necessary for steroidogenesis. With the here conducted study, possible effects of β-HB on bGC will be further elucidated.

3 Materials and Methods

3.1 Cell Culture

3.1.1 Media

All media preparations were conducted under sterile conditions in a laminar flow cabinet. If not otherwise stated, the medium components were purchased from PAA Laboratories GmbH, Pasching, Austria.

3.1.1.1 Washing Medium

For the washing medium, 48.1 ml DMEM/Ham's F-12 medium (1:1) with L-glutamine was supplemented with 1.4 ml HEPES buffer (1 M) and 500 µl penicillin/streptomycin solution.

3.1.1.2 Standard Medium

For the standard medium, 469.33 ml DMEM/Ham's F-12 medium (1:1) with L-glutamine were supplemented with 14 ml HEPES buffer 1 M, 5 ml penicillin/streptomycin solution (100x), 5 ml ITS (insulin-transferrin-selenium) supplement (100x), 2.5 ml fetal calf serum gold, 2.5 ml Intergonan^{TM} solution (prepared as recommended by the manufacturer, apart from replacing the provided solvent with sterile water, Intervet GmbH, Unterschleissheim, Gemany), 1.667 µl fatty acid free bovine serum albumin solution 10 % in PBS (made of BSA fatty acid free powder), and 5 µl androstenedione solution (1 µg/ml in ethanol, 4-androstene-3,17-dione powder, Sigma Aldrich Co. LLC., Taufkirchen, Germany).

3.1.1.3 Standard Medium with Urea

A stock solution containing 5 M urea was produced by dissolving 6.0060 g urea powder (≥ 99.5 % Ph. Eur., Carl Roth GmbH & Co. KG, Karlsruhe, Germany) in DMEM/Ham's F-12 medium (1:1) with L-glutamine to a solution with a total volume of 20 ml. 20 µl of this 5 M urea solution were added to 19.980 µl of standard medium. Standard medium containing urea had a final concentration of 5 mM urea.

3.1.1.4 Standard Medium with β-Hydroxybutyric Acid

A solution containing 0.4 M β-hydroxybutyric acid was produced by dissolving 0.50436 g sodium-β-hydroxybutyric acid (Sigma Aldrich Co. LLC., Taufkirchen, Germany) in DMEM/Ham's F-12 medium (1:1) with L-glutamine to a solution with a total volume of

20 ml. 100 µl of this 0.4 M β-hydroxybutyric acid solution were added to 19.900 µl of standard medium. Standard medium containing β-hydroxybutyric acid had a final concentration of 2 mM β-hydroxybutyric acid.

3.1.2 Establishment of the Bovine Granulosa Cell Perfusion Culture

3.1.2.1 Determination of the HEPES Buffer Concentration for the CO_2 Independent System

To determine the concentration of HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer solution needed to get a stable CO_2 independent pH of 7.40, a 24-well plate was prepared with serum-free medium containing DMEM/Ham's F-12 medium (1:1) with L-glutamine (PAA Laboratories GmbH, Austria), 1 % penicillin/streptomycin solution (100x, PAA Laboratories GmbH, Pasching, Austria), 1 % ITS supplement (100x, PAA Laboratories GmbH, Pasching, Austria), 0.1% fatty acid free bovine serum albumin solution 10 % in PBS (made of BSA fatty acid free powder, PAA Laboratories GmbH, Austria) and different amounts of HEPES buffer (PAA Laboratories GmbH, Austria), ranging from 200 to 380 mM in steps of 20 mM. After incubation overnight in a CO_2 free atmosphere in saturated humidity at 37.5 °C, the pH of the samples was measured using a pH-Meter with thermometer. This experiment was repeated twice.

3.1.2.2 Isolation of Bovine Granulosa Cells

Bovine ovaries were obtained from the local abattoir (Schlachthof Giessen GmbH, Giessen, Germany) from cows of mixed breed and age. Samples were only taken from animals with physiological diagnostic findings concerning ovaries and uterus [159]. After dissecting the ovaries from the surrounding tissues and washing off remaining contamination from the slaughtering process with tap water, they were placed into 50 ml tubes containing transport medium (phosphate buffered saline produced by using PBS powder (Dulbecco's PBS without Ca and Mg, PAA Laboratories GmbH, Austria) and sterile water as re−commended by the manufacturer with 1 % penicillin/streptomycin solution (100x, PAA Laboratories GmbH, Austria)) to prevent desiccation. The tubes with the ovaries were stored in a Styrofoam™ box and transported at ambient temperature.

In the lab, the follicular fluid from superficially visible follicles was aspirated using a 10 ml syringe and a 26 G needle. The follicular fluid was collected in 11 ml conical

centrifuge tubes in three separate groups, depending on the external diameter of the follicle they were obtained from, measured from the surface of the ovary. The follicles were classified as small (< 4 mm diameter), medium (4-8 mm) and large (> 8 mm), representing different stages of gonadotropin dependence, LH receptor expression and steroidogenic capacity of the included GCs [160]. GCs obtained from small follicles will be further entitled as bGC I, those from medium sized follicles as bGC II and those from large follicles as bGC III, as it will be referred to bGC classes I, II and III. The follicular fluid from small follicles was only aspirated once, whereas the follicular fluid from medium sized and large follicles was aspirated and reinjected repeatedly whilst the follicle wall was gently rubbed to the plain outer wall of the needle to disrupt the multiple layers of granulosa cells. Only follicular fluid from healthy follicles (homogeneous lucid appearance with ample but fine capillarisation) which was classified as physiological (without particles, yellowish translucent)[159] was used for further processing. The follicular fluid was then centrifuged at 200 g for 10 minutes at 4 °C The supernatant was discharged and the remaining cell pellet resuspended in 1 ml washing medium using a Pasteur pipette. To disrupt cell clusters, the cell suspension was pipetted up and down ten times. Two more identical washing steps followed, whereby the last cell pellet was resuspended in standard medium for the investigation in different cell carrier materials and in washing medium for the other experiments.

20 µl of the cell suspension were used to determine the cell number and viability by trypan blue staining in a haemocytometer, while the remaining cell suspension was kept at 37.5 °C in the incubator. For the counting, the cell suspension was mixed with the same amount of 0.4 % trypan blue solution (powder dissolved in PBS, trypan blue cell culture tested, Sigma Aldrich Co. LLC., Taufkirchen, Germany). After 4 minutes incubation time, the suspension was pipetted into a haemocytometer and put under a microscope. After one more minute to let the cells sediment in the chamber, the cell number from 4 large squares was counted for dead (blue stained) and alive (not stained) cells and the average cell number of alive and dead cells per large square was calculated. Cell viability was determined by dividing the average number of alive cells per large square through the average number of total cells per large square. To determine the number of alive cells per μl of cell suspension, the result was multiplied by 2 (dilution factor) and 10 (to get from 0.1 µl volume cell suspension in one large square to 1 µl total).

3.1.2.3 Investigation of Different Materials as Support for Bovine Granulosa Cells

There are many different materials with different pore sizes available as support materials for cell culture. To determine which material best suits for bGC attachment and growth, 12 different materials, which are commonly used as support materials as well as recommended for perfusion culture by the manufacturer of the Tissue Factory™ (minucells and minutissue Vertriebs GmbH, Bad Abbach, Germany), were tested (Table 3-1). Some of the materials were already confectioned in the right size to fit into the provided tension rings, the so called minusheets™ (minucells and minutissue Vertriebs GmbH, Bad Abbach, Germany), others still had to be cut into round pieces with a diameter of 13 mm. If the material was not sterile because of the handling or from shipping on, the pieces were sealed in Melafol™ and autoclaved at 105 °C for 20 minutes. The experiment was repeated thrice, using two samples of each material every time. The support materials were placed in the tension rings. The so assembled cell carriers were placed into a 24-well plate with the black tension ring part at the bottom, because this allows medium flow beneath the tissue carrier via small stands and facilitates later handling. Each well was filled with the amount of prewarmed culture medium that resulted in a total fill of 500 µl after adding the cell suspension, so that the surface of the cell carrier was just covered with medium to prevent the later added cells from floating off the scaffold. Special attention had to be paid to avoiding gas bubbles underneath the support material. Then, 100 000 bGCs containing equal parts of each of the three cell classes (bGC I, II and III) were seeded carefully on each carrier. The cells were cultured at 37.°C with 0 % CO_2 in humidified atmosphere. Because most of the materials were not applicable to examination with standard light microscopy, the cell attachment and distribution had to be inspected using fluorescence methods. Therefore, after 48 h of cell culture, the carriers were washed twice in 750 µl PBS. After that, they were each fixed in 750 µl of ice-cold 70 % ethanol solution (v/v solution of sterile water and pure ethanol $\geq$ 99.5 %, Ph. Eur., Carl Roth GmbH & Co. KG, Karlsruhe, Germany) for 10 minutes. The nuclear staining with 200 µl DAPI solution (0.2 µg/ml, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) was conducted in a dark chamber for 2 minutes. After the incubation, the DAPI solution was removed and remaining dye was thoroughly washed off using 750 µl PBS twice. The supporting materials were then removed from the tension rings and placed with the cell

Table 3-1 Materials tested as support material for bGCs

material	pore size (µm)	product name	product ID	handling	manufacturer
polycarbonate	0.2	Isopore™ membrane filter, transparent, diameter 13 mm	GTTP01300	autoclave	Millipore GmbH, Schwalbach, Germany
polycarbonate	0.4	Isopore™ membrane filter, transparent, diameter 13 mm	HTTP01300	autoclave	Millipore GmbH, Schwalbach, Germany
cellulosenitrate	0.45	cellulosenitrate membrane filter	11304-47	cut out, autoclave	Sartorius Stedim Biotech GmbH, Göttingen, Germany
cellulosenitrate	0.8	cellulosenitrate membrane filter	11306-47	cut out, autoclave	Sartorius Stedim Biotech GmbH, Göttingen, Germany
reg. cellulose	0.2	regenerated cellulose membrane filter	18407-47	cut out, autoclave	Sartorius Stedim Biotech GmbH, Göttingen, Germany
reg. cellulose	0.45	regenerated cellulose membrane filter	18406-47	cut out, autoclave	Sartorius Stedim Biotech GmbH, Göttingen, Germany
glass	-	cover slip circles, diameter 13 mm	CB00130RA1	autoclave	G. Menzl GmbH, Braunschweig, Germany
Thermanox™	-	Thermanox™ cover slips, diameter 13 mm	174950	-	Nunc GmbH, Roskilde, Denmark
Melafol™	-	Melafol™	502	cut out, autoclave	Melag oHG Medizintechnik, Berlin, Germany

PET	1	polyethylene terephthalate millicell hanging cell culture 6 well	PIRP30R48	cut out, autoclave	Millipore GmbH, Schwalbach, Germany
nylon	0.45	nylon WH PL 25 mm	HNWP02500	cut out, autoclave	Millipore GmbH, Schwalbach, Germany
collagen	-	collagen cell carrier	S051-026	-	PAA Laboratories GmbH, Pasching, Austria

side up on a microscope slide. To compensate the sometimes uneven surface of the support material, one drop of immersion oil (Merck KGaA, Darmstadt, Germany) was added on top of the cell side and a coverslip was glued to the microscope slide at the borders using ethyl acetate solved nitrocellulose lacquer (Alcina Nail Colour 10 ml, Dr. Kurt Wolff GmbH & Co. KG, Bielefeld, Germany). Samples were examined under a fluorescence microscope at an excitation wavelength of 459 nm. From each scaffold, 5-7 pictures were taken from randomly chosen areas using a microscope camera and corresponding software (Leica Application Suite^{TM} EZ, version 1.8.0, Leica Microsystems (Schweiz) AG, Heerbrugg, Switzerland). The pictures were analysed using a vector graphics programme. Therefore, a grid was laid over each picture, consisting of a big circle subdivided into four quadrants. Then additional small circles were laid over every stained nucleus. The number of marked nuclei per big circle and per quadrant were grouped and automatically counted, so that the total number of nuclei per circle (represented by the total number of small cycles per big circle) and the number of nuclei per quadrant (represented by the number of small circles per big circle quadrant) were accessible (Figure 3-1). To calculate the total number of cells per cell carrier, the size of the growth area on the cell carrier (59.294 mm² was divided by the size of the area of the big circle (0.058 mm²) and the dividend multiplied with the arithmetic mean of the number of stained nuclei per big circle for each material. This variable is further referred to as cell count. To assess the equal distribution of the cells on the different cell carrier materials, the coefficient of variation of the cell numbers of the four quadrants was calculated. For practicability reasons, this variable is further referred to as CVQ (coefficient of variation of the cell numbers between the quadrants). Medium samples were taken from every well and stored in 3 ml collection tubes at

–20 °C. Medium was later examined using radioimmunoassay (RIA) for E2. The amount of E2 was calculated per single scaffold. Additionally, the correlation between the three parameters cell count, ES and CVQ was described graphically and the Pearson product-moment correlation coefficient (r) and the production of ES per 1 000 cells calculated.

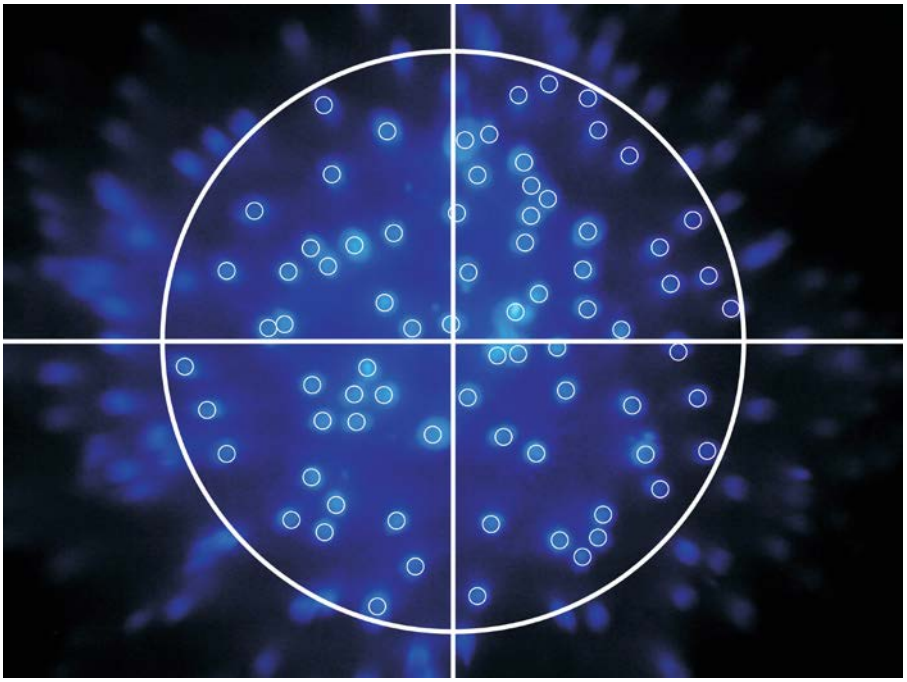

Figure 3-1 Fluorescence microscopy image of bovine granulosa cells grown on a polycarbonate membrane with a pore size of 0.2 µm after nuclear staining with DAPI processed with a vector graphic programme for the cell counting process. Region of interest subdivided in four quadrants.

3.1.2.4 The Perfusion Cell Culture System

The Tissue Factory™ comprises components directly in contact with medium and cells and additional technical devices. The medium was stored in 100 or 250 ml glass bottles closed with a special cap, the so called screw cap innovative™. The screw cap innovative™ has two holes, in which the tube for the medium supply and a syringe filter for inflow of filtered air as compensation for the consumed medium were placed. The medium supply bottles were placed in a small fridge, in which an additional hole was cut directly adjacent to the door to allow sterile placement of the medium tubes and closing of the door without squeezing the tubing. After leaving the fridge, the medium tubes were fixed in a peristaltic pump. Thereafter, the tubes were fitted to the inlet of

Table 3-2 Perfusion culture equipment

item	description	manufacturer
culture container	6 x 13 mm, 60/40/32 mm	minucells and minutissue Vertriebs GmbH, Bad Abbach, Germany
cover lid	acrylic glass cover lid	minucells and minutissue Vertriebs GmbH, Bad Abbach, Germany
fridge	MF-5M TK-280SB	Dometic Waeco International GmbH, Emsdetten, Germany
glass bottle	100 or 250 ml	Schott AG, Mainz, Germany
peristaltic pump	IsmatecTM IPC-N8	IDEX Health and Science GmbH, Wertheim-Mondfeld, Germany
pump tubing	silicone platin 2-stopper white-white	Postnova Analytics GmbH, Landsberg/Lech, Germany
screw cap	screw cap innovative™	minucells and minutissue Vertriebs GmbH, Bad Abbach, Germany
syringe filter	Rotilabo™ syringe filter 0.22 µm, PVDF	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
thermo plate	ME 12501	MEDAX Nagel GmbH, Kiel, Germany
tension rings	minusheet™ 13 mm	minucells and minutissue Vertriebs GmbH, Bad Abbach, Germany
tube connectors	y-barbed 1/16''	Postnova Analytics GmbH, Landsberg/Lech, Germany
tube connectors	male and female luer lock 1/16''	Postnova Analytics GmbH, Landsberg/Lech, Germany
tubes	Tygon™ SI Silicone 3350 platinum	Postnova Analytics GmbH, Landsberg/Lech, Germany

the perfusion chambers near their bottom. The perfusion chambers were placed on a thermo plate that was covered with an acrylic glass lid for optimising temperature stability. The medium flew out over tubes connected near to the top side of the perfusion containers and ended in the collection tubes. As collection tubes, plastic tubes for RIA samples were used, with a hole in the cap to fix the tubing. The tube material was Tygon SI platinum™ with an inner diameter of 1.02 mm and an outer diameter of 2.70 mm. To connect the different tubes and the tubes with the perfusion chambers, different connectors, as listed in the equipment list (Table 3-2), were used.

The support material for the cells must have a round shape and a diameter of 13 mm to be fixed in the tension rings. These so assembled cell carriers are vertically placed in the perfusion chamber, whereby 6 cell carriers fitted into one chamber. The perfusion culture apparatus and details are shown in Figure 3-2. A list of the perfusion culture equipment is given in Table 3-2.

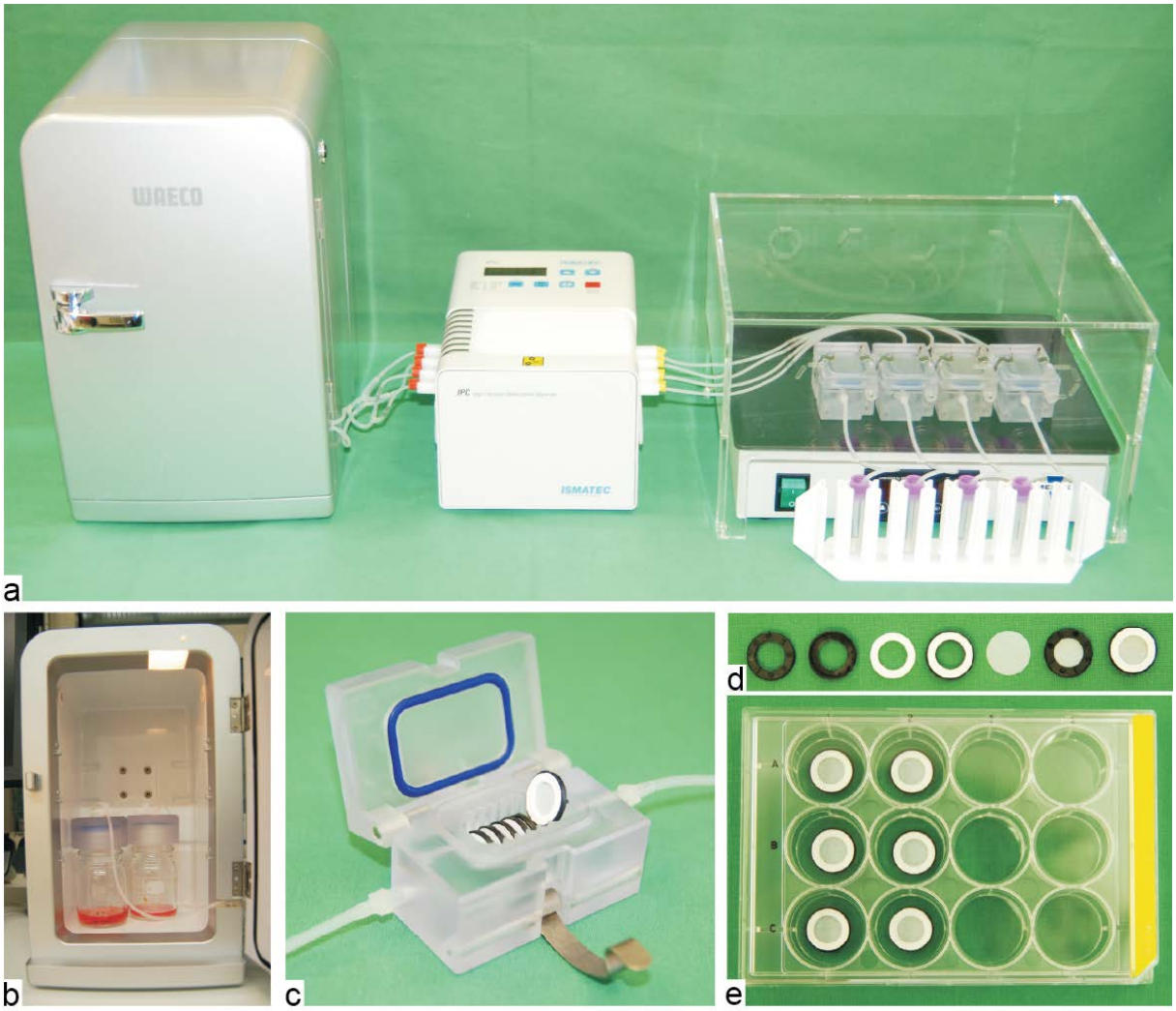

Figure 3-2 The Tissue Factory™ (a) complete perfusion apparatus (b) medium cooler with supply bottles (c) perfusion chamber with cell carriers (d) detailed aspects of the tissue carriers and their assembly (e) preculture of tissue carriers in 12-well plates.

3.1.2.5 Comparison of Static to Perfusion Cell Culture

To obtain comparable conditions, cells for static and dynamic culture were handled exactly the same except the way of medium supply. One part of the cell suspension, containing 2 million cells, was centrifuged at 500 g for 5 minutes and the supernatant was discharged. The cell pellet was resuspended in 500 µl PBS and transferred to 2 ml Eppendorf Safe Lock^{TM} tubes. After two more washing steps using 500 µl PBS and centrifugation at 500 g for 5 minutes and discharge of the supernatant, the cell pellet was immediately frozen at –80 °C for later RNA extraction. For each bGC class (I, II and III), 12 cell carriers mounted with polycarbonate membranes (pore size 0.2 µm) were seeded with 100 000 cells. The cell carriers were precultured for 56 ± 1 hours in static preculture in 12-well plates with 960 µl standard medium under humidified atmosphere without CO_2 at 37.5 °C to allow cell attachment. The duration of the static preculture was slightly altered by the time when the isolation of the GC was finished, because the point of time where the culture was further processed was fixed. After the preculture period, the carriers were taken out of the 12-well plates and washed in prewarmed PBS three times. A mixed sample of the preculture medium was taken from every cell class and frozen at –20 °C for later radioimmunoassay (RIA) for total estrogens (ES, comprising E2 and estrone) and P4.

Six carriers of each cell class were put into new 24-well plates with 833.3 µl prewarmed standard medium. The 833.3 µl medium in the static culture were changed every 8 hours and the used medium was collected in collection tubes and frozen at –20 °C for later RIA for total estrogens (ES) and P4.

For the perfusion culture, the perfusion apparatus was prepared and the tubes and supply bottle filled with medium. All three perfusion chambers were filled with prewarmed medium and 6 washed carriers of the same bGC class were placed in each perfusion container. The containers were closed and the system connected to the medium cooler, the peristaltic pump and the warming plate with lid. Then the perfusion was started with a flow rate of 5 ml per 8 hours per container. For the medium collection in the perfusion system, the perfusate was collected from each container separately in collection tubes. These tubes were changed every 8 hours and the tubes with used medium frozen at –20 °C for later RIA for ES and P4. For data analysis, the amounts of ES and P4 were calculated per 6 cell carriers per 8 hours from both systems. Additionally, the amount of ES and P4 produced in the last interval was

normalised with the cell count and the amount of ES and P4 produced per 1 000 cells calculated, as well as the ratio of ES to P4 of the values produced in the last culture interval.

Both culture types were maintained for 64 hours. After that time, one cell carrier of every cell class and every culture method was examined for cell count using fluorescence microscopy as described before in chapter 3.1.2.3. For data analysis, the cell count was calculated as cell count per 6 cell carriers. The remaining 5 cell carriers were used to extract the cells' RNA. Therefore, the carriers were washed thoroughly in PBS and the polycarbonate membrane was separated from the tension rings. The membranes with the cells were put upside down into 3 cm Petri dishes containing prewarmed trypsin solution (1:250, PAA Laboratories GmbH, Pasching, Austria) and incubated at 37.5 °C for 20 min. After the incubation, the carriers were scraped 5 times with a cell scraper on the cell side to detach remaining cells. The carriers and the scraper were rinsed using PBS (PAA Laboratories GmbH, Pasching, Austria) after scraping. The trypsin-PBS-cell solution was transferred into an 11 ml centrifuge tube and immediately centrifuged in a precooled centrifuge at 500 g for 5 minutes at 2 °C. The supernatant was discharged and the pellet resuspended in PBS. The PBS-cell solution was put into 2 ml Eppendorf Safe Lock^{TM} tubes and centrifuged again at 1000 g for 5 minutes at 2 °C. The supernatant was discharged and the pellet frozen at –80 °C. This experiment was repeated four times.

3.1.3 Influence of Agents Possibly Linking Nutrition to Reproduction on Bovine Granulosa Cell Perfusion Cultures

3.1.3.1 Investigations on the Influence of Urea on Granulosa Cells

To investigate the influence of urea in a concentration as it occurs during negative energy balance post partum (5 mM, [161–163]) on bGCs, only bGC of class I and III were included in the trial. One part of the cell suspension of bGC I and III, containing 2 million cells, was centrifuged at 500 g for 5 minutes and the supernatant discharged. The cell pellet was resuspended in 500 µl PBS and transferred to 2 ml Eppendorf safe lock tubes. After two more washing steps using 500 µl PBS and centrifugation at 500 g for 5 minutes and discharge of the supernatant, the cell pellet was immediately frozen at –80 °C for later RNA extraction. From the remaining cell suspension, 100 000 cells of each class were seeded on polycarbonate membranes with a pore size of 0.2 µm,

and 12 carriers were used per class and experiment. The seeding and preculture procedure were the same as described before in the experiment for the comparison of static and perfusion culture, whereby the duration of the static preculture was 55 ± 1 hours here. After the static preculture, the cell carriers were distributed in four perfusion containers in groups of six of the same cell class as described before, so that, up to this moment, two identical containers were present for each cell class. One of the two containers of every cell class was connected to a medium supply only containing standard medium, the other to a medium supply which could be switched between standard medium and standard medium containing urea (Figure 3-4). The perfusion was started with a flow of 5 ml medium per 8 hours per perfusion chamber. In the first 8 hours of perfusion culture (time 0 - 8 hours), both containers of each cell class were only supplied with standard medium. Then the medium collection tubes were changed and the medium supply was switched for one of the two perfusion containers of the same cell class to standard medium containing urea. This kind of medium supply was maintained for the following 10 hours (time 8 - 18 hours). Assuming an ideal medium intermixture, this resulted in a continuous rise of the urea concentration in the perfusion container supplied with standard medium with urea in the first 8 hours (time 8 - 16 hours). After this time, the standard medium was completely replaced by standard medium with urea, which resulted in a concentration plateau of 5 mM for the following 2 hours (16 - 18 hours). Then the medium supply was switched back to a supply only with standard medium, resulting in a continuous decline of urea concentration in the perfusion container.

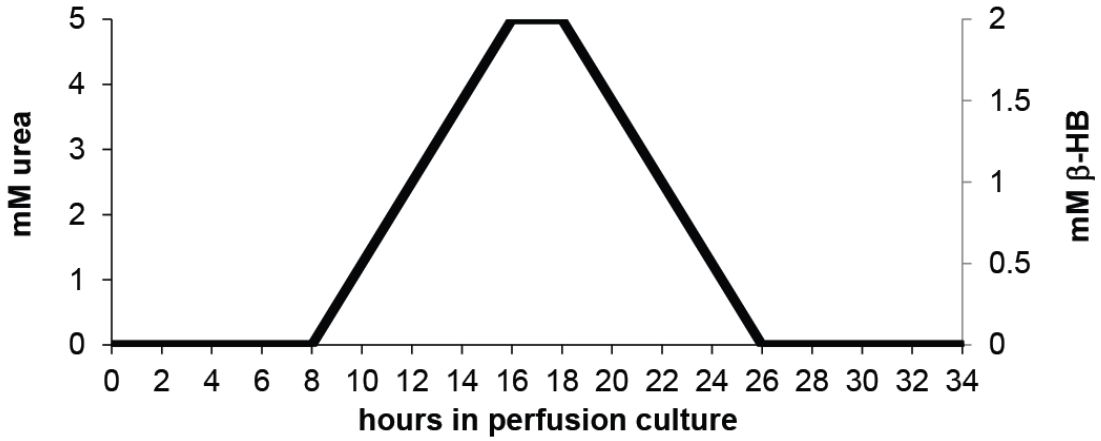

Figure 3-3 Concentration of urea and β-hydroxybutyric acid (β-HB) in the perfused culture perturbed with metabolites.

The supply with standard medium was maintained for the following 18 hours (time 18 - 34 hours). The thus reached concentration of the metabolite is shown in Figure 3-3. To analyse the time-related influence of urea, medium samples were taken every two hours by changing the medium collection tubes every two hours in the time frame 8 - 22 hours. After that, one more 8 hours sample was taken between time 24 and 32 and one more 2 hours sample was taken between time 32 and 34 hours. The perfusion culture was stopped after 34 hours. All medium samples were immediately frozen at – 20 °C for later RIA for ES and P4. For the data analysis, the amounts of ES and P4 were calculated per 6 cell carriers per 2 hours from both cultures. Additionally, the amount of ES and P4 produced in the last interval was normalised with the cell count and the amount of ES and P4 produced per 1 000 cells calculated, as well as the ratio of ES to P4 of the values produced in the last culture interval. One cell carrier of every perfusion container was examined for cell count using fluorescence microscopy as described before in chapter 3.1.2.3, the cells of the other 5 cell carriers were isolated for later RNA extraction as described before in chapter 3.1.2.5. This experiment was repeated four times.

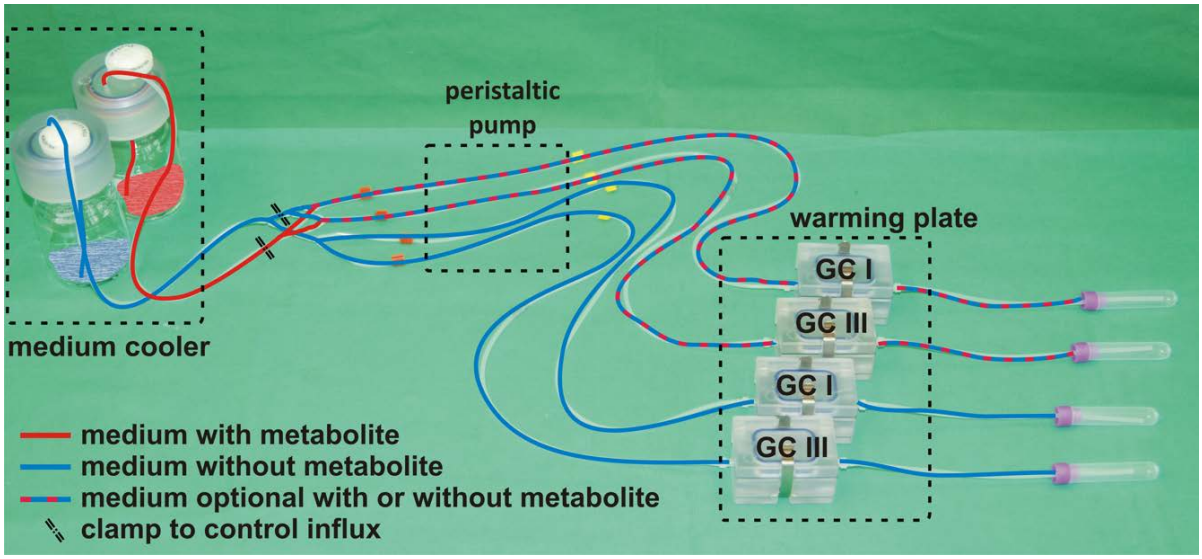

Figure 3-4 Schematic set of the perfusion apparatus for the investigation of the influence of urea and β-HB on bGC function.

3.1.3.2 Investigations on the Influence of β-Hydroxybutyric Acid on Granulosa Cells

To investigate the influence of β-hydroxybutyric acid (β-HB) in a concentration as it occurs during negative energy balance in subketotic cows (2 mM, threshold to distinguish between subketotic and healthy cows is 1.4 mM [164,165]) on bGC, exactly

the same experiment was conducted as described before for the influence of urea on bGC, apart from replacing 5 mM of urea with 2 mM of β-hydroxybutyric acid. The concentration curve of β-HB is shown in Figure 3-3. This experiment was repeated four times as well.

3.2 Real-time RT-PCR for Key Receptors and Enzymes Linked to Steroidogenesis

3.2.1 Extraction of RNA from bGCs

The RNA from the frozen cell pellets was extracted using a Quiagen RNeasy™ mini kit, comprising all reagents and consumables for the extraction except β-mercapto ethanol (Quiagen GmbH, Hilden, Germany). All steps were performed at room temperature; centrifugation was performed at 23 °C, as recommended by the manufacturer. First, the cell samples were thawed. Then, 350 µl buffer RLT containing 1 % β-mercapto ethanol (14.3 M, Sigma Aldrich Co. LLC., Taufkirchen, Germany) were added and the mixture pipetted thoroughly up and down to disrupt the cells. The lysate was then vortexed for 1 minute. Then, 350 µl 70 % ethanol were added and thoroughly mixed by pipetting up and down. The sample was transferred to a RNeasy™ MinElute™ spin column placed in a 2 ml collection tube and then centrifuged at 10 000 g for 15 seconds. The flow through was discharged. 350 µl buffer RW1 were added to the spin column and a centrifugation step at 10 000 g for 15 seconds followed. The flow through was discharged. For the digestion of DNA remains, a DNAse solution was prepared using 10 µl DNAse I stock solution and 70 µl buffer RDD for each sample. The 80 µl of DNAse solution were added directly to the RNeasy™ MinElute™ spin column membrane and left for 15 minutes at room temperature on the bench top. Then, 50 µl buffer RW1 were added followed by a centrifugation at 10 000 g for 15 seconds. After this step, flow through and collection tube were discharged. The spin column was placed in a new 2 ml collection tube and 500 µl Buffer RPE were added. After discarding the flow through, 500 µl 80 % ethanol were added to the spin column. After a centrifugation step at 10 000 g for 2 minutes collection tube and flow through were discarded. The spin column was placed in a new 2 ml collection tube. This combination was centrifuged at 13 000 g for 5 min with open lid. After putting the spin column in a new 1.5 ml collection tube, 14 µl of RNAse free water were added directly to the centre of the spin column membrane. With the last centrifugation step at 13 000 g for 1

minute, the RNA sample was eluted and collected in the 1.5 ml collection tube. The spin column was discarded and the RNA concentration in the samples measured using a photometer. Therefore, 1 µl of RNA sample was diluted in 9 µl of RNAse free water, mixed thoroughly and transferred to an UVette^{TM}, which was inserted to the photometer for measurement of RNA content. The photometric measurement of the RNA content was repeated five times and the mean calculated and used as basis for calculating the RNA content for the reverse transcription.

3.2.2 Reverse Transcription of RNA into cDNA

For the reverse transcription, the RNA samples were diluted with RNAse free water to a concentration of 100 ng in 10 µl. The reverse transcription master mix contained for each sample 12 µl $MgCl_2$ solution (Applied Biosystems, Warrington, UK), 6µl PCR buffer (10x PCR Gold^{TM} buffer without $MgCl_2$, Applied Biosystems, Foster City, USA), 24 µl dNTP mix (Applied Biosystems, Warrington, UK), 3 µl random hexamers (Applied Biosystems, Foster City, USA), 3 µl RNAseinhibitor (RiboLock RNAse inhibitor, 40 u/µl, Fermentas GmbH, St. Leon-Rot, Germany) and 3 µl reverse transcriptase (MultiScribe Reverse Transcriptase^{TM}, 50 U/µl, Applied Biosystems, Foster City, USA). The reverse transcription was performed using 51 µl reverse transcription master mix and 9 µl diluted RNA sample. PCR steps included 8 minutes at 21 °C, 15 minutes at 42 °C, 5 minutes at 99 °C and 5 minutes at 5 °C, ending with 4 °C forever. The so produced cDNA samples were stored frozen at –20 °C.

3.2.3 Real-time RT-PCR detecting Aromatase, 3β-Hydroxysteroid Dehydrogenase, FSH-Receptor and LH-Receptor

The primers that were used for the real-time RT-PCR are described in table 3 and were purchased from Eurogentec GmbH, Köln, Germany. For the real-time PCR master mix, 10 µl Sybr Green^{TM} solution (IQ SYBR Green Supermix, Bio-Rad Laboratories, Inc., Munich, Germany) were mixed with 0.6 µl forward and 0.6 µl reverse primer solution (5 pmol) and 7.8 µl DNAse free water for every sample. Each sample was done in duplicate. In the wells of the PCR plate, 1 µl of cDNA was added to 19 µl of real-time PCR master mix. On each plate, the genes of interest and the housekeeping gene β-actin were amplified together for the examined samples. The real-time cycle began with 3 min at 95 °C, step #2 was 10 seconds at 95 °C, step #3 1 minute at 60 °C, followed by the plate read. Then, step #2, #3 and the plate read were repeated 39

times. After another 10 seconds at 95 °C, the melt curve was conducted from 65 to 95 °C, in steps of 0.5 °C for 5 seconds with following plate read. A standard curve to determine the efficiency of the primers was conducted by pooling the cDNA samples and making a four step dilution comprising 1:1, 1:10, 1:100 and 1:1000 dilution of the pooled cDNA sample in DNAse free water. The calculated efficiency for each primer is shown in table 3. For the real time data analysis, plates containing the same gene of interest were united to one gene study. The relative gene expression corrected for the efficiency of the reaction and for the expression of the housekeeping gene β-actin was calculated using the BioRad™ software (BioRad™ CFX Manager, Version 1.6.541.1028, 2008, Bio Rad Laboratories, Inc., Munich, Germany). The relative expression was scaled to the lowest expression.

The relative quantity $\Delta C_{(t)}$ for any sample (GOI) was calculated with this formula:

$$\text{Relative Quantity}(RQ)_{sample\ (GOI)} = E_{GOI}{}^{C_{t(min)} - C_{t(sample)}}$$

E = efficiency of primer and probe set. The efficiency is calculated with the formula (% efficiency × 0.01) + 1, where 100% efficiency = 2.

$C_{t(min)}$ = average C_t for the sample with the lowest average C_t for *GOI*

$C_{t(sample)}$ = average C_t for the sample

GOI = gene of interest (one target)

The normalised expression $\Delta\Delta C_{(t)}$ is the relative quantity of the target (gene) normalized to the quantities of the reference targets (housekeeping gene). The normalised expression was calculated using the following formula:

$$\text{normalised expression } \Delta\Delta C_{(t)sample(GOI)} = \frac{RQ_{sample(GOI)}}{RQ_{sample(Ref)}}$$

RQ = relative quantity of a sample

Ref = reference target in an experiment that includes one or more reference targets in each sample (housekeeping gene).

When the experiment does not include controls, the normalized expression (NE) for each target (gene) is scaled by dividing the expression level of each sample by the lowest level of expression in all samples. The software sets the lowest level of expression to a value of 1, and re-scales all the sample expression levels. The lowest scaling was calculated by the following formula:

$$scaled\ normalised\ expression = \frac{normalised\ expression_{sample(GOI)}}{normalised\ expression_{lowest\ sample(GOI)}}\ .$$

Table 3-3 Real-time RT-PCR Primers. No efficiency was calculated for the LH-receptor primers, because LH-receptor expression was only detected in few samples and the value was thus statistically not considered. (for = forward, rev = reverse)

primer	sequence	origin	efficiency
ß-actin for	TAT-TGC-TGC-GCT-CGT-GGT-CG	Shenavai 2012 [166]	92.3 %
ß-actin rev	TGA-CGA-TGC-CGT-GCT-CAA-TGG		
aromatase for	GCG-AAA-TCT-ATG-CTG-TCT-TC	own laboratory design	100.7 %
aromatase rev	GCT-CTC-CTT-CTC-AAA-CCA-G		
3ß-HSD for	TGT-TGG-TGG-AGG-AGA-AGG-ATC-TG	Nimz et al. 2009 [167]	81.5 %
3ß-HSD rev	GCA-TTC-CTG-ACG-TCA-ATG-ACA-GAG		
FSH-receptor for	AGC-CCC-TTG-TCA-CAA-CTC-TAT-GTC	Luo and Wiltbank 2006 [168]	92.3 %
FSH-receptor rev	GTT-CCT-CAC-CGT-GAG-GTA-GAT-GT		
LH-receptor for	TCC-CTC-GGT-TAA-AAT-ACC-TAA-GC	Robert et al. 2003 [169]	-
LH-receptor rev	GTA-GCC-CAT-AAT-GTC-TTC-ACA-GG		

3.3 Radioimmunoassay

3.3.1 Collection, Preparation and Storage of Media Samples for Radioimmunoassays

Spent medium was collected in 3 ml tubes and immediately frozen at –20 °C.

3.3.2 Radioimmunoassay for 17β-Estradiol

The medium from the investigation of different cell carrier materials was analysed for 17β-estradiol using radioimmunoassay on the basis of a sequential assay. The samples were tested in the hormone analysis laboratory of the Clinic of Obstetrics, Gynaecology and Andrology of Large and Small Animals of the Justus-Liebig-University Giessen. The method used was established by Hoffmann and co-workers [170] and validated in this laboratory. Due to high estradiol-17-β levels in the samples, they were diluted 1:10 before analysis. 250 µl of diluted medium sample were twice extracted using 2.5 ml toluene (for analysis, Merck KGaA, Darmstadt, Germany). Therefore, sample and solvent were mixed thoroughly for 15 minutes using a rotating mixer. Then, the sample was spinned shortly and the aqueous phase frozen off using an alcohol dry ice bath. The supernatant was transferred into a RIA test tube and the solvent evaporated using a vortex evaporator. After the extraction, the samples were transferred into 100 µl BSA phosphate buffer and ready to start the

radioimmunoassay. 400 µl diluted antiserum were added to the samples and incubated at 4 °C overnight. The used antiserum was GI-E2βI and produced by repeated immunisation of a rabbit with 1,3,5,(10)-estratriene-3,17β-diol-6-O-carboxy-methyloxim-BSA. The final dilution of the antiserum was 1:17 000. The association constant of the antibody was 11.19 × 10^9 l/mol. The cross reactivity was 1.3 % for estrone, 0.68 % for estriol and below 0.01 % for androstenedione, cortisol, DHEA, pregnenolone, progesterone, testosterone and 5 α-dihydro-testosterone. On next day, 100 µl ^{3}H-labeled estradiol (approximately 9500 cpm) were added, mixed thoroughly and incubated for 45 minutes at 4 °C. The so started competitive reaction between ^{3}H-labeled estradiol and estradiol from the sample for the binding to the antibody was stopped after 45 minutes by binding left free hormone with 200 µl ice-cold charcoal suspension. The charcoal sample mixture was mixed thoroughly and incubated for 30 minutes at 4 °C. It was then centrifuged for 15 minutes at 2220 g at 0 °C. The supernatant from this centrifugation was mixed with 3 ml scintillator and after 10 minutes rest the sample was transferred in the Coulter counter for measurement of the ^{3}H beats. The standard curve ranged between 0.5 and 32 pg per 100 µl. A blank reagent sample, bull plasma and two positive controls diluted in bull plasma (5 and 15 pg) were used as control. The intraassay variation coefficient and the interassay variation coefficient – averaged from former doctoral theses using the same test in the same laboratory, because too few tests were performed for this work to calculate these values - were 11.9 % and 13.9 %, respectively, whereby the reagent blank value in these works was a mean of 1.76 ± 4.05 pg [171,172].

3.3.3 Radioimmunoassay for Total Estrogens

The medium of the investigation of the comparison between static and perfusion culture and of the influence of metabolites on bGC function was analysed for its content of total estrogens (comprising estrone and 17β-estradiol) using radioimmunoassay on the basis of an equilibrium reaction. The samples were tested in the hormone analysis laboratory of the Clinic of Obstetrics, Gynaecology and Andrology of Large and Small Animals of the Justus-Liebig-University Giessen. 12.5, 25, 50 or 100µl of medium sample were used for extraction, depending on the expected hormone content. The medium sample was twice extracted using 2.5 ml toluene (for analysis, Merck KGaA, Darmstadt, Germany). Therefore, sample and solvent were mixed thoroughly for 15 minutes using a rotating mixer. Then, the sample

was spinned shortly and the aqueous phase frozen off using an alcohol dry ice bath. The supernatant was transferred into a RIA test tube and the solvent evaporated using a vortex evaporator. After the extraction, the samples were transferred into 100 µl BSA phosphate buffer and ready to start the radioimmunoassay. 400 µl of diluted antiserum and 100 µl of ^{3}H-labeled 17β-estradiol (approximately 9500 cpm) were added and mixed for 20 min at 37 °C. Thereafter, the mixture was incubated in an ice bath for 60 minutes at 4 °C. The used antiserum was GI E1-Ges. and produced by repeated immunisation of a rabbit with 17β-estradiol-hemisuccinat-BSA as antigen. The final dilution of the antiserum was 1:80 000. The antibody reactivity was 100 % for estrone and 80 % for 17β-estradiol, the cross reactivity was below 0.1 % for androstenedione and testosterone. The so started competitive reaction between ^{3}H-labeled estradiol and estradiol and estrone from the sample for the binding to the antibody was stopped by binding left free hormone with 200 µl ice-cold charcoal suspension. The charcoal sample mixture was mixed thoroughly and incubated for 10 minutes at 4 °C. It was then centrifuged for 15 minutes at 2220 g at 0 °C. The supernatant from this centrifugation was mixed with 3 ml scintillator and after 10 minutes rest the sample was transferred in the coulter counter for measurement of the ^{3}H beats. The standard curve ranged between 5.45 and 871.62 pg per 100 µl. A blank reagent sample and two positive controls, one containing estrone and the other plasma from a pregnant cow, both diluted in bull plasma, were used as control. The interassay variation coefficient was 28.1 %. The reagent blank value was a mean of 7.59 ± 3.26 pg.

3.3.4 Radioimmunoassay for Progesterone

The medium of the investigation of the comparison between static and perfusion culture and of the influence of metabolites on bGC function was analysed for progesterone using radioimmunoassay on the basis of an equilibrium reaction. The samples were tested in the hormone analysis laboratory of the Clinic of Obstetrics, Gynaecology and Andrology of Large and Small Animals of the Justus-Liebig-University Giessen after the method described by Hoffmann and co-workers in 1973 [173]. 100 µl medium sample were twice extracted using 2.0 ml hexane (for analysis, Merck). Therefore, sample and solvent were mixed thoroughly for 15 minutes using a rotating mixer. Then, the sample was spinned shortly and the aqueous phase frozen off using an alcohol dry ice bath. The supernatant was transferred into a RIA test tube and the solvent evaporated using a vortex evaporator.

After the extraction, the samples were transferred into 100 µl BSA phosphate buffer and ready to start the radioimmunoassay. 400 µl diluted antiserum and 100 µl of ^{3}H-labeled progesterone (approximately 7200 cpm) were added, mixed thoroughly for 30 seconds and then preincubated for 20 minutes in a water bath at 37 °C. Thereafter, the sample was incubated for 60 minutes at 4 °C. The antiserum used was GI P4 IV and produced by repeated immunisation of a rabbit with 11α-OH-progesterone-HS-RSA. The final dilution of the antiserum was 1:50 000. The association constant of the antibody was 3.42×10^{9} l/mol. The cross reactivity was below 0.01 % for androstenedione, DHEA, E2, estron, cortisol and 17α-hydroxy-pregnenolone, 0.37 % for testosterone, 0.49 % for 17α-hydroxy-progesterone and 0.69 % for pregnenolone. The competitive reaction between ^{3}H-labelled progesterone and progesterone from the sample for the binding to the antibody was then stopped by binding left free hormone with 200 µl ice-cold charcoal suspension. The charcoal sample mixture was mixed thoroughly and incubated for 10 minutes at 0 °C. It was then centrifuged for 15 minutes at 2 220 g and 0 °C. The supernatant from this centrifugation was mixed with 3 ml scintillator and after 10 minutes rest the sample was transferred in the coulter counter for measurement of the ^{3}H beats. The standard curve ranged between 12.58 and 805.02 pg per 100 µl. A blank reagent sample and two samples of cow plasma, one with known low and one with known high progesterone value, were used as control. The interassay variation coefficient was 7.1 %. The intraassay variation coefficient, averaged from former doctoral theses using the same test in the same laboratory, was 5.7 % [171,172]. The reagent blank value was a mean of 17.72 ± 5.81 pg.

3.4 Consumables and Laboratory Equipment

3.4.1 Consumables

Table 3-4 Consumables

item	description	manufacturer
desinfectant	Meliseptol™ rapid	B.Braun Melsungen AG, Melsungen, Germany
glass bottles	25/100/200/500 ml	Schott AG, Mainz, Germany
gloves	nitrile gloves N-DEX™	Showa Best Gloves, Paris, France
Melafol™	transparent sterilisation package	Melag oHG Medizintechnik, Bremen, Germany
microscope slide	76 x 26, cleaned, ready to use	IDL GmbH und Co. KG, Nidderau, Germany
needle	0.45 x 2 mm BL/LB, 26 G x 1''	B.Braun Melsungen AG, Melsungen, Germany
Pasteur pipettes	ISO 9001 certified	Hirschmann Laborgeräte, Eberstadt, Germany
PCR pipet tips	10/200/1000 µl	Nerbe Plus GmbH
Petri dishes	Falcon™ 3001	Becton Dickinson, Franklin Lakes, USA
pipet tips	10/200 µl	Sarstedt, Nümbrecht, Germany
pipet tips	1000 µl	Ratiolab GmbH, Dreieich, Germany
safe lock tubes	2 ml	Eppendorf AG, Hambug, Germany
screw cap tubes	50 ml BD Falcon™ tubes	BD Biosciences, New Jersey, USA
serological pipettes	5/10 ml	SPL Life Sciences, Pocheon, Korea
serological pipettes	25 ml	Jet Biofil, Guangzhou, China
syringe	Injekt™, 10 ml, Luer Solo	B.Braun Melsungen AG, Melsungen, Germany
Uvette™	centre height 8.5 mm	Eppendorf AG, Hamburg, Germany

3.4.2 Laboratory Equipment

Table 3-5 Laboratory equipment

item	description	manufacturer
aspiration pump	Vacusafe™ comfort	IBS integra bioscience, Fernwald, Germany
autoclave	Systec 3850 ELC	Systec GmbH Labortechnik, Wettenberg, Germany
balance	Pj 300	Mettler-Toledo GmbH, Gießen, Germany
centrifuge	5702 RH	Eppendorf AG, Hamburg, Germany
centrifuge	Mikro 22 R	Hettich, Tuttlingen, Germany
diswasher	Mielabor 67783 Multitronic	Miele & Cie. KG, Gütersloh, Germany
electronic dispenser	Pipetboy™ comfort	IBS integra bioscience, Fernwald, Germany
fluorescence filter	A 513516	Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany
fluorescence microscope	Leitz Orthoplan™	Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany
haemocytometer	Modell Neubauer, 0.0025 mm^2, depth 0.100 mm	Labor Optik GmbH, Bad Homburg, Germany
incubator	Heracell™	Heraeus, Hanau, Germany
laminar flow cabinet	LaminAir™ HA 2448 GS	Heraeus, Hanau, Germany
microscope	CME	Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany
microscope	DM IL	Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany
microscope camera	EC3	Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany
pH-meter	pH-meter with thermometer	MAGV Laborbedarf + Laborgeräte, Rabenau-Londorf, Germany
photometer	Bio Photometer	Eppendorf AG, Hamburg, Germany
pipet	Reference™, adjustable pipet, 1000, 200 and 50 µl	Eppendorf AG, Hamburg, Germany

real-time thermocycler	BioRad C1000 Thermal Cycler, CFX real-time system	Bio-Rad Laboratories, Inc., Munich, Germany
sterilising oven	UL 80	Memmert GmbH & Co. KG, Büchenbach, Germany
thermocycler	T Personal	Biometra GmbH, Göttingen, Germany
thermocycler	T 1	Biometra GmbH, Göttingen, Germany
vortexer	Reax^{TM} control	Heidolph, Schwabach, Germany
water purification system	Milli Q^{TM}	Millipore GmbH, Schwalbach, Germany
water bath	Type 3048	Köttermann, Hänigsen, Germany

3.5 Statistical Methods

The statistical analysis of the collected data was done in cooperation with the Workgroup for Biomathematics and Data Processing of the Faculty of Veterinary Medicine of the Justus-Liebig University Giessen. The statistical software used for the data analysis was BMDP/Dynamic, Release 8.1 (Statistical Solutions Ltd., Cork, Ireland). Some variables were logarithmically transformed (to the basis 10, $\log_{10}$) for the statistical analysis due to their distribution or high variability. The value zero had to be replaced for the $\log_{10}$ transformation, and for the relative expression, it was replaced with 0.5 (the lowest occurring value was 1, because the data were scaled to the lowest), and for ES, it was replaced with 0.08 pg, half of the analytic detection threshold ($\bar{x}_{\alpha(\text{blank value})} + 3 \times \text{standard deviation}$).

For the statistical analysis of the influence of the carrier material on E2, cell count and CVQ, the parameter E2 and CVQ were $\log_{10}$ transformed due to their high variability. Then, a one-factorial analysis of variance with repeated measurements was conducted for each parameter with respect to the cell carrier material. Additionally, a pairwise comparison of the results after Student-Newman-Keuls was calculated.

For the statistical analysis of the influence of the static and the perfused cell culture on the production of ES and P4, the cell count and the relative expression of FSH-R, LH-R, P450arom and 3β-HSD, all parameters were $\log_{10}$ transformed. A two-factorial analysis of variance with repeated measurements for the parameters cell count and

relative expression of FSH-R, LH-R, P450arom and 3β-HSD with respect to the factors cell class and system, and a three-factorial analysis of variance with repeated measurements for the parameters ES and P4 with respect to the factors cell class, system and culture time was conducted.

For the statistical analysis of the influence of a metabolite (urea and 3β-HSD) on the production of ES and P4, the cell count and the relative expression of FSH-R, LH-R, P450arom and 3β-HSD, all parameters were $\log_{10}$ transformed. Then, a two-factorial analysis of variance with repeated measurements for the parameters cell count and relative expression of FSH-R, LH-R, P450arom and 3β-HSD with respect to the factors cell class and metabolite, and a three-factorial analysis of variance with repeated measurements for the parameters ES and P4 with respect to the factors cell class, metabolite and culture time was conducted.

For the data description, the arithmetic mean and the standard deviation ($\bar{x}_a \pm \mathrm{SD}$) were calculated for normally distributed variables. If the distribution of a variable was skewed to the right, the data were $\log_{10}$ transformed and the geometric mean and the dispersion factor ($\bar{x}_g$, DF) with its positive and negative aberration were used for the data description [174,175].

dispersion factor DF = $10^{s_{\lg(x)}}$

with $s_{\lg(x)}$= standard deviation of the $\log_{10}$ transformed results

positive aberration = $\bar{x}_g \times (DF - 1)\mathrm{DF}$

negative aberration = $\bar{x}_g \times \left(1 - \frac{1}{\mathrm{DF}}\right)$.

4 Results

4.1 Establishment of the Bovine Granulosa Cell Perfusion Culture

4.1.1 Determination of HEPES-Buffer Concentration for the CO_2 Independent Culture System

The pH measured at different concentrations of HEPES buffer in the culture medium from both experiments and the arithmetic mean of the two experiments are presented in Figure 4-1. With increasing HEPES buffer concentration, the medium pH decreased. The measured pH values ranged between 7.28 and 7.63.

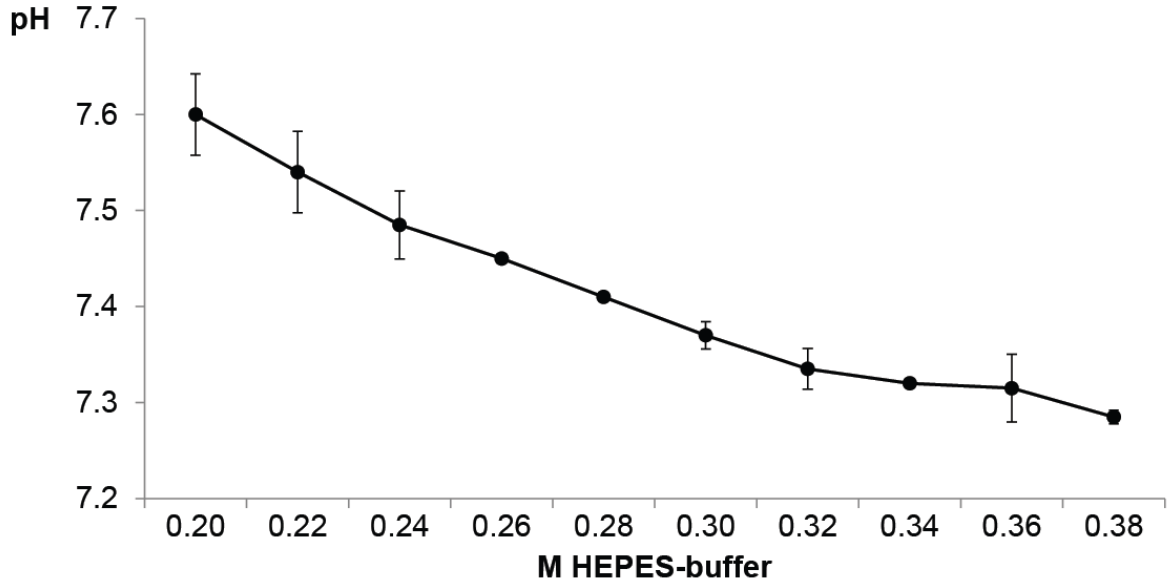

Figure 4-1 Arithmetic mean and standard deviation of the medium pH at different concentrations of HEPES buffer.

4.1.2 Isolation of Bovine Granulosa Cells

For each experiment, 24 ± 6 ovaries were collected at the local abattoir and 115 ± 36 small, 15 ± 3 medium and 9 ± 2 large follicles punctured. From the number of small follicles per experiment, an average of (22.48 ± 6.82) × 10^6 cells could be isolated, from medium follicles (21.29 ± 5.05) × 10^6 cells and from large follicles (30.85 ± 10.56) × 10^6 cells. Viability of cells from small follicles was 23.88 ± 6.64 %, of cells from medium follicles 37.19 ± 9.79 % and of cells from large follicles 27.28 ± 9.04 %. The average number of vital cells that could be isolated per follicle was 49 334 ± 23 208 for small follicles, 559 726 ± 258 296 for medium follicles and 1 012 243 ± 487 882 for large

follicles. In total, 362 ovaries were used for this study and 1722 small, 108 medium and 127 large follicles punctured.

4.1.3 Investigation in Different Materials Concerning their Applicability as Granulosa Cell Carriers

4.1.3.1 Cell Count on Different Cell Carrier Materials

The distribution of the variable cell count was skewed to the right, consequently the geometric mean and the dispersion factor were calculated for the data description ($\bar{x}_g$, DF). For better clarity, the geometric mean and the results of the three experimental repeats are shown in Figure 4-2.

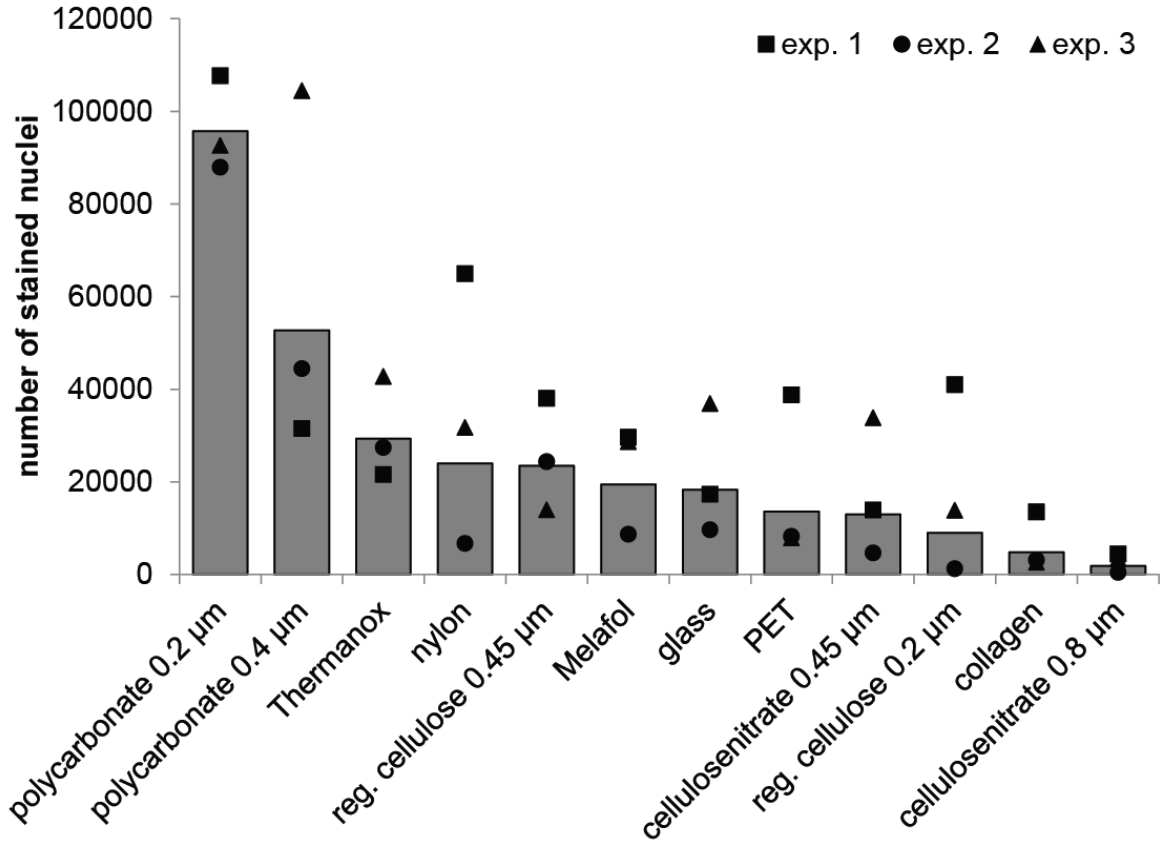

Figure 4-2 Single experimental results and geometric mean of the number stained nuclei per scaffold on the 12 different cell carrier materials. Pairs of columns labelled with the same letter are not significantly different from each other, pairs of columns labelled without a common letter are significantly different from each other.

Polycarbonate with a pore size of 0.2 µm (polycarbonate 0.2) had the highest cell count (95 714, 1.11), followed by polycarbonate with a pore size of 0.4 µm (polycarbonate 0.4, 52 677, 1.85), Thermanox™ (29 341, 1.42), nylon (23 963, 3.20), regenerated cellulose with a pore size of 0.45 µm (reg. cellulose 0.45, 23 468, 1.65), Melafol™ (19 439, 2.02), glass (18 318, 1.96), polyethylene terephthalate (PET, 13 602, 2.48), cellulosenitrate with a pore size of 0.45 µm (cellulosenitrate 0.45, 12 994, 2.70), regenerated cellulose with a pore size of 0.2 µm (reg. cellulose 0.2, 8 967, 5.90), collagen (4 789, 2.46) and cellulosenitrate with a pore size of 0.8 µm (cellulosenitrate 0.8, 1 855, 3.61).

The data were $\log_{10}$ transformed for the statistical analysis. There was a significant influence of the cell carrier material on the cell number ($p = 0.000\ 2$). The single results of the pairwise comparison after Student-Newman-Keuls are shown in Table 4-2 and

*Table 4-1 Results of the pairwise comparison after Student-Newman-Keuls for the geometric mean of the cell numbers on the different cell carrier materials. PET = Polyethylene terephthalate, '–' = not significant, ** = significant with p = 0.01, * = significant with* p *=0.05.*

	collagen	reg. cellulose 0.2 µm	cellulosenitrate 0.45 µm	PET	glass	Melafol™	reg. cellulose 0.45 µm	nylon	Thermanox™	polycarbonate 0.4 µm	polycarbonate 0.2 µm
cellulosenitrate 0.8 µm	--	*	*	*	*	*	**	**	*	**	**
collagen		--	--	--	--	--	--	--	--	*	**
reg. cellulose 0.2 µm			--	--	--	--	--	--	--	--	*
cellulosenitrate 0.45 µm				--	--	--	--	--	--	--	--
PET					--	--	--	--	--	--	--
glass						--	--	--	--	--	--
Melafol™							--	--	--	--	--
reg. cellulose 0.45 µm								--	--	--	--
nylon									--	--	--
Thermanox™										--	--
polycarbonate 0.4 µm											--

included in Figure 4-2 using a letter code, where pairs of columns labelled without a common letter are significantly different from each other. Polycarbonate 0.2 was highly significantly different from cellulosenitrate 0.8 and collagen (p = 0.01) and significantly different from reg. cellulose 0.2 (p = 0.05). Polycarbonate 0.4 was highly significantly different from cellulosenitrate 0.8 (p = 0.01) and significantly different from collagen (p = 0.05). Nylon and reg. cellulose 0.45 were also highly significantly different from cellulosenitrate 0.8. The remaining materials with exception of collagen were significantly different from cellulosenitrate 0.8 (p = 0.01). Other pairwise comparisons were not significant.

4.1.3.2 Distribution of Cells on Different Cell Carrier Materials

*Table 4-2 Results of the pairwise comparison after Student-Newman-Keuls for the coefficient of variation on the different cell carrier materials. PET = polyethylene terephthalate, '–' = not significant, ** = significant with* p = *0.01, * = significant with* p = *0.05*

	reg. cellulose 0.45 µm	nylon	polycarbonate 0.4 µm	Thermanox™	Melafol™	glass	cellulosenitrate 0.8 µm	cellulosenitrate 0.45 µm	PET	reg. cellulose 0.2 µm	collagen
polycarbonate 0.2 µm	--	--	--	--	--	--	--	--	--	--	*
cellulosenitrate 0.45 µm		--	--	--	--	--	--	--	--	--	--
nylon			--	--	--	--	--	--	--	--	--
polycarbonate 0.4 µm				--	--	--	--	--	--	--	--
Thermanox™					--	--	--	--	--	--	--
Melafol™						--	--	--	--	--	--
glass							--	--	--	--	--
cellulosenitrate 0.8 µm								--	--	--	--
reg. cellulose 0.45 µm									--	--	--
PET										--	--
reg. cellulose 0.2 µm											--

For the CVQ, the arithmetic mean and the standard deviation were calculated for the

data description ($\bar{x}_a \pm \mathrm{SD}$). For better clarity, the arithmetic mean and the results of the three single experiments are shown in Figure 4-3.

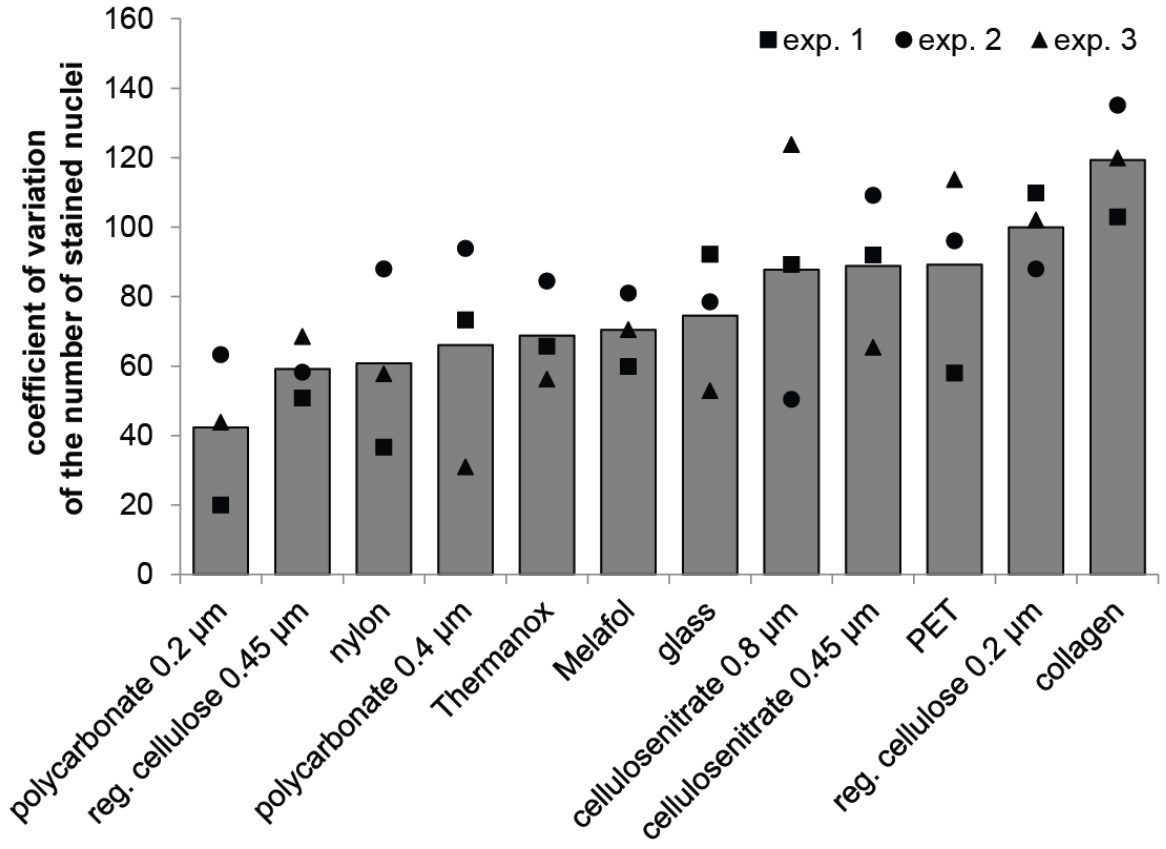

Figure 4-3 Single experimental results and arithmetic mean of the coefficient of variation for the 12 different cell carrier materials. Pairs of columns labelled with the same letter are not significantly different from each other, pairs of columns labelled without a common letter are significantly different from each other.

Polycarbonate 0.2 had the lowest CVQ (42.36 ± 21.71), followed by regenerated cellulose 0.45 (59.15 ± 8.90), Nylon (60.78 ± 25.78), polycarbonate 0.4 (66.01 ± 32.09), Thermanox™ (68.79 ± 14.36), Melafol™ (70.42 ± 10.57), glass (74.53 ± 19.94), cellulosenitrate 0.8 (87.79 ± 36.69), cellulosenitrate 0.45 (88.81 ± 22.02), PET (89.22 ± 28.48), regenerated cellulose 0.2 (99.92 ± 11.08), and collagen (119.30 ± 16.10).

There was a significant influence of the cell carrier material on the distribution of the cells on the different cell carrier materials, measured as CVQ ($p = 0.0237$). The results of the pairwise comparison after Student-Newman-Keuls are shown in Table 3-1 and included in Figure 4-3 using a letter code, where pairs of column labelled without a common letter are significantly different from each other. The only significant

difference between the CVQ on the different materials was found between collagen and polycarbonate 0.2 (p = 0.05).

4.1.3.3 *Amount* of 17β-estradiol Produced on Different Carrier Materials

For the measurement of E2, only two experimental repeats were accessible for the materials polycarbonate 0.2, polycarbonate 0.4, cellulosenitrate 0.45, cellulosenitrate 0.8, reg. cellulose 0.2 and reg. cellulose 0.45. For the other materials, all three experimental repeats could be included in the analysis. The distribution of the variable E2 was skewed to the right, so the geometric mean and the dispersion factor were calculated for the data description ($\bar{x}_g$, DF). For better clarity, the geometric mean and the results of the three single experiments are shown in Figure 4-4.

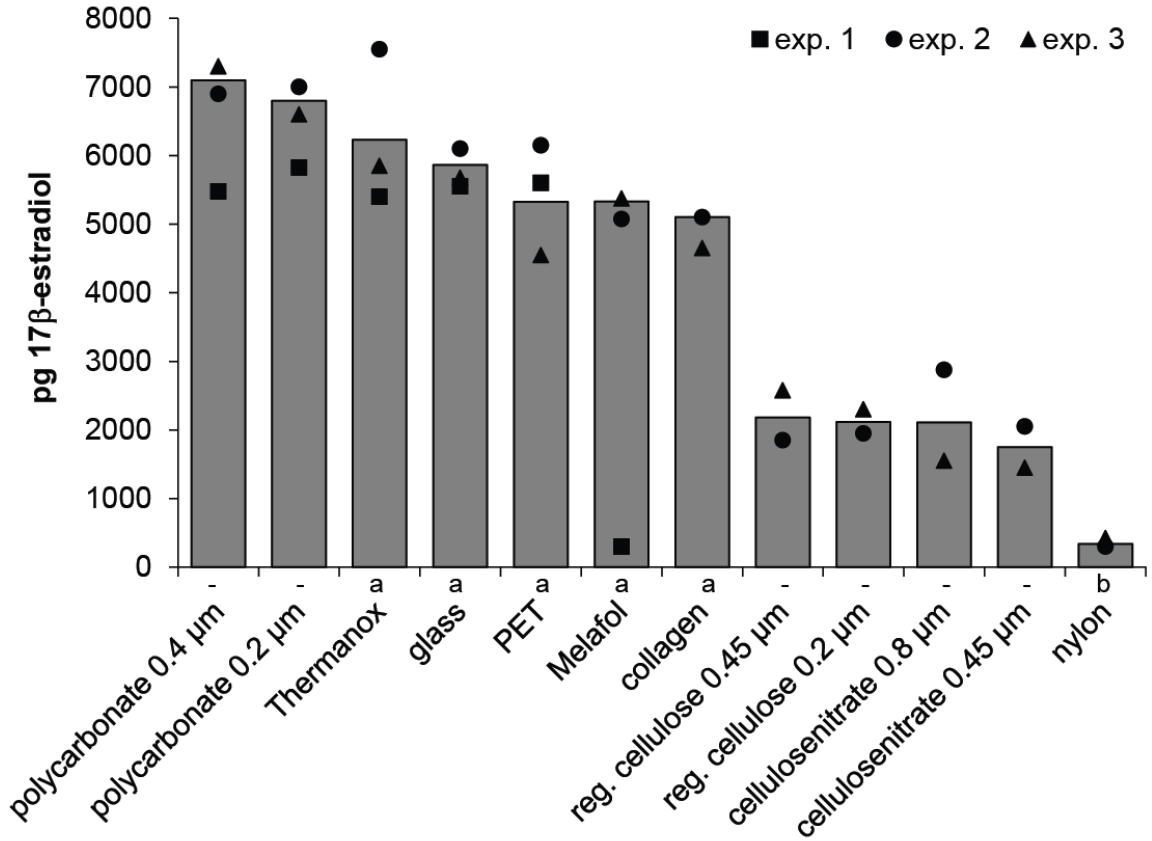

Figure 4-4 Single experimental results and geometric mean of the production of 17β-estradiol per cell carrier on the 12 different cell carrier materials. Pairs of columns labelled with the same letter are not significantly different from each other; pairs of columns labelled without a common letter are significantly different from each other. Columns labelled with '-' were not included in the pairwise comparison test.

The highest amount of E2 was produced by the bGC grown on polycarbonate 0.4 (7098.75 pg, 1.04), followed by polycarbonate 0.2 (6 800.00 pg, 1.04), Thermanox™ (6 291.67 pg, 1.18), glass (5 866.67 pg, 1.04), PET (5 366.65 pg, 1.16), Melafol™ (5 333.33 pg, 1.05), collagen (5 116.67 pg, 1.10), cellulosenitrate 0.8 (2 212.50 pg, 1.55) and regenerated cellulose 0.45 (2 212.500 pg, 1.26), regenerated cellulose 0.2 (2 125.00 pg, 1.12) and cellulosenitrate 0.45 (1 750 pg, 1.28).

Because of their right-skewed distribution, the data were $\log_{10}$ transformed for the statistical analysis. There was a significant influence of the cell carrier material on the production of E2 ($p < 0.0001$). The pairwise comparison after Student-Newman-Keuls was only possible for the materials with three experimental repeats. These results are shown in Table 4-4 and included in Figure 4-4 using a letter code, where pairs of columns labelled without a common letter are significantly different from each other. Thermanox™, glass, Melafol™, PET and collagen were highly significantly different from nylon (*p* = 0.01), but no other significant differences were detected in the pairwise comparison.

*Table 4-3 Results of the pairwise comparison after Student-Newman-Keuls for the production of E2 on the different cell carrier materials. PET = polyethylene terephthalate, '–' = not significant, ** = significant with* p = *0.01, * = significant with* p = *0.05.*

	collagen	PET	Melafol™	glass	Thermanox™
nylon	**	**	**	**	**
collagen		--	--	--	--
PET			--	--	--
Melafol™				--	--
glass					--

4.1.3.4 Correlation between Cell Count, Cell Distribution and Production of 17β-estradiol

To identify possible correlations between the three measured parameters, they were opposed in pairs of two for each material on the x and y axis of a diagram. Then a linear trend line was added to the diagram to detect tendencies of correlation. After that, the Pearson product-moment correlation coefficient (r) was calculated. A negative correlation could be seen between the cell count and the cell distribution (r = –0.75). This means, that the higher the cell count was, the more equally distributed the cells were on the material (Figure 4-5 a).

The cell count on a distinct cell carrier material correlated positively with the production of E2 on the same material (r = 0.52). This means, that the higher the cell count was, the more E2 was produced on the material (Figure 4-5 b). A slightly negative correlation could be seen between the cell distribution and the production of E2 (r = –0.15). This means, that the more equally distributed the cells were on the material, the more E2 was produced (Figure 4-5 c).

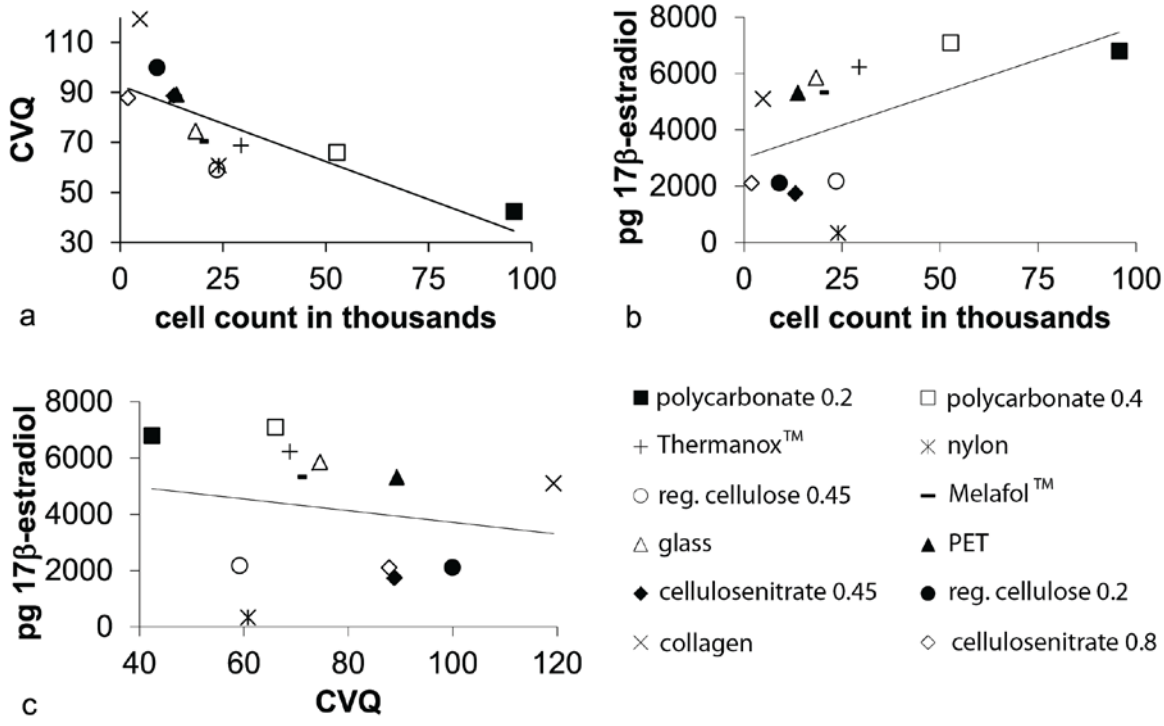

Figure 4-5 Correlation between cell count, CVQ and E2 on the different cell carrier materials.

4.1.3.5 Production of 17β-estradiol per Cell Count

The production of E2 per 1 000 cells, expressed as geometric mean and dispersion factor ($\bar{x}_g$, DF) of the experimental repeats, results in the highest productivity on cellulosenitrate 0.8 (1 759.60 pg, 6.73), followed by collagen (1 706.59 pg, 2.26), PET (656.54 pg, 2.47), reg. cellulose 0.2 (504.88 pg, 4.8), Melafol™ (331.58 pg, 1.93), glass (312.19 pg, 2.03), Thermanox™ (194.17 pg, 1.47), cellulosenitrate 0.45 (137.29 pg, 5.18), reg. cellulose 0.45 (118.35 pg, 1.87), polycarbonate 0.4 (104.14 pg, 1.76), polycarbonate 0.2 (75.31 pg, 1.08) and nylon (24.53 pg, 3.12)(Figure 4-6).

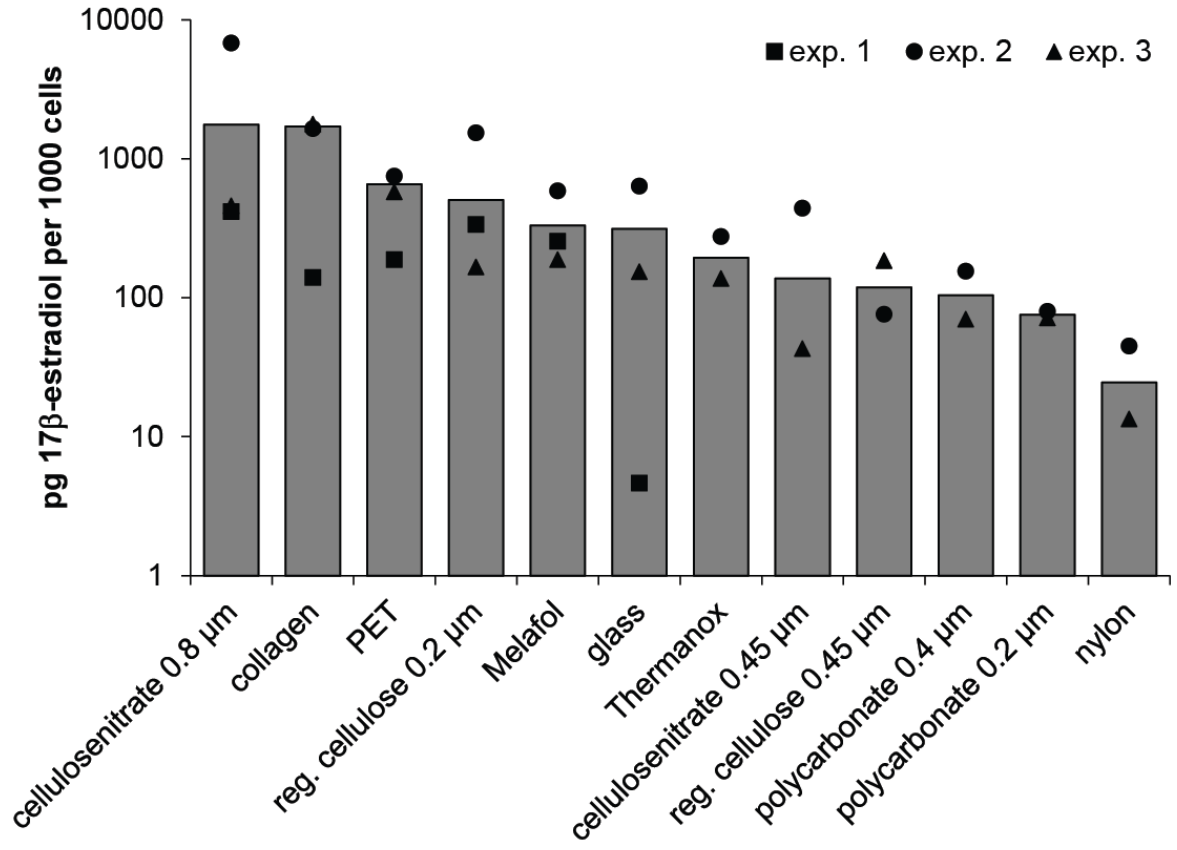

Figure 4-6 Geometric mean and single experimental results of the production of 17β-estradiol per 1000 cells on the different cell carrier materials. Geometric mean and single experimental results of the production of 17β-estradiol per 1000 cells on the different cell carrier materials.

4.1.4 Comparison between Static and Perfusion Cell Culture System

4.1.4.1 Cell Numbers

The variable cell count, calculated per 6 cell carriers, was skewed to the right, so the geometric mean and the dispersion factor were calculated for the data description ($\bar{x}_g$, DF) and used in the diagram (Figure 4-7). For bGC I, more cells were counted in the static system (179 334, 4.40) than in the perfusion system (158 858, 5.36). In contrast, bGC II and bGC III had a higher cell count in the perfusion system (339 205, 1.17 and 191 670, 1.73, respectively) than in the static system (279 959, 1.24 and 95 473 3.04, respectively), with the greatest difference between the systems in bGC III, were only half as much cells were detectable after the static culture in comparison to the perfused culture. Concerning the cell classes, the highest numbers of cells attached from bGC II, followed in case of the static system by bGC I and then bGC II and in case of the perfused system by bGC II and then by bGC I (Figure 4-7). The data were $\log_{10}$ transformed for the statistical analysis. The influence of the cell class and the system on the cell count were not significant (p = 0.3799 and p = 0.2300, respectively). There was also no significant interaction between the cell class and the system (p = 0.1337).

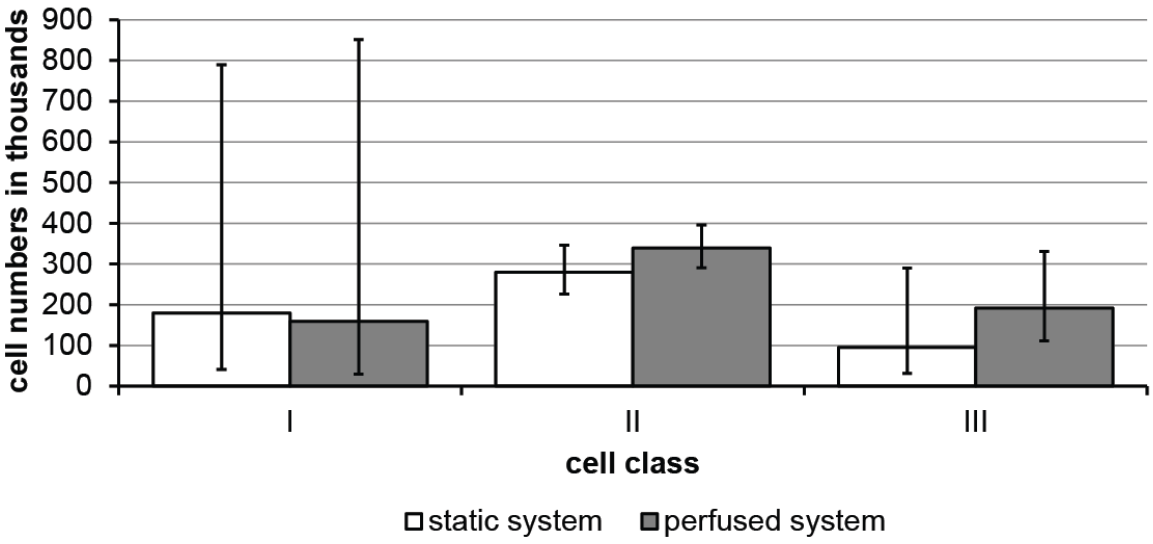

Figure 4-7 Geometric mean and deviation of the cell numbers of bGC I, II and III per 6 cell carriers of the four experimental repeats after the culture in the static and in the perfused system.

4.1.4.2 Production of Total Estrogens and Progesterone

The distribution of the variables ES and P4, calculated per 6 cell carriers per 8 hours, was skewed to the right, so the geometric mean and the dispersion factor were calculated for the data description ($\bar{x}_g$, DF). For better clarity, these values are presented on a logarithmic scale to the basis 2 in the corresponding diagrams.

4.1.4.2.1 Total Estrogens

For bGC I, the production of ES started with an average of 589.74 pg (1.82) during the static preculture (Figure 4-8). In the following three 8 hour periods, the production of ES declined to levels about half as high as the initial ones, whereby this decline was more distinct in the static than in the perfusion system (212.93 pg, 4.82 and 267.89 pg, 2.67, respectively). In the next 8 hours, the production of ES rose slightly in the perfused system and considerably in the static system (335.92 pg, 1.89 and 406.08 pg, 2.81, respectively). From this point on, the production of ES constantly declined in the perfused system, but was almost stable in the static system for 8 hours before a constant increase of ES production took place in this system. At the end of the culture, the production of ES by bGC I was more than 5 times higher in the static than in the perfused system (1 060.75, 4.68 and 184.54 pg, 1.86, respectively).

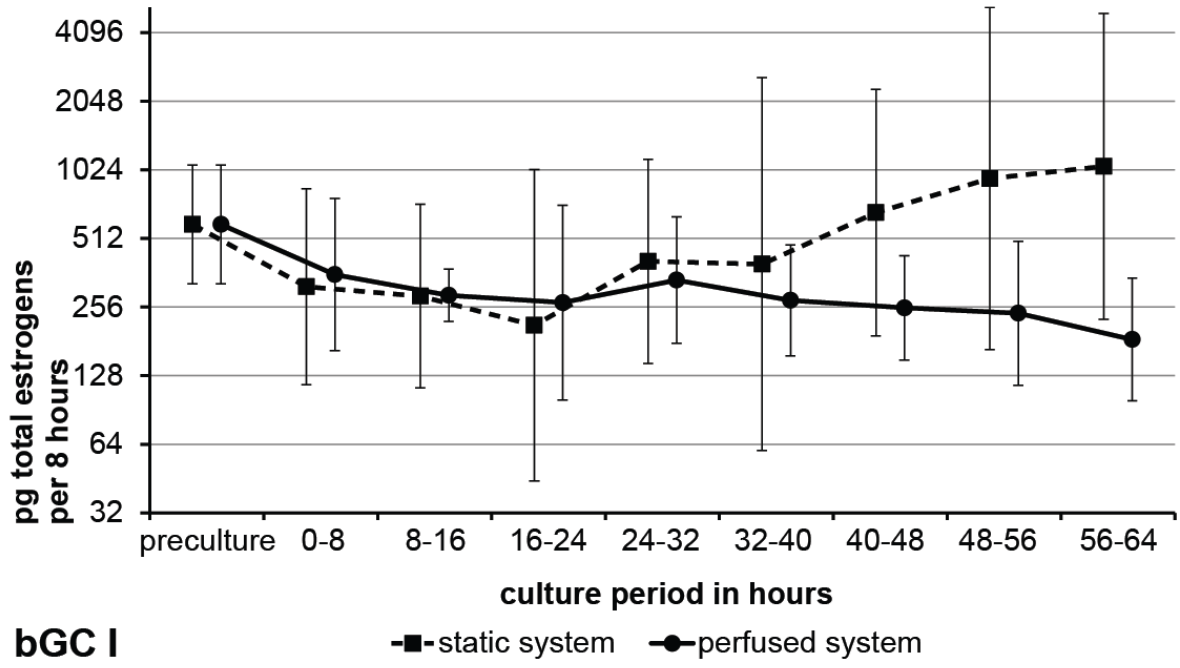

Figure 4-8 Geometric mean and deviation of the production of total estrogens by bGC I in the static and in the perfused system.

For bGC II, the production of ES started with an average of 2 633.15 pg (1.81) during the static preculture and was about 4.5 times higher than for bGC I (Figure 4-9). During the next two 8 hour intervals, it dropped to levels around 500 pg, whereby the production of ES was slightly higher in the perfused than in the static system. Over the following 8 hours, the production of ES further declined, with the cells in the perfused system still producing more ES than the cells in the static system (475.27, 1.40 and 257.05 pg, 2.11, respectively). In the following interval, between 24 and 32 hours, the production of ES slightly rose in the static system, whereby it was still declining in the perfused system, making the amount of produced ES for the first time higher in the static than in the perfused system. From this time on, the production of ES was further rising in the static system, whereby it further declined in the perfused system. At the end of the culture, the production of ES by bGC II was about three times higher in the static than in the perfused system (540.08 pg, 2.39 and 179.9 pg, 1.80, respectively).

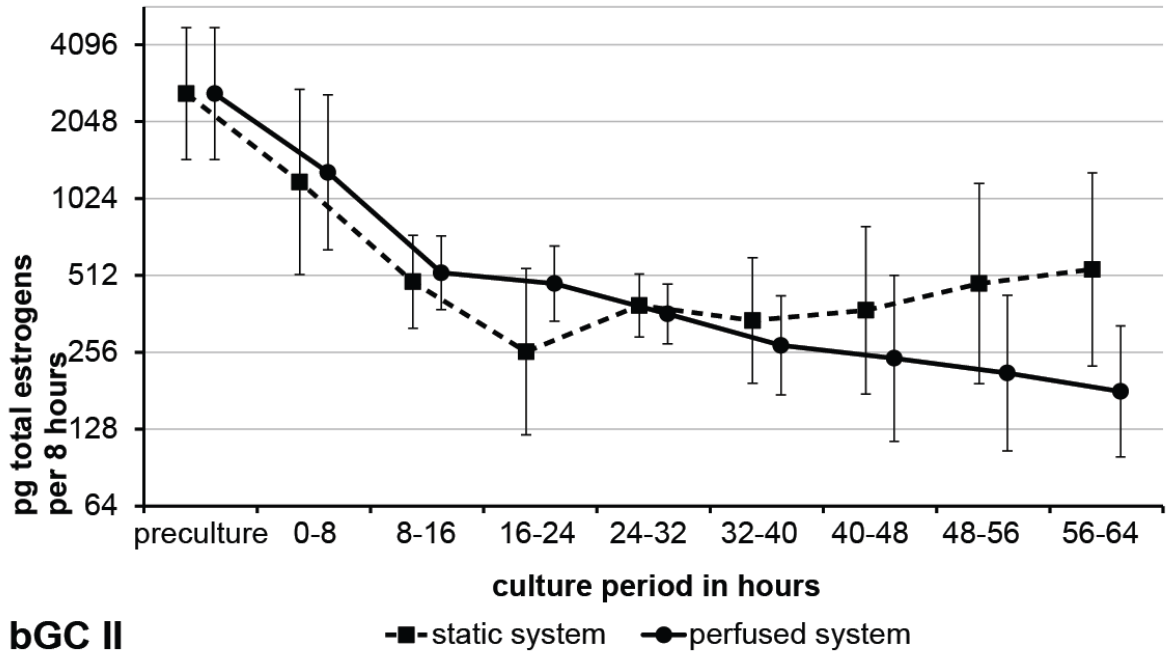

Figure 4-9 Geometric mean and deviation of the production of total estrogens by bGC II in the static and in the perfused system.

For bGC III, the production of ES started with an average of 14 901.16 pg (1.53) during the static preculture and was about 25 times higher than for bGC I (Figure 4-10). In the perfused system, the production of ES declined rapidly during the first 8 hours in the new system to levels around 3 800 pg and remained at this level for the next following

8 hours. In the static system, the drop of ES production to comparable levels happened in two almost equal steps during the first 16 hours in the new system, so that after this time, both systems had a nearly equal level of ES production. From this time on, there was a constant decline in the production of ES in both systems, whereby the reached levels of ES were lower in the static than in the perfused system until the second last 8 hour interval. After that, the production of ES was slightly higher in the static than in the perfused system. At the end of the culture, the production of ES by bGC III was slightly higher in the static system than in the perfused system (885.67 pg, 2.01 and 653.95 pg, 1.27, respectively).

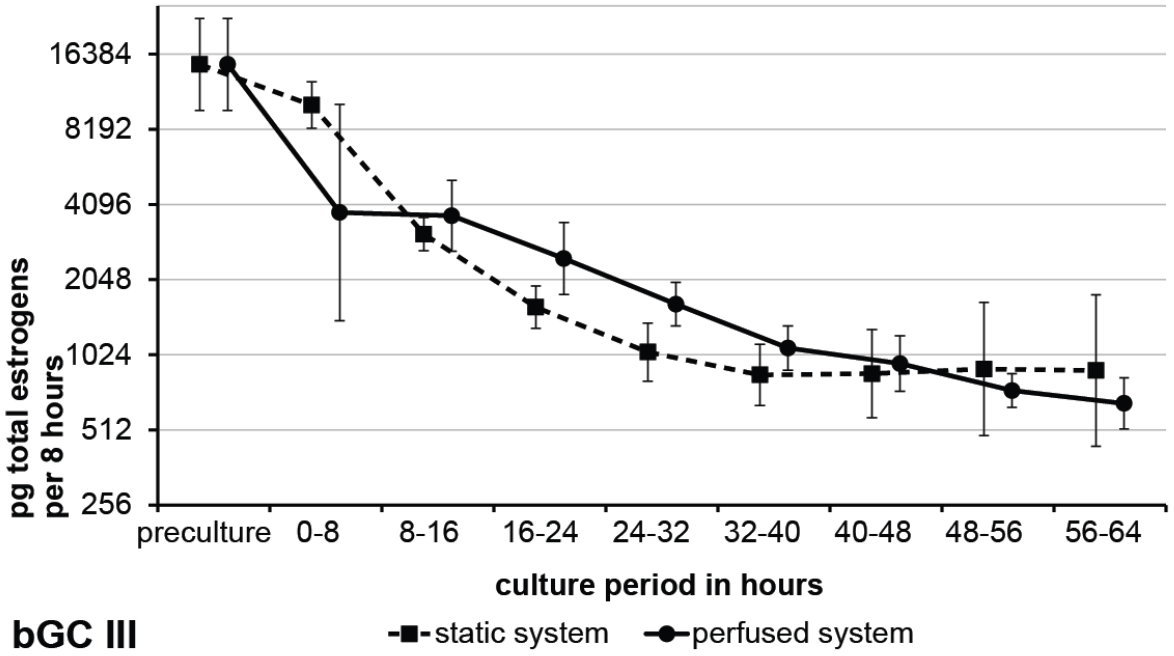

Figure 4-10 Geometric mean and deviation of the production of total estrogens by bGC III in the static and in the perfused system.

Because of their right-skewed distribution, the data were $\log_{10}$ transformed for the statistical analysis. A three-factorial analysis of variance, including the factors cell class, culture system and culture time, was conducted. The influence of the cell class on the production of ES was significant ($p = 0.0055$), as well as the influence of culture time, which was highly significant ($p = 0.0001$). The influence of the applied system on the production of ES was not significant ($p = 0.4589$). There was no significant interaction between cell class and applied system ($p = 0.4557$). The interactions between cell class and time and between applied system and time were significant ($p =$

0.0001 and $p = 0.0002$, respectively). There was also a significant interaction between all three influencing variables cell class, system and time ($p = 0.0011$).

As an additional parameter, the production of ES in the last measured interval was normalised using the cell count obtained at the culture end (Figure 4-11). The production of ES per 1 000 cells was calculated for each experimental repeat, and the arithmetic mean and the standard deviation calculated. The relation between the static and the perfused system stayed the same with the amount of ES produced per 1000 cells being generally higher in the static than in the perfused system for all cell classes. The highest amounts of ES produced per 1 000 cells were reached by bGC III in both systems, whereby the production in the static system was more than twice as high as in the perfused system (10.52 ± 6.70 pg and 4.10 ± 3.17 pg, respectively). The second highest amounts were produced by bGC I in both systems, whereby the amounts reached in the static system were also more than twice as high as in the perfused system (6.46 ± 3.05 pg and 2.47 ± 3.52 pg, respectively). The lowest amounts of ES per 1 000 cells were measured from bGC II, whereby the amount of ES produced in the static system was almost four times higher than in the perfused system (2.40 ± 1.64 pg and 0.63 ± 0.40 pg, respectively).

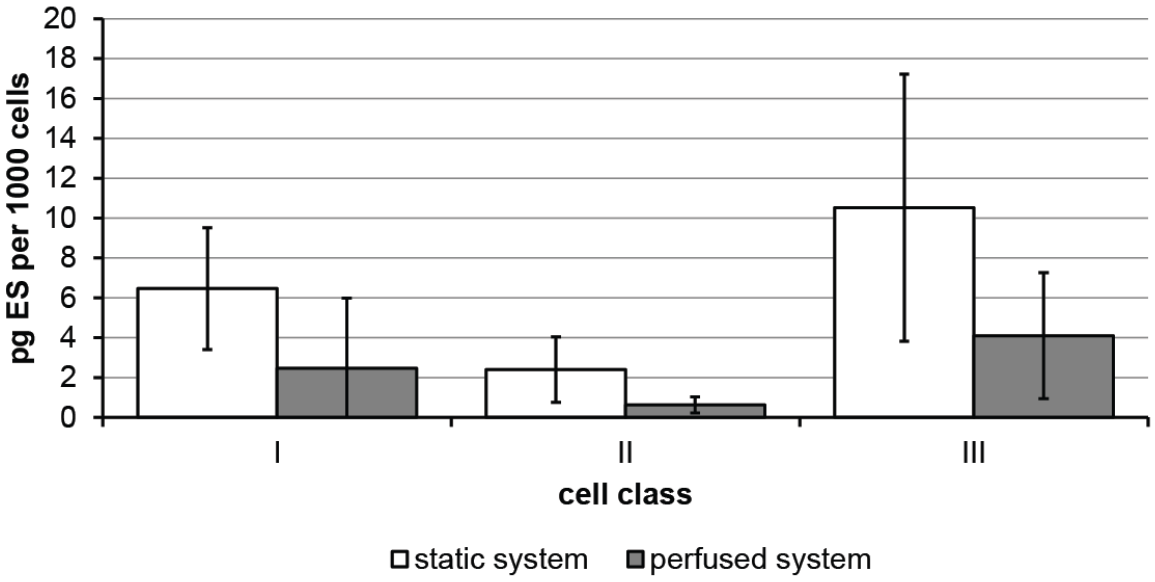

Figure 4-11 Arithmetic mean and standard deviation of the production of total estrogens per 1 000 cells by bGC I, II and III in the static and in the perfused system.

4.1.4.2.2 Progesterone

For bGC I, the production of P4 started with an average of 76 946.24 pg (1.45) in the static preculture (Figure 4-12). During the first 8 hours in the new culture system, it dropped to less than the half amount in the static system (34 190.18 pg, 30.96) and to less than 2 % of the preculture level in the perfused system (1 475.18 pg, 47.44). During the following culture period, the cells in the static system produced, after a slight initial decline, stable amounts varying between35 000 and 40 000 pg. The production of P4 from the cells in the perfused system rose, except two slight declines between two culture intervals, steadily from 2000 to about 3000 pg towards the end of the culture period. At the end of the culture period, the amount of P4 produced by bGC I in the static system was about 13 times higher than in the perfused system (38 010.75 pg, 42.83 and 2 914.2 pg, 53.50, respectively).

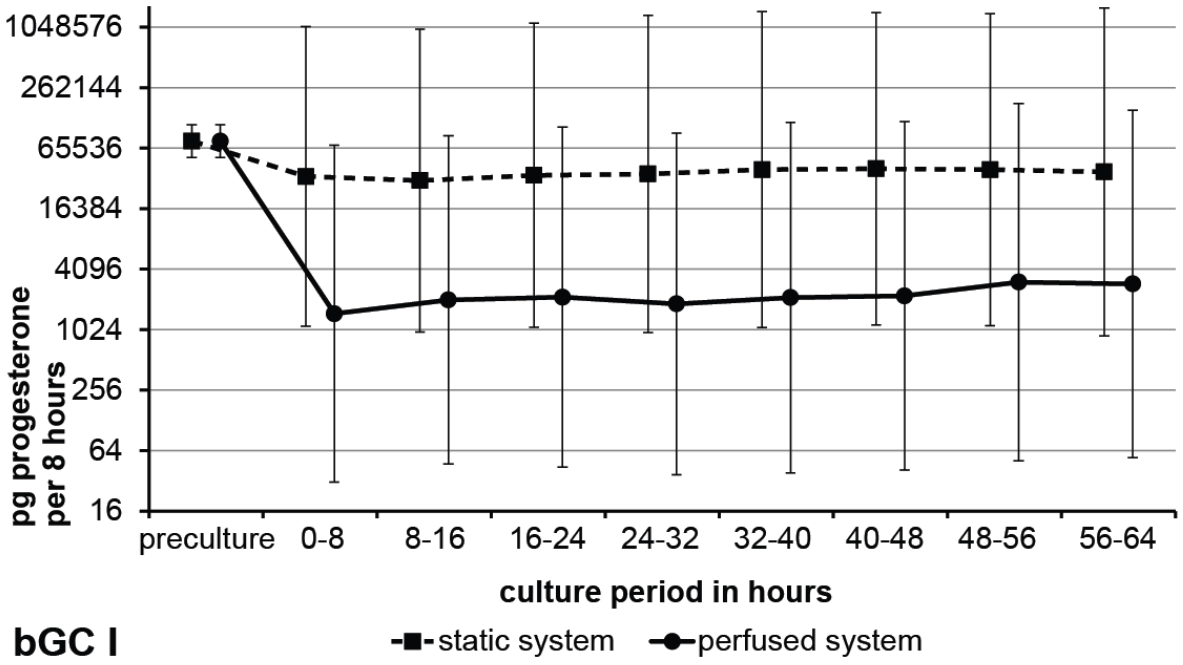

Figure 4-12 Geometric mean and deviation of the production of progesterone by bGC I in the static and in the perfused system.

For bGC II, the production of P4 started with an average of 94 489.35 pg (1.61) in the static preculture and was about 1.23 times higher than from bGC I (Figure 4-13). During the first 8 hours in the new system, it dropped to less than the half amount in the static system (35 530.01 pg, 31.59) and to less than 2 % of the preculture level in the perfused system (1 753.59 pg, 47.38). In the static system, the amount of P4 declined

to about 31 500 pg in the next following 8 hours, but increased then, with one exception in the second last measurement, steadily to almost 50 000 pg at the end of the culture period. In the perfused system, the production of P4 rose again, with some plateau phases after the sharp initial decline, to amounts up to more than 4 000 pg. At the end of the culture period, the amount of P4 produced by bGC II was in the static system more than 11 times higher than in the perfused system (49 498.72 pg, 44.64 and 4 304.8 pg, 70.04, respectively).

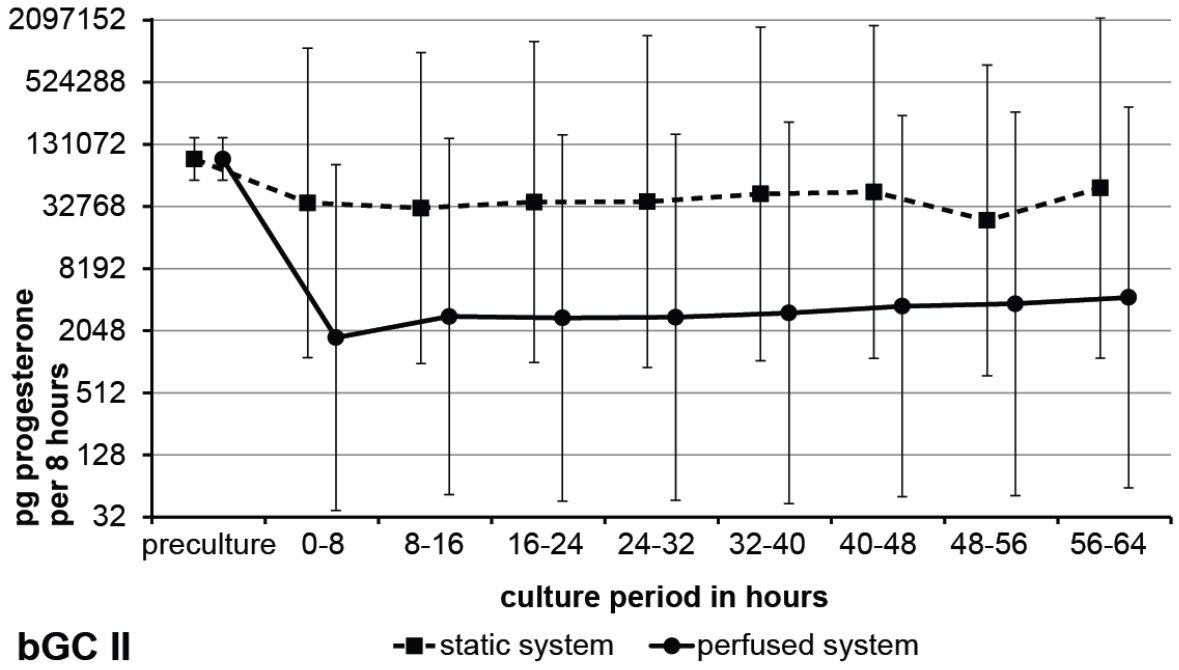

Figure 4-13 Geometric mean and deviation of the production of progesterone by bGC II in the static and in the perfused system.

For bGC III, the production of P4 started with an average of 97 986.79 pg (1.31) in the static preculture and was about 1.27 times higher than from bGC I (Figure 4-14). The production of P4 declined during the first 8 hours in the static system to levels lower than half of the preculture levels, and in the perfused system to amounts about 2 % of the preculture level. In the static system, the amount of produced P4 still declined slightly in the following 8 hour interval, and increased steadily to amounts higher than 50 000 pg from then on. In the perfused system, the production of P4 altered between 2 000 and 2 400 pg in the next three following time intervals and rose then steadily to more than 4 000 pg. At the end of the culture period, the amount of P4 produced from

bGC III was more than 12 times higher in the static than in the perfused system (52051.15 pg, 23.56 and 4229.6 pg, 73.47, respectively).

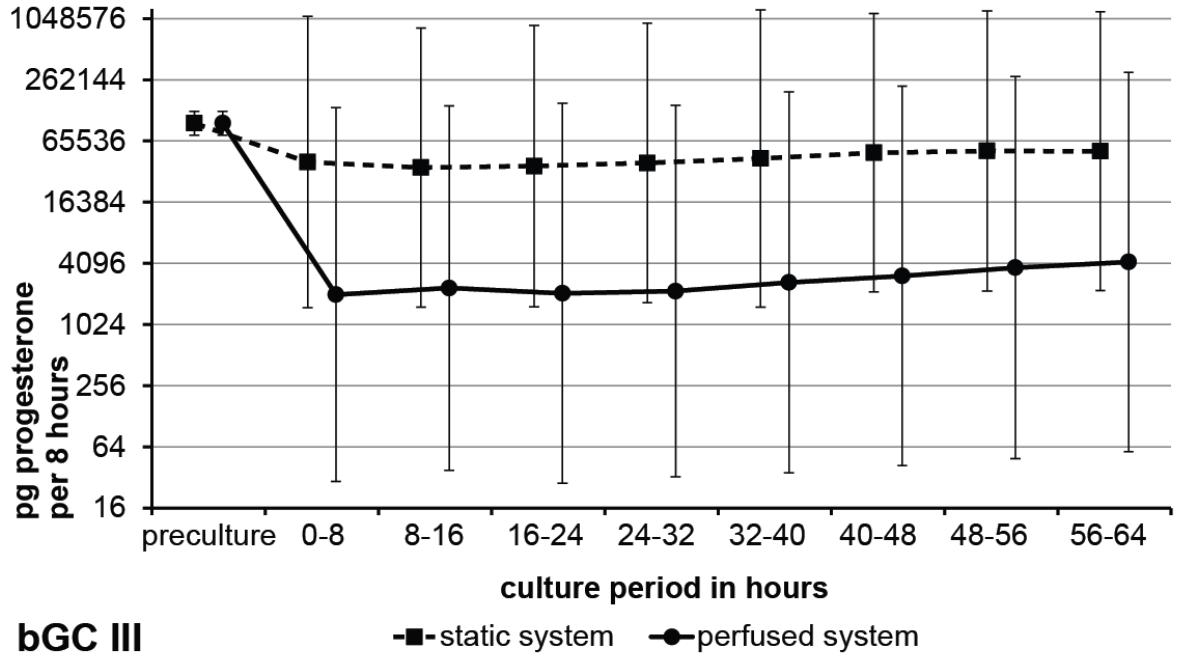

Figure 4-14 Geometric mean and deviation of the production of progesterone by bGC III in the static and in the perfused system.

Because of their right-skewed distribution, the data were $\log_{10}$ transformed for the statistical analysis. A three-factorial analysis of variance, including the factors cell class, culture system and culture time, was conducted. The cell class had no significant influence on the production of P4 ($p = 0.5168$), as well as the culture time ($p = 0.2685$). There was a significant influence of the applied system on the P4 production of the cells ($p = 0.0055$). There was no significant interaction between cell class and system ($p = 0.5894$) and cell class and time ($p = 0.6110$), but between applied system and time ($p = 0.0001$). No significant interaction could be detected between all three influencing variables cell class, system and time ($p = 0.8638$).

As additional parameter, the production of P4 in the last measured interval was normalised using the cell count obtained at the culture end (Figure 4-15). The production of P4 per 1 000 cells was calculated for each experimental repeat, and the arithmetic mean and the standard deviation calculated. The relation between the static and the perfused system stayed the same, with the amount of P4 produced per 1000 cells being generally higher in the static than in the perfused system in the same cell

class. The highest amounts of P4 produced per 1 000 cells were reached by bGC III in both systems, whereby the production in the static system was more than fourteen times higher as in the perfused system (2404.19 ± 1662.72 pg and 171.51 ± 153.35 pg, respectively). The second highest amounts were produced by bGC I in both systems, whereby the amounts reached in the static system were more than seven fold higher as in the perfused system (1063.87 ± 140.22 pg and 140.22 ± 101.04 pg, respectively). The lowest amounts of P4 per 1 000 cells were measured from bGC II, whereby the amount produced in the static system was also seven fold higher than in the perfused system (921.76 ± 836.31 pg and 129.16 ± 146.16 pg, respectively).

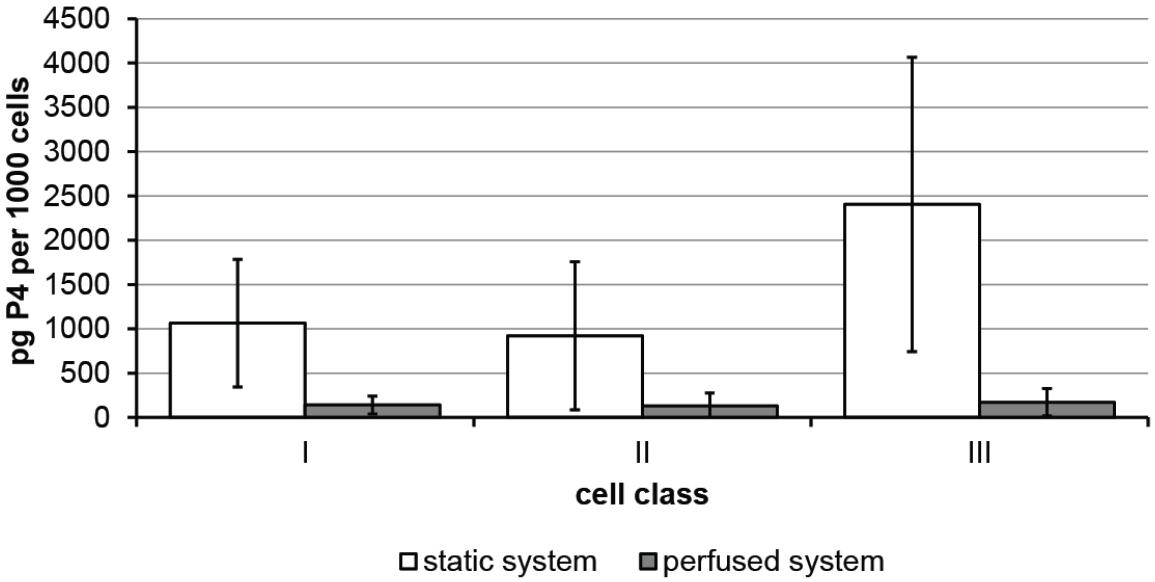

Figure 4-15 Arithmetic mean and standard deviation of the production of progesterone per 1 000 cells by bGC I, II and III in the static and in the perfused system.

4.1.4.2.3 Ratio of Total Estrogens to Progesterone

The ratio of ES to P4 was calculated by dividing the amount of ES with the amount of P4 produced during the last eight-hour interval. The geometric mean of the ratio of ES to P4 was higher in the perfusion than in the static system (mean perfusion system: 0.09, mean static system: 0.02). In the perfusion system, bGC III had the highest ratio (0.15), followed by bGC I (0.06) and bGC II (0.04). In the static system, bGC I had the highest ratio (0.03), followed by bGC III (0.02) and bGC II (0.01).

4.1.4.3 Expression of Key Genes for Steroidogenesis

The variables of the gene expression showed a very high variability and were therefore not only $\log_{10}$ transformed for the statistical analysis, but the geometric mean and the dispersion factor ($\bar{x}_g$ (DF)) calculated for the data description are, for better clarity, also presented on a logarithmic scale.

4.1.4.3.1 Aromatase

In the preculture samples tested for P450arom, the relative expression was the higher the larger the follicle was from which the cells were isolated (Figure 4-16).

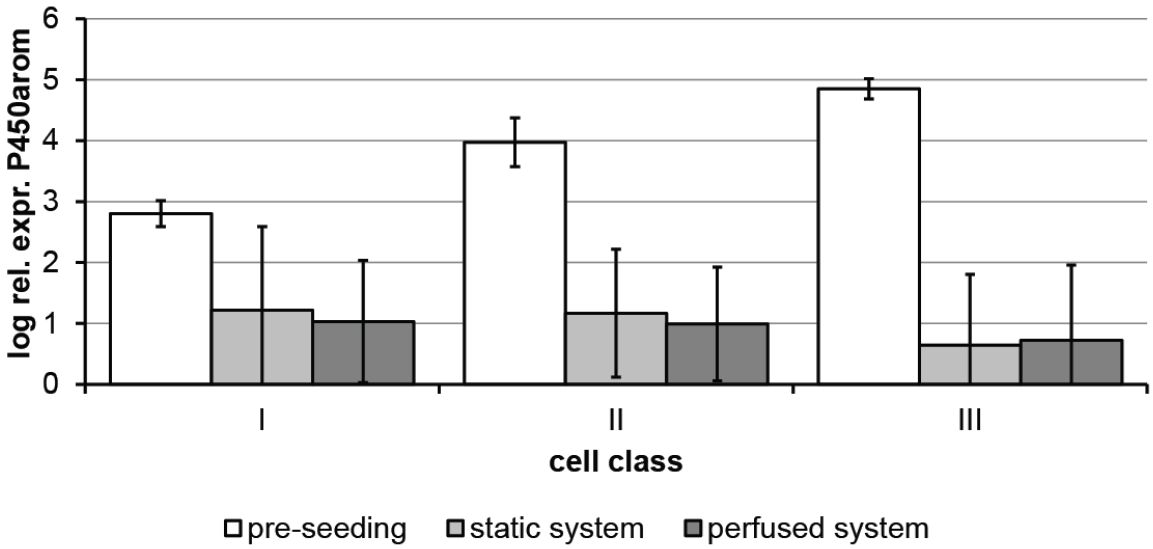

Figure 4-16 Logarithm of the relative expression of P450arom by bGC I, II and III in pre-culture samples and after the culture in the static and perfused system.

The relative expression in bGC III was about 7.5 times higher than in bGC II and more than 100 times higher than in bGC I (70 790.49 (1.46), 9398.62 (2.50) and 635.74 (1.63), respectively). At the end of the culture period, the relative expression of P450arom was in both the static and the perfused culture much lower than in the pre-seeding samples. Post culture, P450arom was in bGC I about 1.5 times higher expressed in the static than in the perfused system (16.48 (23.69) and 10.80 (10.08), respectively), and in comparison to the pre-seeding sample, the relative expression after the culture in the static system was about 40 times lower. In bGC II, P450arom was also expressed at a higher level after the culture in the static than in the perfused system (14.76 (11.17) and 9.84 (8.55), respectively), whereby the relative expression in the static system was more than 600 times lower compared to the pre-seeding

samples. In bGC III, the difference between preculture and end of culture P450arom expression was the highest, comprising more than four orders of magnitude. In bGC III, the relative expression of P450arom was, in contrast to bGC I and II, slightly higher after the culture in the perfused system than in the static (5.31 (17.15) and 4.40 (14.51), respectively). Between the three cell classes, the relative expression of P450arom at the end of the culture was highest in bGC I, followed by bGC II and then by bGC III in both applied system.

Statistically, the culture time had a significant influence on the relative expression of P450arom (p = 0.0264). The differences between the cell classes and the systems were not significant (p = 0.3367 and p = 0.2026, respectively). A significant interaction was proven between cell class and culture time (p = 0.0107), whereby there was no significant interaction between culture system and culture time (p = 0.2026) as well as no significant interaction between cell class, system and time (p = 0.1959).

4.1.4.3.2 3β-Hydroxysteroid Dehydrogenase

The relative expression of 3β-HSD in the pre-seeding samples was highest in bGC III, followed by bGC I and bGC II (4.79 (1.26), 2.17 (1.40) and 1.87 (1.64), respectively)(Figure 4-17). The samples from the end of the culture period all showed a distinctively higher relative expression of 3β-HSD than the preculture samples. Also, the relative expression was higher in the static than in the perfused system over all cell classes. In bGC I, the relative expression of 3β-HSD was more than 12 times higher in the static system and more than 3 times higher in the perfusion system compared to the pre-seeding sample (26.85 (1.21) and 7.04 (6.16), respectively). In bGC II, the relative expression of 3β-HSD was more than 16 times higher in the static system and more than 8 times higher in the perfused system compared to the pre-seeding sample (29.67 (1.58) and 16.23 (1.58), respectively). The relative expression of 3β-HSD in bGC III was more than 5 times higher in the static and about 3 times higher in the perfused system in comparison to the pre-seeding sample (26.03 (1.67) and 14.51 (2.35), respectively). The relative expression of 3β-HSD at the end of the culture period was comparable for all cell classes in the static system. In the perfused system, the relative expression of 3β-HSD at the end of the culture period was highest in bGC II, followed by bGC III and bGC I.

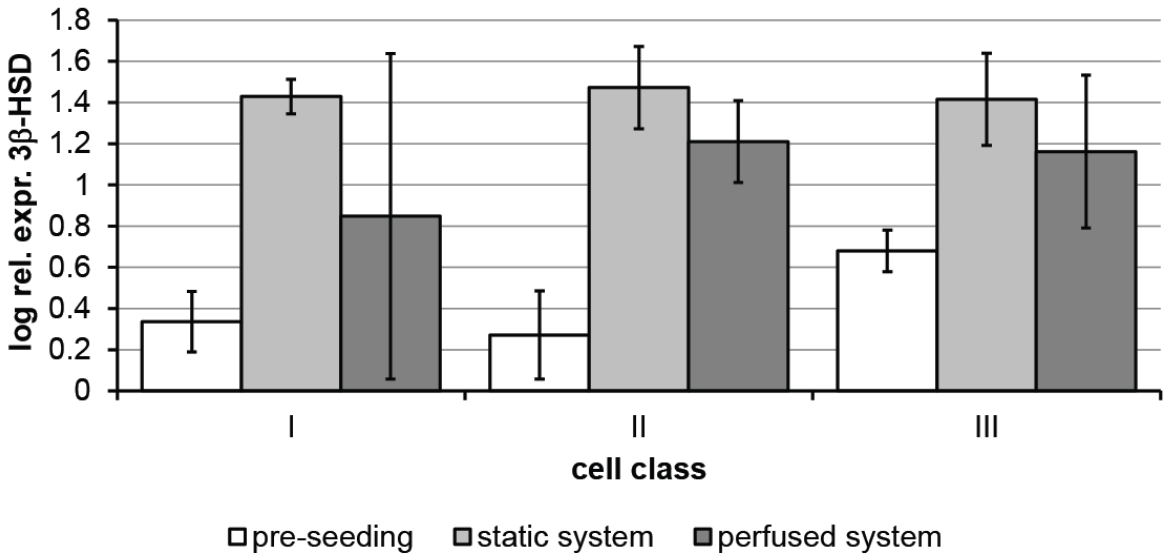

Figure 4-17 Logarithm of the relative expression of 3β-HSD by bGC I, II and III in pre-culture samples and after the culture in the static and perfused system.

Statistically, the influence of the cell class and the system on the relative expression was not significant (p = 0.2295 and p = 0.3074, respectively), whereby the influence of the time on the relative expression was just under the significant level (p = 0.0544). No significant interaction could be proven between the system and the culture time, between cell class and system, cell class and time and all three factors cell class, system and time (p = 0.3074, p = 0.8881, p = 0.3461 and p = 0.8881, respectively).

4.1.4.3.3 FSH-Receptor

The relative expression of the FSH-R in the pre-seeding samples was the higher the larger the follicle was from which the cells were obtained, with a relation of nearly 1 : 1.5 : 2 for bGC I : bGC II : bGC III (876.45 (1.45), 1315.62 (1.48) and 1761.53 (1.38), respectively). For all three cell classes, the post culture relative expression was much lower than the pre-seeding one, whereby the relative expression was the highest in bGC I, followed by bGC II and bGC III. In all post culture samples, the relative expression of FSH-R was higher in the perfused system than in the static system. In bGC I, the relative expression after the static culture was almost 160 times lower than in the pre-seeding sample (5.52 (8.07)), and the relative expression in the perfused system about 1.2 times higher than in the static system (6.55 (6.06)). The difference between perfused and static culture samples was broader for bGC II, where the relative expression of FSH-R in cells from the static system was only half as high as in cells from the perfused system (1.86 (4.70) and 3.61 (3.90), respectively).

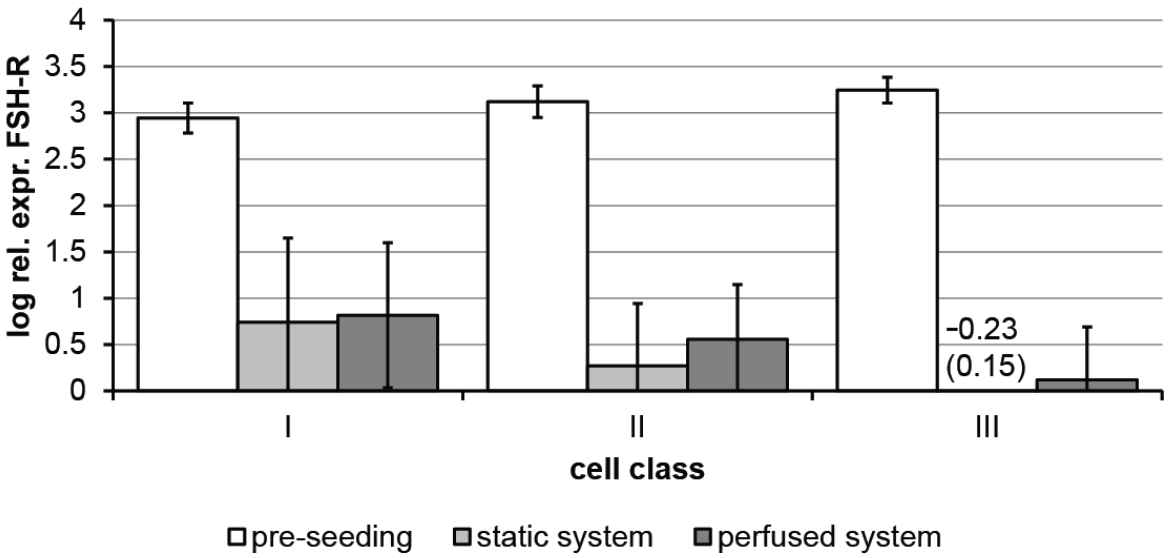

Figure 4-18 Logarithm of the relative expression of FSH-R by bGC I, II and III in pre-culture samples and after the culture in the static and perfused system, values for bGC III after culture in the static system inserted ($\bar{x}_a$ (SD)).

In comparison to the pre-seeding sample, the relative expression in the perfused system was more than 350 times lower post culture. After the static culture of bGC III, only very low amounts of FSH-R could be detected (0.59 (1.41)), whereby after the perfused culture still thrice as much relative expression could be detected (1.32 (3.70)). The difference between pre-seeding and post culture was most explicit in bGC III, with the post culture relative expression being more than 1500 times lower than the pre-seeding relative expression (Figure 4-18).

For the relative expression of FSH-R, no significance was detectable for the influence of the cell class (p = 0.3512), the system (0.1904) and for interactions between cell class and system (p = 0.8520), system and time (p = 0.1904) and for cell class, system and time (p = 0.8520). The culture time had a significant influence on the relative expression of FSH-R (p = 0.0146) and an interaction between cell class and culture time was just under the significant level (p = 0.0526).

4.1.4.3.4 LH-Receptor

The expression of LH-R was only detectable in pre-seeding samples of bGC III and had a very high variability (1.93 (132.90)) (Figure 4-19).

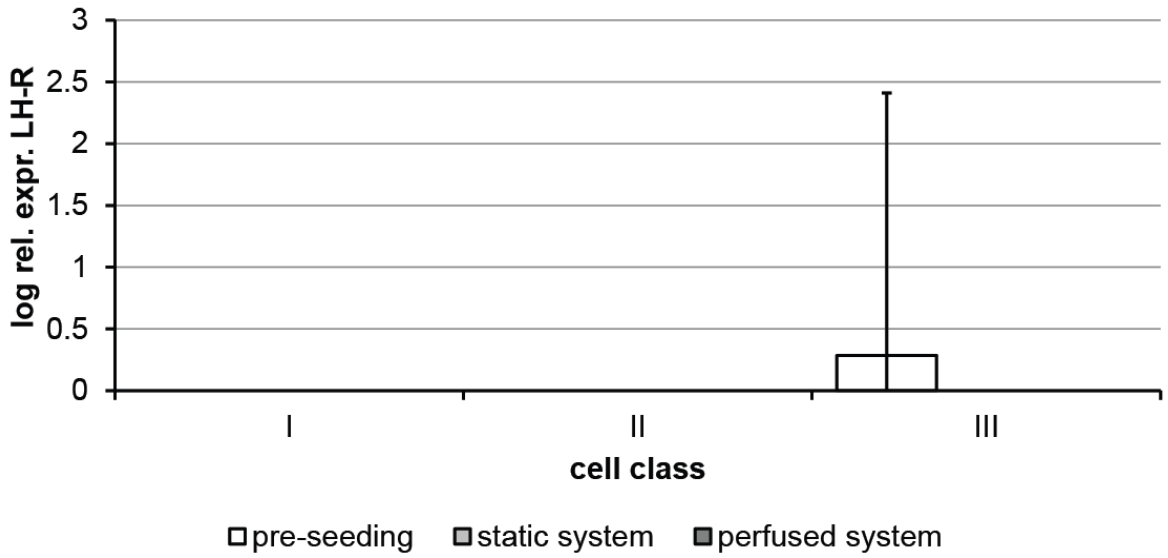

Figure 4-19 Logarithm of the relative expression of LH-R by bGC I, II and III in pre-culture samples and after the culture in the static and perfused system.

4.2 Influence of Metabolites Possibly Linking Nutrition to Reproduction During Negative Energy Balance

4.2.1 Influence of Urea on Granulosa Cell Function and Cell Numbers

4.2.1.1 Cell Numbers

The variable cell count, calculated per 6 cell carriers, was skewed to the right, so the geometric mean and the dispersion factor were calculated for the data description ($\bar{x}_g$ (DF)) and the diagram (Figure 4-20).

For bGC I, the amount of cells counted after culture with urea was about 10 % lower than after the culture without urea (214 933 (1.66) and 243 182 (2.68), respectively). For bGC III, the cell count also was higher after the culture without urea, whereby here almost twice as much cells grew in comparison to the culture with urea (363 744 (1.53) and 189 684 (3.86)). Overall, the highest number of cells grew of bGC III in the culture without urea, followed by the culture of bGC I without urea, by the culture of bGC I with urea and the culture of bGC III with urea. Because of their right-skewed distribution, the data were $\log_{10}$ transformed for the statistical analysis. The influence of the cell class and the metabolite on the cell count were not significant (p = 0.5273 and p = 0.0932, respectively). There was also no significant interaction between the cell class and the system (p = 0.5221).

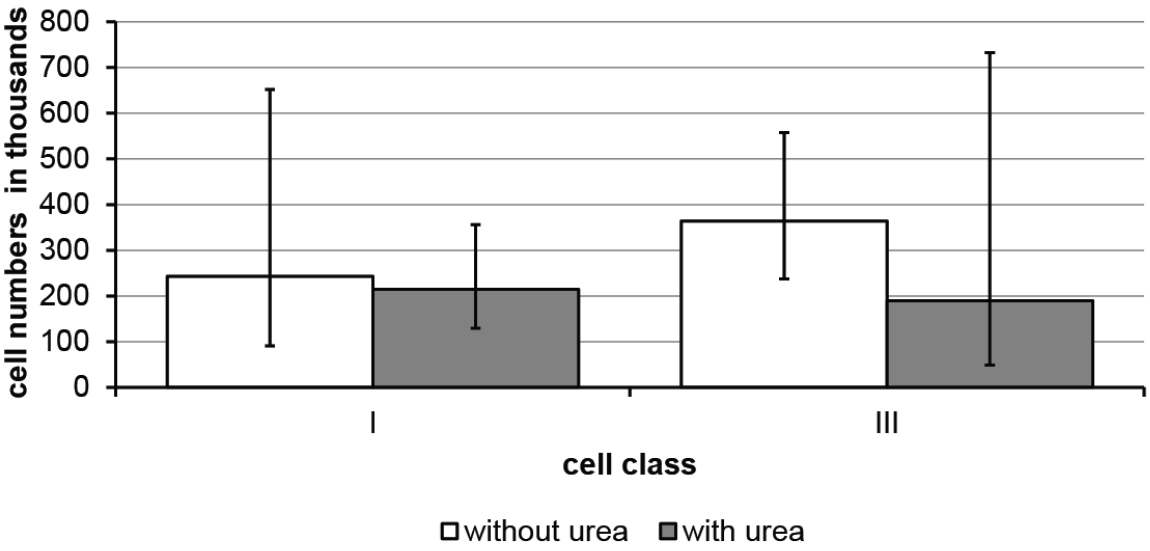

Figure 4-20 Geometric mean and deviation of the cell numbers of bGC I and III after culture with or without urea.

4.2.1.2 Production of Estrogens and Progesterone

The distribution of the variables ES and P4, calculated per 6 cell carriers per 2 hours, was skewed to the right, so the geometric mean and the dispersion factor were calculated for the data description ($\bar{x}_g$, DF). For better clarity, these values are presented on a logarithmic scale to the basis 2 in the corresponding diagrams.

4.2.1.2.1 Total Estrogens

The production of ES from bGC I started with an average of 389.71 pg (2.71) per 2 hours during the static preculture (Figure 4-21). During the first two-hour interval in the perfused system, the production of ES dropped to about 20 % of this value in the culture without urea (65.14 pg, 3.5) and to about 30 % of it in the culture with urea (109.25 pg, 1.68). After that low point, the production of ES rose again during the next 2 hours to values about 1.5, respectively 2 times higher than before (118.30 pg, 1.79 without urea and 143.0837 pg, 1.54 with urea, respectively). After this rise, it declined again in both culture types over the following 4 hours to values slightly lower than at the first low point (50.80 pg, 6.29 without urea and 92.72 pg, 2.02 with urea, respectively). During this rise and fall, the production of ES was always lower in the culture containing urea than in the culture without urea. In the next three two-hour intervals, a similar rise and fall of the production of ES could be monitored as over the six hours before, with the difference that, after half of the time, the production of ES in the culture

with urea started to be lower than the production of ES in the culture without urea.

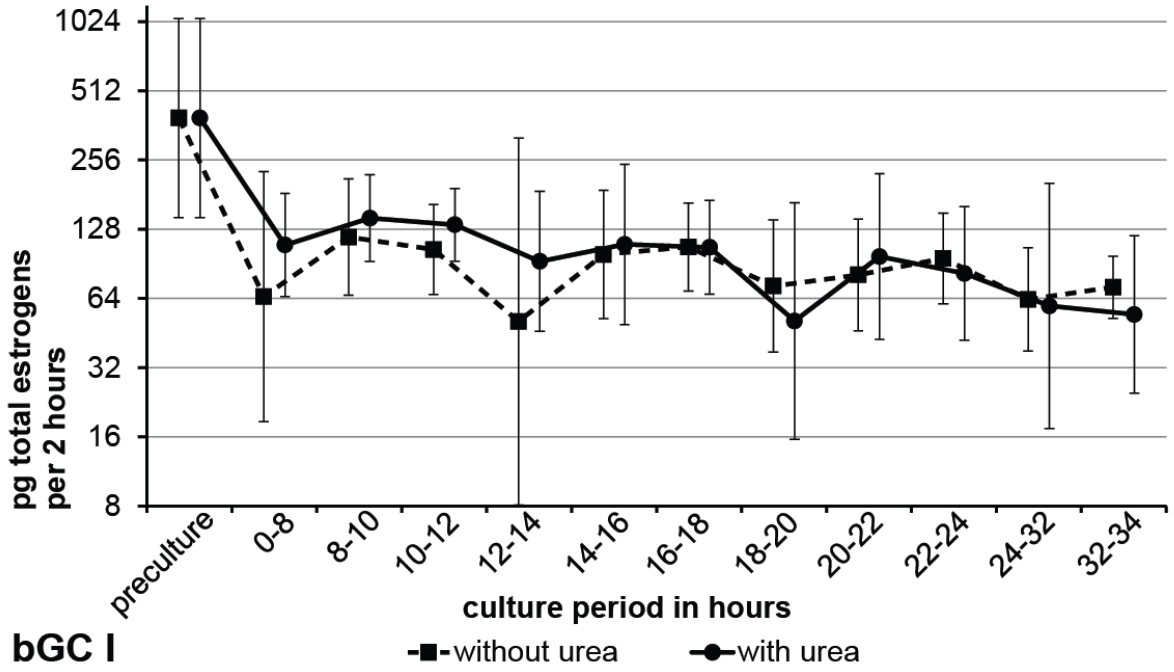

Figure 4-21 Geometric mean and deviation of the production of total estrogens by bGC I cultured with or without urea.

Also, the maximum values reached during this period were slightly lower and reached similarly, after 2 hours in the culture with urea and, retarded, after 4 hours in the culture without urea (109.91 pg, 2.23 with urea and 107.18 pg, 1.55 without urea, respectively). Accordingly, also the low point was lower at the end of this interval (50.82 pg, without urea, 92.72 pg with urea). In the following two, respectively four hours, the ES production rose again in the culture with urea and without urea (97.35 pg, 2.29 and 95.57 pg, 1.57, respectively). Because the next time interval monitored was the 8 hours over night interval, these 6 hour patterns could not be further followed. During the 8 hours interval, the production of ES averaged over 2 hours was low and similar to the low point values before (63.35 pg, 1.68 in the culture without urea and 59.37 pg, 3.42, in the culture with urea). During the last two-hour interval in the morning, the production of ES was still lower in the perfused system with urea than in the one without urea, whereby these values were also quite similar to the low point values (71.56 pg, 1.36 without urea and 54.49 pg, 2.20 with urea).

If the data from the four experimental repeats are examined separately, the oscillating pattern described before during the two-hour interval monitoring of ES production is

most clearly observable in the experiments where the preculture production was not higher than 500 pg (Figure 4-22 a, b and c).

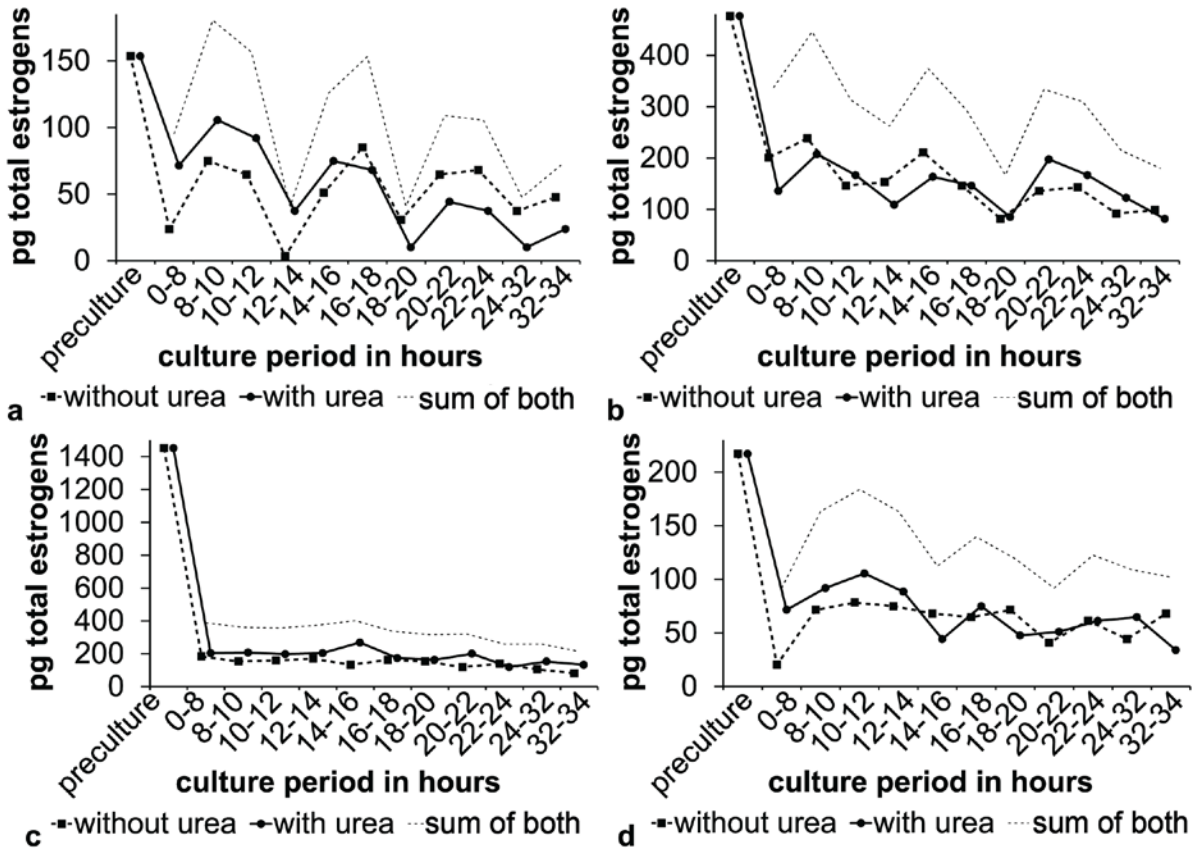

Figure 4-22 Production of total estrogens by bGC I cultured with or without urea and the sum of both values, (a) experiment 1, (b) experiment 2, (c) experiment 3, (d) experiment 4.

bGC III produced an average of 4 162.97 pg (1.34) ES per 2 hours during the static preculture, which is more than 10 times the amount that was produced by bGC I (Figure 4-23). After the transfer to the perfused system, the production during the first two-hour interval was only 30 % as high as the averaged amount in the preculture (1 144.15 pg, 1.16 without urea and 1 224.31 pg, 1.37 with urea). During the rest of the culture period, the amount of ES steadily declined, with exception of the last measurement in the culture with urea. In the earliest four two-hour intervals, the production of ES was first slightly lower in the culture without urea, then in the culture with urea, and then twice in the culture without urea. After that, the amount of ES measured was always lower in the culture with urea, whereby the values were similar, with exception of the second last measurement, where the production of ES was almost only half as high in the culture with urea than in the culture without urea (218.91

pg, 2.78 and 393.40 pg, 1.14, respectively). At the end of the culture, the cells in the culture without urea produced 286.41 pg (1.27) ES and in the culture with urea 267.65 pg ES (1.45).

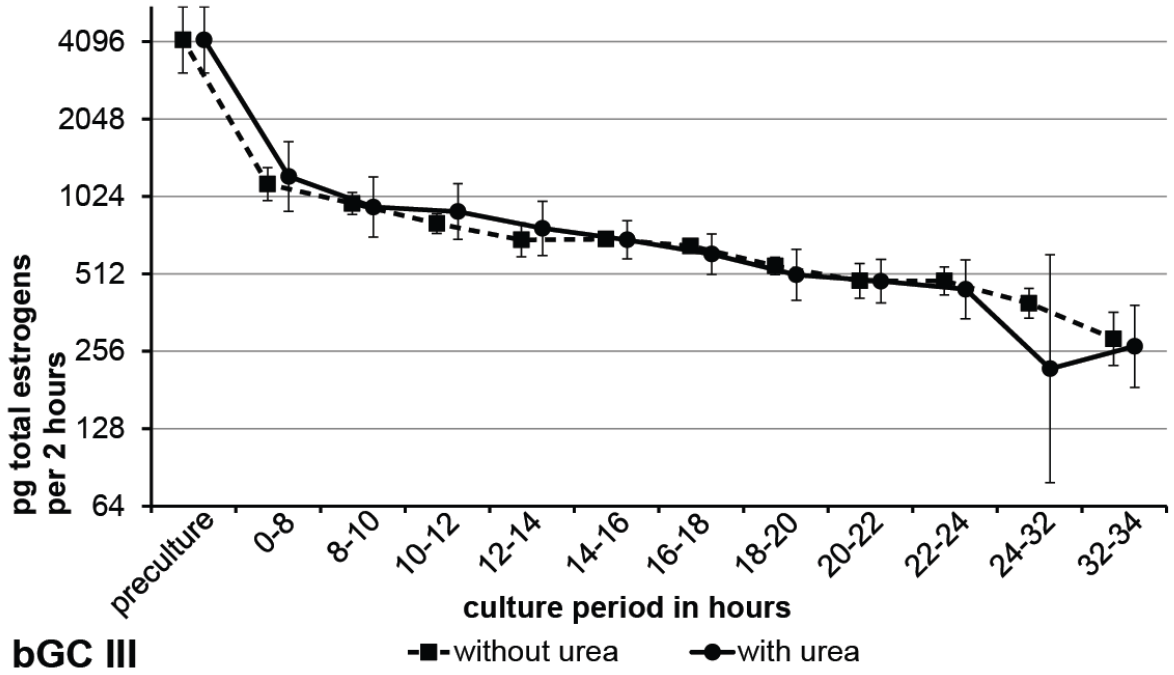

Figure 4-23 Geometric mean and deviation of the production of total estrogens by bGC III cultured with or without urea.

Due to their right-skewed distribution, the data were $\log_{10}$ transformed for the statistical analysis. The cell class had a significant effect on the production of ES (p = 0.0066), whereby no significant effect of urea on the production of ES could be confirmed (p = 0.8256). A highly significant effect on the ES production had the culture time (p = 0.0001), and also the interaction between cell class and time was significant (p = 0.0034). No significant interaction could be found between cell class and urea (p = 0.2859), urea and culture time (p = 0.3080) and all three factors cell class, urea and time (p = 0.5024).

As an additional parameter, the production of ES in the last measured interval was normalised using the cell count obtained at the end of the culture (Figure 4-24). The production of ES per 1 000 cells was calculated for each experimental repeat, and the arithmetic mean and the standard deviation calculated. In bGC I, the relation between the culture without and with urea stayed the same, with the amount of ES produced per 1000 cells being higher in the culture without urea than in the culture with urea (0.36 ± 0.30 pg and 0.27 ± 0.13 pg, respectively). In bGC III, the production of ES not

normalised for the cell count was higher in the culture without urea than in the culture with urea, but after normalisation to cell numbers, this relation switched, and the production of ES was lower in the culture without urea than with urea (0.80 ± 0.19 pg and 2.17 ± 2.58 pg, respectively).

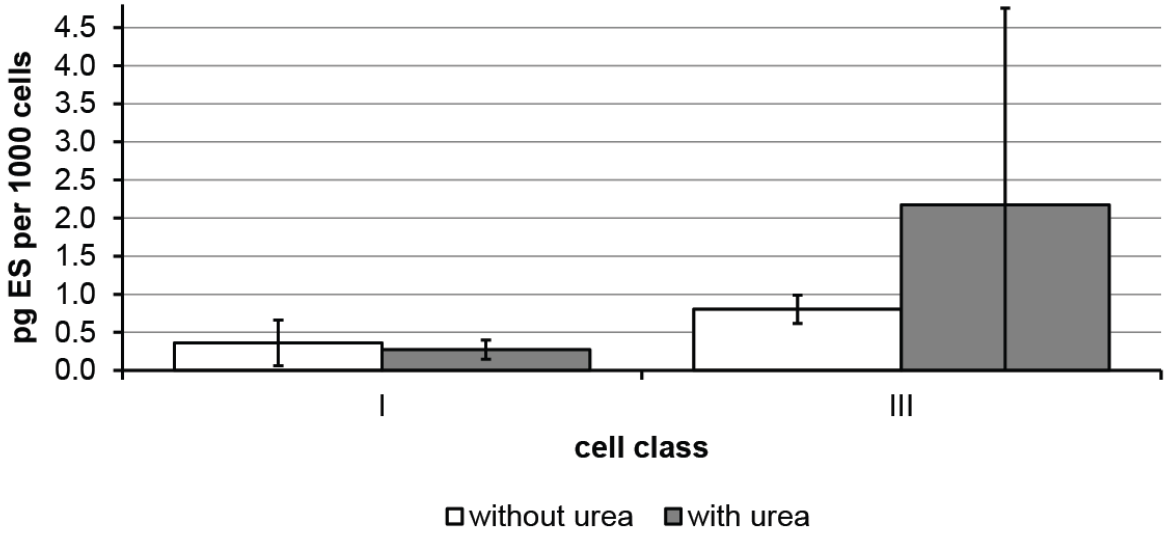

Figure 4-24 Arithmetic mean and standard deviation of the production of total estrogens per 1 000 cells by bGC I and III after the culture with or without urea.

4.2.1.2.2 Progesterone

The production of P4 of bGC I in the static preculture was 32 146.23 pg (1.73) per 2 hours (Figure 4-25). During the first two-hour interval in the perfused system, the cells produced only about 15 % in the culture without urea and about 10 % in the culture with urea in comparison to the preculture (4 855.37 pg, 4.03 and 3 483.54 pg, 4.07, respectively). During the whole culture period, the production of P4 declined in both culture types. Thereby the amount of P4 produced in the culture with urea was, with one exception of about 45 % in the second two-hour interval, always 70 - 85 % of the amount produced in the culture without urea. In the last two-hour interval, the cells in culture without urea produced 3 063.11 pg (1.45) and with urea 2 395.27 pg P4 (1.82). The production of P4 in the static preculture was with an average of 28 670.18 pg (1.47) per 2 hours lower in bGC III than in bGC I (Figure 4-26). In the first eight-hour interval in the perfused system, the produced amounts of P4 dropped to 10 % and 8 % of the preculture values in the system without and with urea (3076.09 pg, 5.64 and 2258.24 pg, 4.08, respectively).

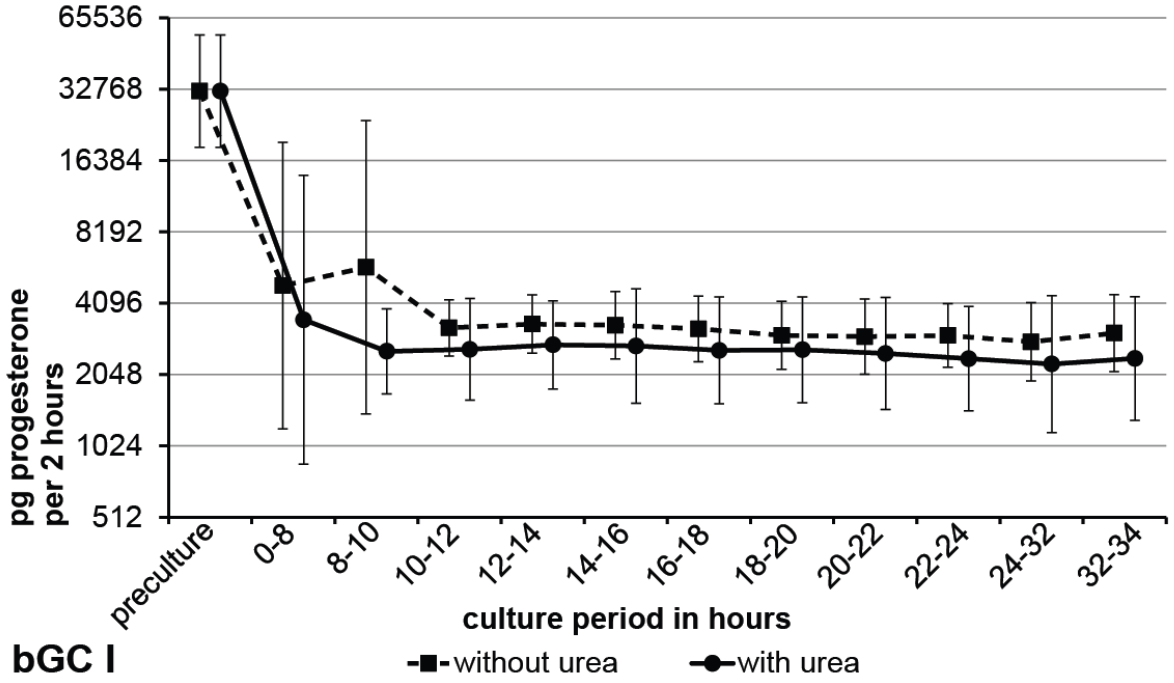

Figure 4-25 Geometric mean and deviation of the production of progesterone by bGC I cultured with or without urea.

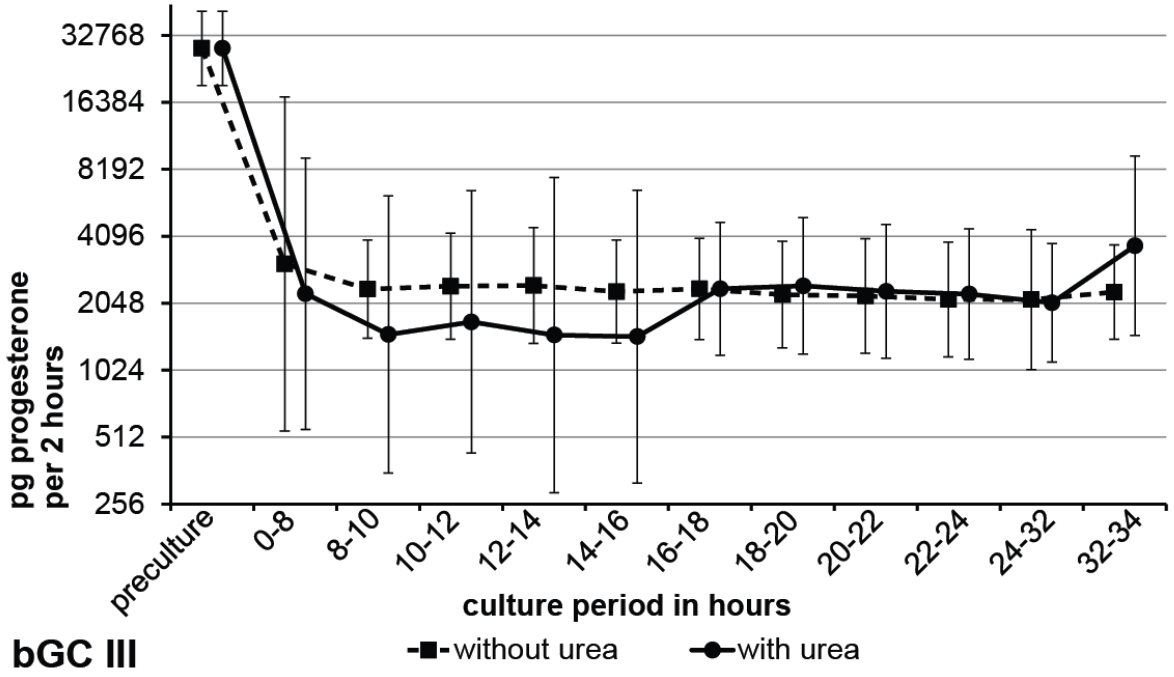

Figure 4-26 Geometric mean and deviation of the production of progesterone by bGC III cultured with or without urea.

During the following four two-hour intervals, the production of P4 was higher in the culture without than in the culture with urea, whereby the production was slightly declining to 2 308.88 pg (1.70) in the culture without urea, and 1 448.16 pg (4.56) in the

culture with urea between 14 and 16 hours. In the following two-hour interval, the production of P4 in the system containing urea rose more than 1.5 times (2377.64 pg, 1.99), whereby the production of P4 in the system without urea only showed a slight increase (2374.28 pg, 1.69). From this time interval on and over the following three ones, the production of P4 was higher in the culture with urea than in the culture without urea, but still declining in both systems. During the overnight period, the production of P4 averaged per 2 hours was again slightly higher in the culture without urea than in the culture with urea (2 127.06 pg, 2.06 and 2056.56 pg, 1.85, respectively). In the last two-hour interval, the production of P4 rose again in both culture types, whereby it reached levels in the culture with urea that were higher than during all 10 intervals before (3 712.552 pg, 2.53) and also higher than the P4 production in the culture without urea (2 296.74 pg, 1.63).

Due to their right-skewed distribution the data were $\log_{10}$ transformed for the statistical analysis. There was a highly significant effect of the culture time on the production of P4 (p = 0.0001). No significant influence had the cell class (p = 0.3479) and the addition of urea (p = 0.6324). The interaction between cell class and urea, cell class and culture time and also urea and culture time was not significant (p = 0.7684, p = 0.3317 and p = 0.3841, respectively). Moreover, no significant interaction could be found between cell class, metabolite and time (p = 0.7955).

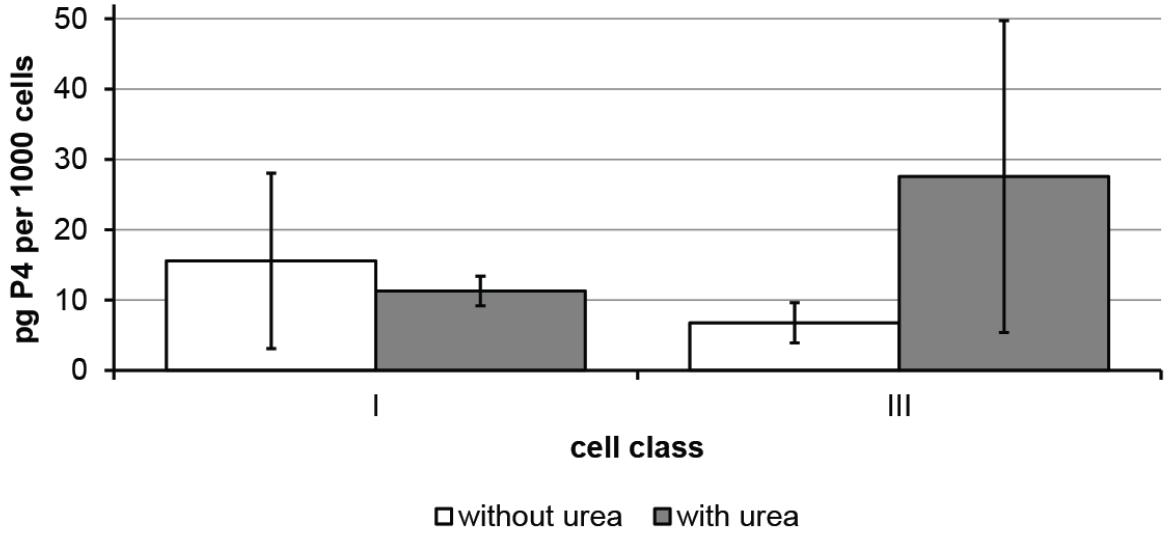

Figure 4-27 Arithmetic mean and standard deviation of the production of progesterone per 1 000 cells by bGC I and III after the culture with or without urea.

As additional parameter, the production of P4 in the last measured interval was normalised using the cell count obtained at the culture end (Figure 4-27). The production of P4 per 1 000 cells was calculated for each experimental repeat, and the arithmetic mean and the standard deviation calculated. The relation between the culture with and without urea stayed the same as in the data not corrected for the cell count. The production of P4 per 1 000 cells was highest in bGC III cultured with urea, and this value was more than four times higher than in the culture of bGC III without urea (27.57 ± 22.18 pg and 6.78 ± 2.88 pg, respectively). In bGC I, the production of P4 per 1 000 cells was almost 1.5 times higher after the culture without urea than after the culture with urea (15.59 ± 12.48 pg and 11.30 ± 2.12 pg, respectively).

4.2.1.2.3 Ratio of Total Estrogens to Progesterone

The ratio of ES to P4 was calculated by dividing the amount of ES with the amount of P4 produced during the last two-hour interval. The ratio of ES to P4 was higher in the culture without than in the culture with urea (mean without urea: 0.07, mean with urea: 0.05). In the culture without urea, the ratio of ES to P4 was higher in bGC III (0.12) than in bGC I (0.02). In the culture with urea, this ratio was also higher in bGC III (0.07) than in bGC I (0.02).

4.2.1.3 Expression of Key Genes for Steroidogenesis

The variables of the gene expression showed a very high variability and were therefore not only $\log_{10}$ transformed for the statistical analysis, but the geometric mean and the dispersion factor ($\bar{x}_g$ (DF)) calculated for the data description are, for better clarity, also presented on a logarithmic scale.

4.2.1.3.1 Aromatase

In the pre-seeding samples, the relative expression of P450arom was more than 40 times higher in bGC III than in bGC I (32 006.7 (1.43) and 736.43 (2.46), respectively) (Figure 4-28). After the culture, the relative expression of P450arom was much lower, whereby the relative expression was still higher in bGC III than in bGC I. In bGC I, there was only a small difference between the culture without and with urea, both being about 100 times lower than the pre-seeding samples (7.52 (2.20)and 7.03 (3.02), respectively). In bGC III, the relative expression of P450arom after the culture with urea was about 1.5 times higher than after the culture without urea (17.16 (3.03) and

11.25 (9.28), respectively), but still only 0.05 % as high as the expression in the pre-seeding sample.

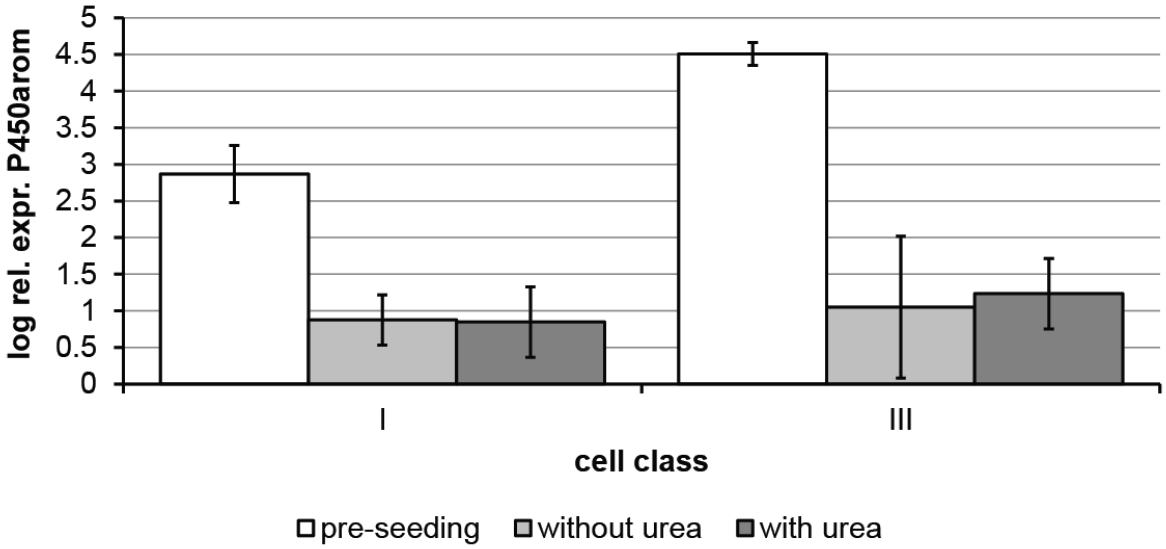

Figure 4-28 Logarithm of the relative expression of P450arom by bGC I and III before and after culture with or without urea.

The cell class had a significant influence on the relative expression of P450arom (p = 0.0090), as well as the culture time (p = 0.0013). No significant influence of urea on the relative expression of aromatase could be confirmed (p = 0.6842). Between cell class and time was a significant interaction (p = 0.0083), whereby interactions between cell class and urea, urea and culture time and between all three factors cell class, urea and culture time were not significant (p = 0.5809, p = 0.6942 and p = 0.5809, respectively).

4.2.1.3.2 3β-Hydroxysteroid Dehydrogenase

The relative expression of 3β-HSD in the pre-seeding samples was about twice higher in bGC III than in bGC I (4.21 (1.53) and 1.92 (1.18), respectively)(Figure 4-29). At the end of the culture, bGC I cultured without urea expressed nearly 6 times and bGC I cultured with urea more than 8 times higher levels of 3β-HSD compared to the pre-seeding sample (11.25 (1.23) and 16.30 (1.73), respectively). In bGC III, the relative expression at the culture end was also more than 4 times higher compared to the pre-seeding samples, whereby the relative expression of 3β-HSD was higher after the culture without urea than after the culture with urea (18.48 (1.60) and 16.38 (1.64), respectively). The relative expression was similar in bGC I and bGC III after the culture containing urea.

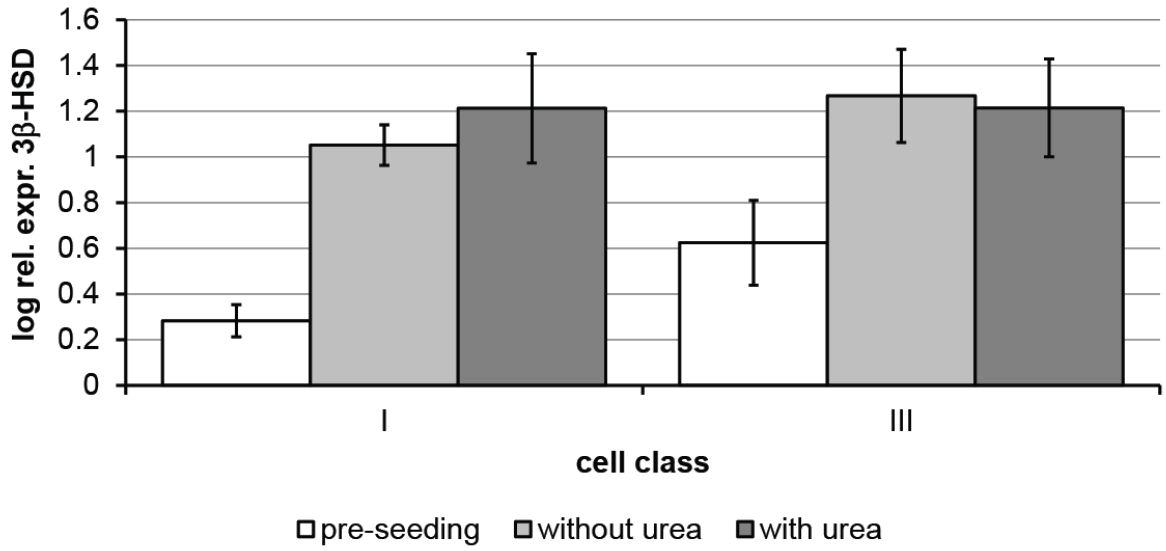

Figure 4-29 Logarithm of the relative expression of 3β-HSD by bGC I and III before and after culture with or without urea.

A significant influence of the cell class and the culture time on the relative expression of 3β-HSD was proven (p = 0.0182 and p = 0.0005, respectively). The influence of urea on the relative expression was not significant (p = 0.2683). No significance was also detected for the interaction of cell class and urea (p = 0.2683), cell class and culture time (p = 0.1643), urea and culture time (p = 0.2683) and for all three factors together (p = 0.2122).

4.2.1.3.3 FSH-Receptor

The relative expression of the FSH-R in the pre-seeding samples was about 1.5 times higher in bGC III than in bGC I (1412.43 (1.80) and 911.89 (1.22), respectively)(Figure 4-30). The relative expression at the end of the culture was markedly lower compared to the pre-seeding relative expression. In bGC I, the relative expression was only about 10 % of the pre-seeding expression after the culture without urea (10.15697 (2.34)), and the relative expression after the culture with urea was less than 50 % compared to the culture without urea (4.17 (4.45)). In bGC III, a very low relative expression of FSH-R could be detected after the culture without urea (1.13 (2.96)), and the relative expression after the culture containing urea was comparable to the relative expression in bGC I after the culture with urea (4.83 (5.32)). Even if the expression of FSH-R in bGC III was higher after the culture with urea compared to the culture without, it was still only less than 0.5 % of the relative expression in the preculture sample. The highest post culture relative expression was found in bGC I cultured without urea,

followed by bGC I and bGC III cultured with urea and then by bGC III cultured without urea.

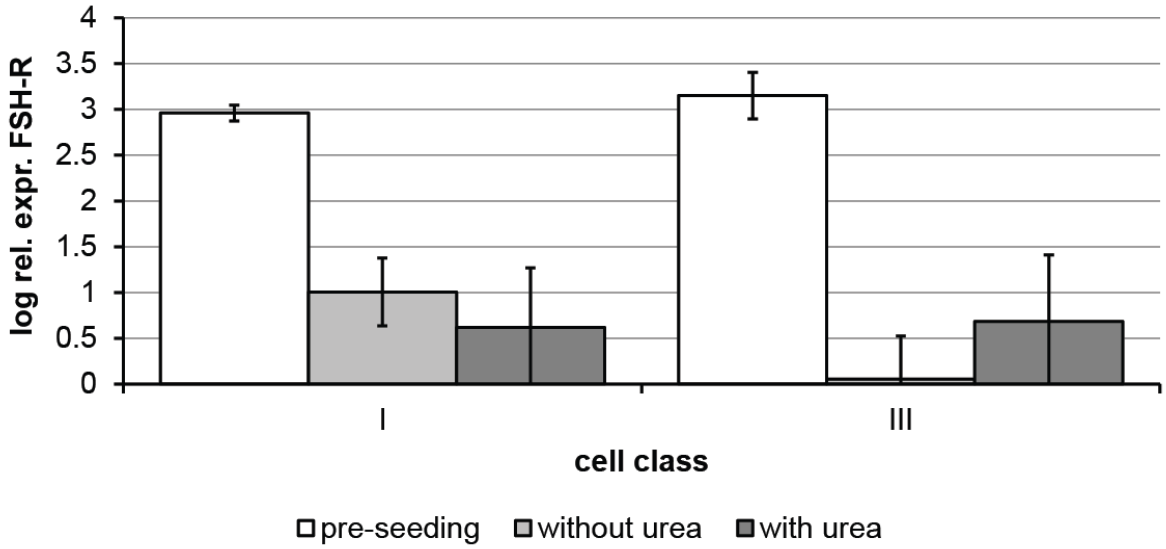

Figure 4-30 Logarithm of the relative expression of FSH-R by bGC I and III before and after culture with or without urea.

The culture time had a significant influence on the relative expression of the FSH-R (p = 0.0016). No significant influence was detected for the cell class and urea (p = 0.1827 and p = 0.7846, respectively). The interaction between cell class and urea was just under the significant level (p = 0.0580), as was the interaction between cell class, urea and culture time (p = 0.0580). The interaction between cell class and culture time and between urea and culture time were not significant (p = 0.1044 and p = 0.7846, respectively).

4.2.1.3.4 LH-Receptor

The expression of LH-R was only detectable in pre-seeding samples of bGC III and had a very high variability (1.10 (1.89)) (Figure 4-31).

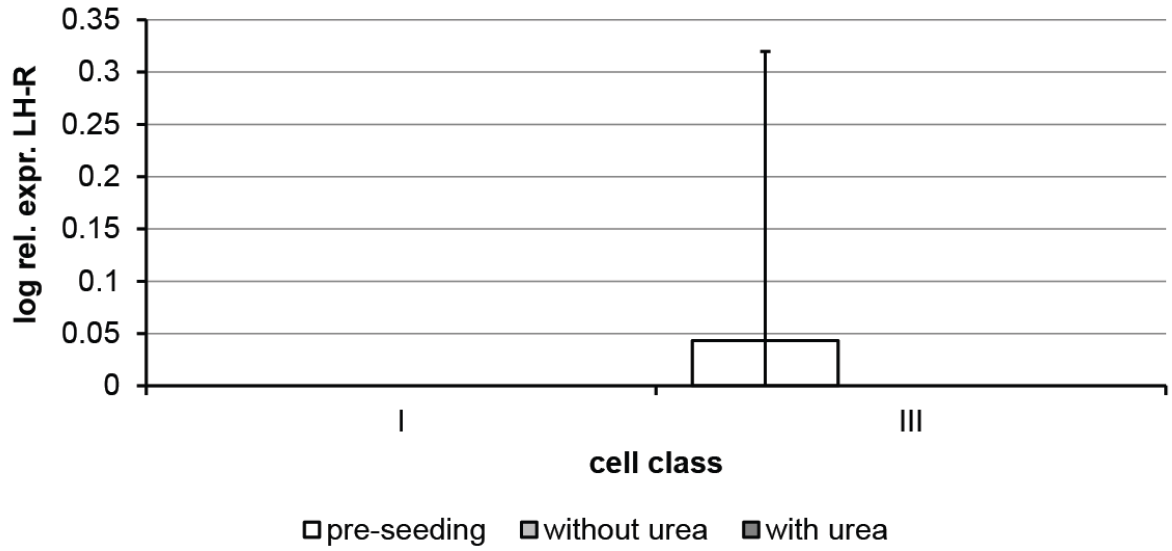

Figure 4-31 Logarithm of the relative expression of LH-R by bGC I and III before and after culture with or without urea.

4.2.2 Influence of β-Hydroxybutyric Acid on Granulosa Cell Function and Cell Numbers

4.2.2.1 Cell Numbers

The distribution of the variable cell count, calculated per 6 cell carriers, was skewed to the right, so the geometric mean and the dispersion factor were calculated for the data description ($\bar{x}_g$, DF). In both cell classes, the cell numbers were lower if cultured in the presence of β-HB (Figure 4-32). The numbers of bGC I cultured without β-HB were about 1.5 times higher than the number of bGC I cultured in the presence of β-HB (421 178.8 (1.27) and 283 075.5 (1.27), respectively). For bGC III, the difference between the culture without and with β-HB was only about 10 % (506 717.4 (1.04) and 463 191.9 (1.21), respectively). Generally, the cell count was higher in the culture of bGC III than in the culture of bGC I. Due to their right-skewed distribution, the data were $\log_{10}$ transformed for the statistical analysis. The influence of the cell class on the cell numbers was just below the significant level (p = 0.0552). No significance was detected for the influence of β-HB on the cell count (p = 0.1579) and for the interaction between cell class and β-HB (p = 0.1743).

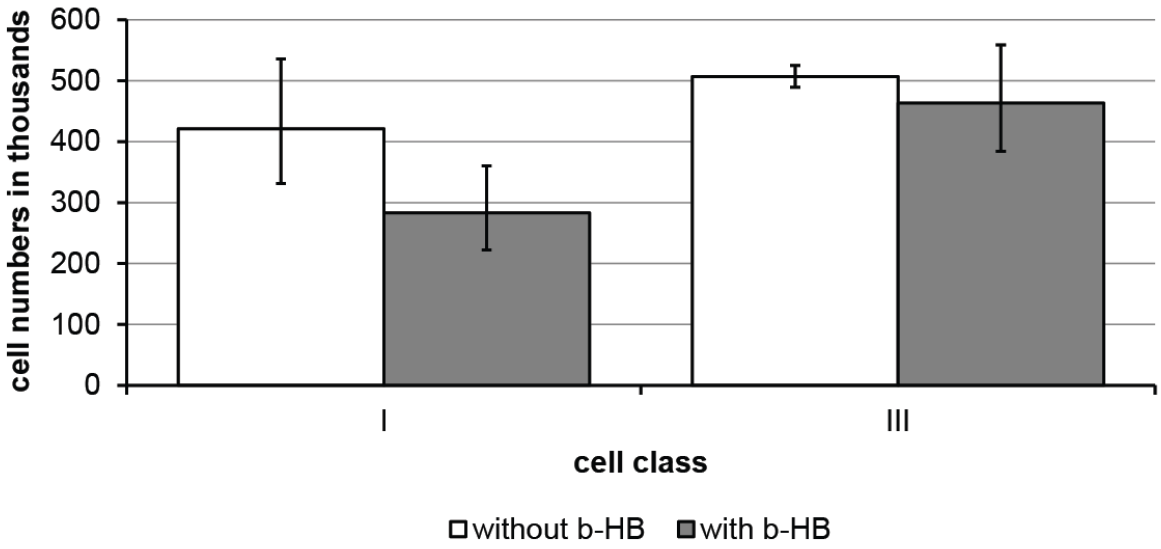

Figure 4-32 Cell numbers of bGC I and III after the culture with or without β-HB (b-HB).

4.2.2.2 Production of Estrogens and Progesterone

The distribution of the variables ES and P4, calculated per 6 cell carriers per 2 hours, was skewed to the right, so the geometric mean and the dispersion factor were calculated for the data description ($\bar{x}_g$, DF). For better clarity, these values are presented on a logarithmic scale to the basis of 2 in the corresponding diagrams.

4.2.2.2.1 Total Estrogens

bGC I produced an average amount of 260.30 pg (1.91) ES per 2 hours during the static preculture period (Figure 4-33). After the transfer to the perfusion culture system, the production of ES per 2 hours declined in the culture period 0-8 hours to about 20 % of the preculture amount in the culture without β-HB and to about 30 % of the preculture amount in the culture containing β-HB (58.09 pg, 2.51 and 79.76 pg, 2.14, respectively). In the following two-hour period (8-10), the production of ES increased more than 10 % in both systems (72.47 pg, 1.80 without β-HB and 89.09 pg, 1.78 with β-HB, respectively). In the culture without β-HB, the amount of ES in the following two-hour period (10-12) declined about the same 10 % again (58.09 pg, 2.22), whereby this decline was followed by an increase in ES production in the time interval between 12 and 16 hours, ending with a similar ES production as in the culture period 8-10 hours (73.46 pg, 1.63). During the two two-hour intervals between 16 and 20 hours, the amount of ES produced in the system without β-HB declined again over 30

% (48.73 pg, 2.40).This decline was again followed by a rise between 20 and 24 hours culture time, reaching the highest amount of ES produced during the whole perfusion culture without β-HB (76.77 pg, 1.61). In the following overnight interval between 24-32 hours, the average amount of ES produced during 2 hours was more than 50 % lower than the average levels reached before (29.16 pg, 4.21). Even lower, but with a very high dispersion factor, was the amount produced during the last interval 32-34 hours (9.74 pg, 24.84). With exception of the 22-24 hours interval, the production of ES was always lower in the system without β-HB, whereby the last culture period (32-34) was similar in both culture types. In the culture with β-HB, the first decline in the perfused system happened during the two time intervals between 10 and 14 hours and the nadir was lower than during the first interval in the perfused system (72.57 pg, 1.60). After a short rise between 14-16 hours, that only reached levels similar to the first interval in the perfused system (79.41 pg, 1.48), the production declined again to an even lower amount in the following two-hour interval (63.07 pg, 1.55). Between 18 and 22 hours, the production of ES increased again about 20 % (75.69 pg, 1.06). Over the rest of the culture period, the production of ES steadily decreased and ended with a low production of ES in the last two-hour interval that was similar to the amount in the culture without β-HB and also had a high dispersion factor (9.52 pg, 24.54).

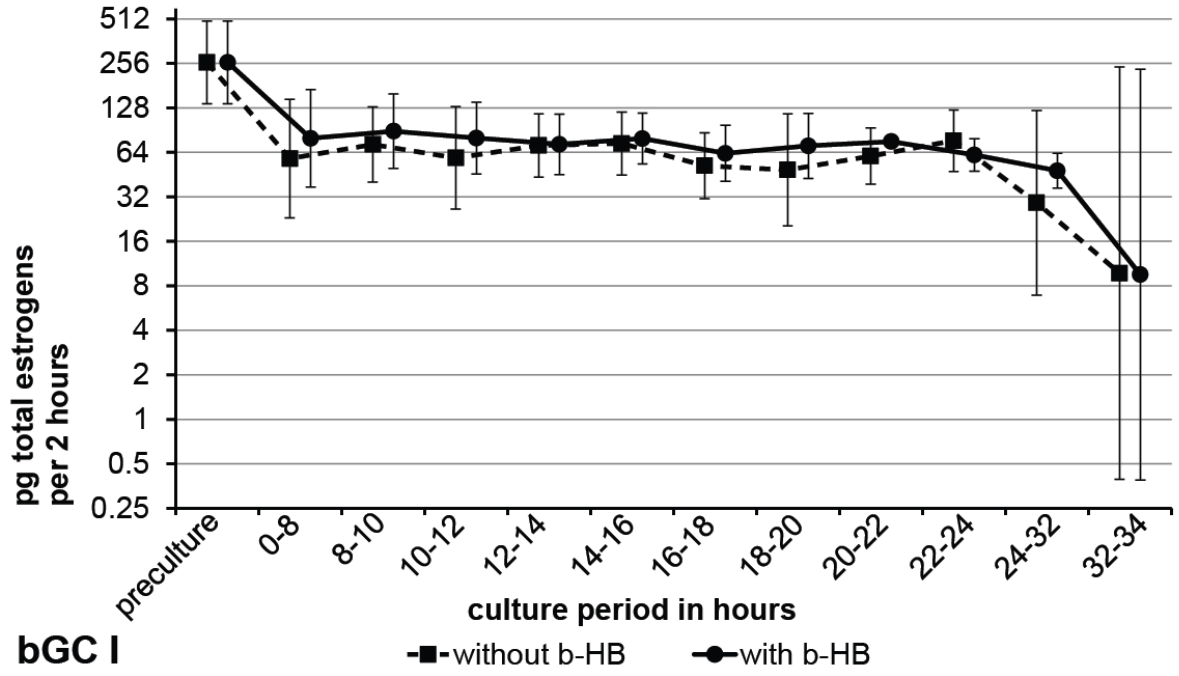

Figure 4-33 Geometric mean and deviation of the production of total estrogens by bGC I in the culture with or without β-HB (b-HB).

As for bGC I examined in the culture with and without urea in chapter 4.2.1.2.1, a time-dependent oscillation can be detected in the production of ES from bGC I, which is more clearly to see if the experimental repeats are examined separately, even if it is not as distinct as in the experiment with or without urea. The pattern here can be as well seen better in the experiments where the preculture production was not higher than 500 pg (Figure 4-34 a, b and c).

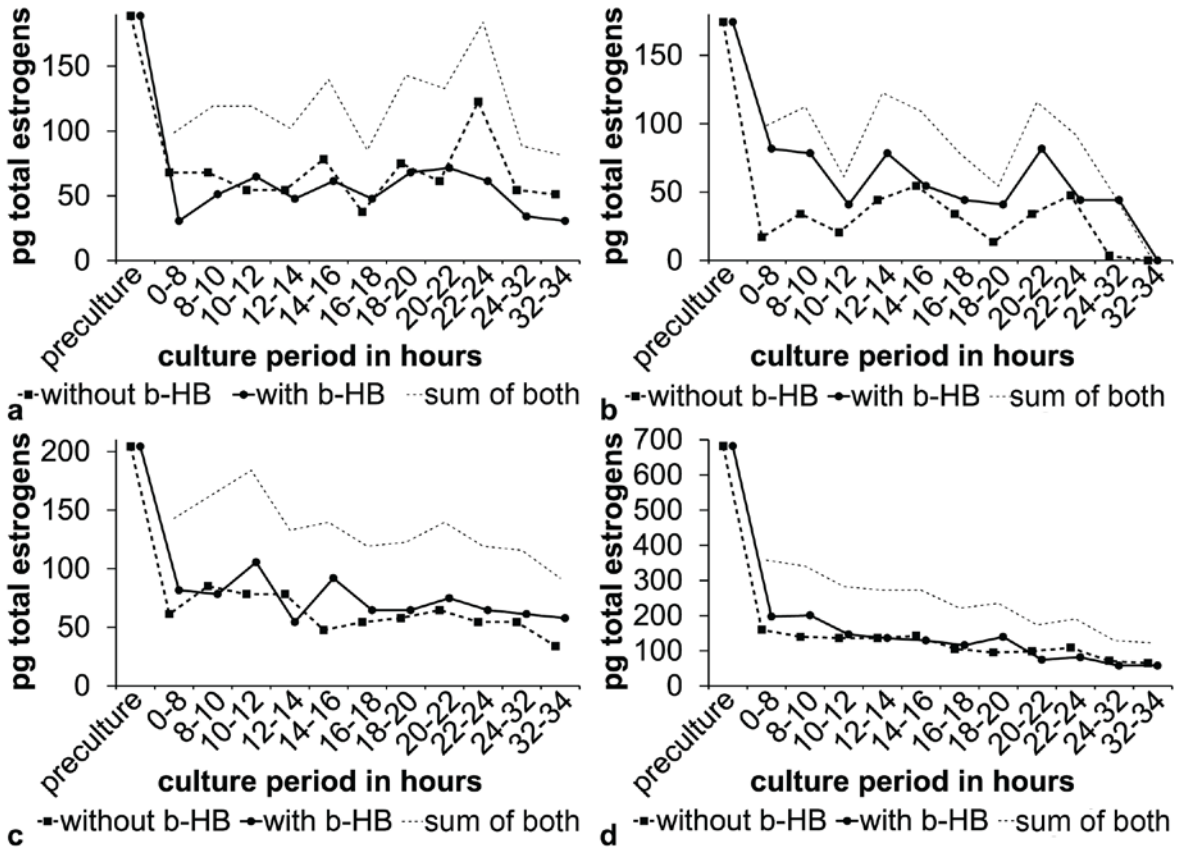

Figure 4-34 Production of total estrogens by bGC I cultured with or without β-HB (b-HB) and the sum of both values, (a) experiment 1, (b) experiment 2, (c) experiment 3, (d) experiment 4.

The average amount of ES produced by bGC III per 2 hours during the preculture period was more than 20 times higher than the amount produced by bGC I (10 634.61 pg, 2.84)(Figure 4-35). During the first culture period in the perfused system, the production of ES was only about 15 % as high as during the preculture period. During the whole culture time, the production of ES steadily declined in both culture types, finally ending in amounts of about 30 % of the amount produced at the begin of the perfused culture (506.98 pg, 1.12 without β-HB and 497.90 pg, 1.16 with β-HB). Thereby, the amount of ES produced in the system without β-HB was very similar to

the amount of ES produced in the system with β-HB, whereby differences did not exceed 11 %. With exception of the culture period 22-24 and 32-34 hours, the production of ES was always marginally lower in the culture without β-HB.

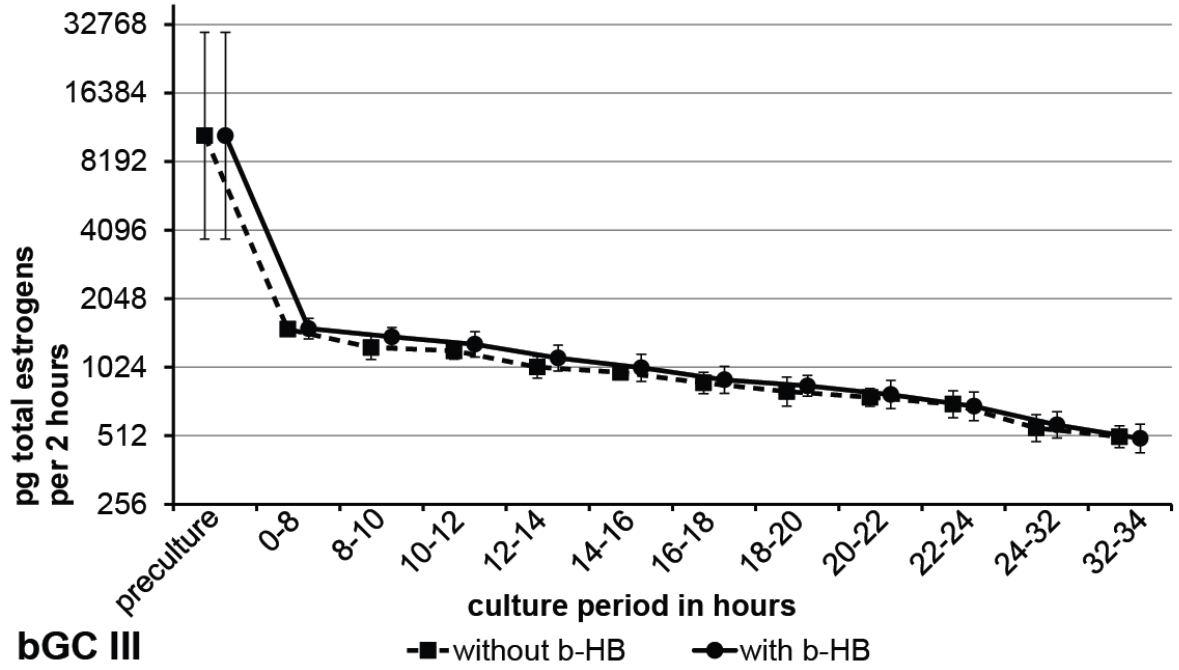

Figure 4-35 Geometric mean and deviation of the production of total estrogens by bGC III in the culture with or without β-HB (b-HB).

Because of their right-skewed distribution, the data were $\log_{10}$ transformed for the statistical analysis. The influence of the culture time on the production of ES was highly significant (p = 0.0001). A significant interaction was also proven between cell class and culture time (*p* = 0.0304). The cell class and β-HB had no significant influence on the production of ES (*p* = 0.7632 and *p* = 0.5327, respectively). Also, interactions between cell class and β-HB, β-HB and culture time and between all three factors were not significant (*p* = 0.4877, *p* = 0.4957 and *p* = 0.6995, respectively).

As an additional parameter, the production of ES in the last measured interval was normalised using the cell count obtained at the culture end. The production of ES per 1 000 cells was calculated for each experimental repeat, and the arithmetic mean and the standard deviation of these values calculated ($\bar{x}_a \pm \mathrm{SD}$). In bGC III, the relation between the culture without and with urea stayed the same as in the data not normalised for the cell count, with the amount of ES produced per 1000 cells being higher in the culture with β-HB than in the culture without β-HB (1.07 ± 0.11 pg and 1.00 ± 0.08 pg, respectively)(Figure 4-36). In bGC I, the production of ES not

normalised for the cell count was slightly higher in the culture without β-HB than in the culture with β-HB, but after the correction for the cell numbers, this relation switched, and the production of ES was slightly lower in the culture without β-HB than with β-HB (0.09 ± 0.08 pg and 0.12 ± 0.10 pg, respectively).

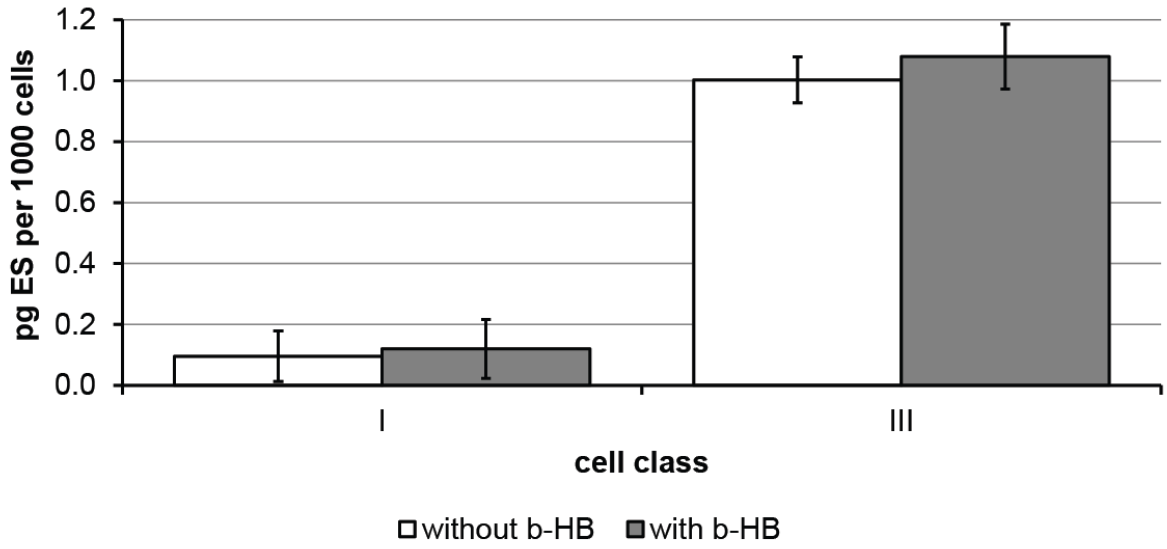

Figure 4-36 Arithmetic mean and standard deviation of the production of total estrogens per 1 000 cells by bGC I and III after the culture with or without β-HB (b-HB).

4.2.2.2.2 Progesterone

The production of P4 from bGC I during the preculture period averaged 29 194.7 pg (1.67) per 2 hours (Figure 4-37). During the first 8 hours in the perfused system, the production of P4 dropped to about 14 % of the preculture amount in the culture without and with β-HB (4 175.18 pg, 1.45 and 4 102.48 pg, 1.73, respectively). In the two culture periods between 8 and 12 hours, the production of P4 increased again about 20 % in the culture without β-HB (5 030.54 pg, 1.36) and about 15 % in the culture with β-HB (4 829.69, 1.76). In the culture without β-HB, the production of P4 decreased over the next three culture periods, but the low point reached at 16-18 hours was higher in comparison to the first perfusion interval (4 435.68 pg, 1.35). After a slight rise followed by an equivalent drop and again a smaller rise between 18 and 24 hours, the production of P4 in the culture without β-HB reached levels during the overnight period 24-32 hours (4 110.85 pg, 1.3), that were lower as the ones detected before. In the final two-hour interval, the amount of P4 had an average level compared to the values from

the perfused culture measured before (4 460.19 pg, 1.30). In the culture with β-HB, a slight decline in P4 production happened between 12 and 16 hours culture time, followed by a slight rise during the following two-hour period. The decline over the next two two-hour periods resulted in a production level of P4 that lies between the two lowest points reached before (4 522.05 pg, 1.72). After a short rise in P4 production in the culture period 22-24 hours, the lowest production of P4 was reached during the over-night period (4 002.00 pg, 1.64). In the last interval between 32 and 34 hours, the production of P4 reached average levels comparable to the whole perfusion culture period (4 531.02 pg, 1.64). Up to the 12-14 hours interval, the production of P4 was slightly lower in the culture with β-HB compared to the culture without β-HB. Between 14 and 24 and 32 and 34 hours, the production of P4 was higher in the culture with β-HB. Overnight, the amount of P4 produced in the culture without β-HB was higher.

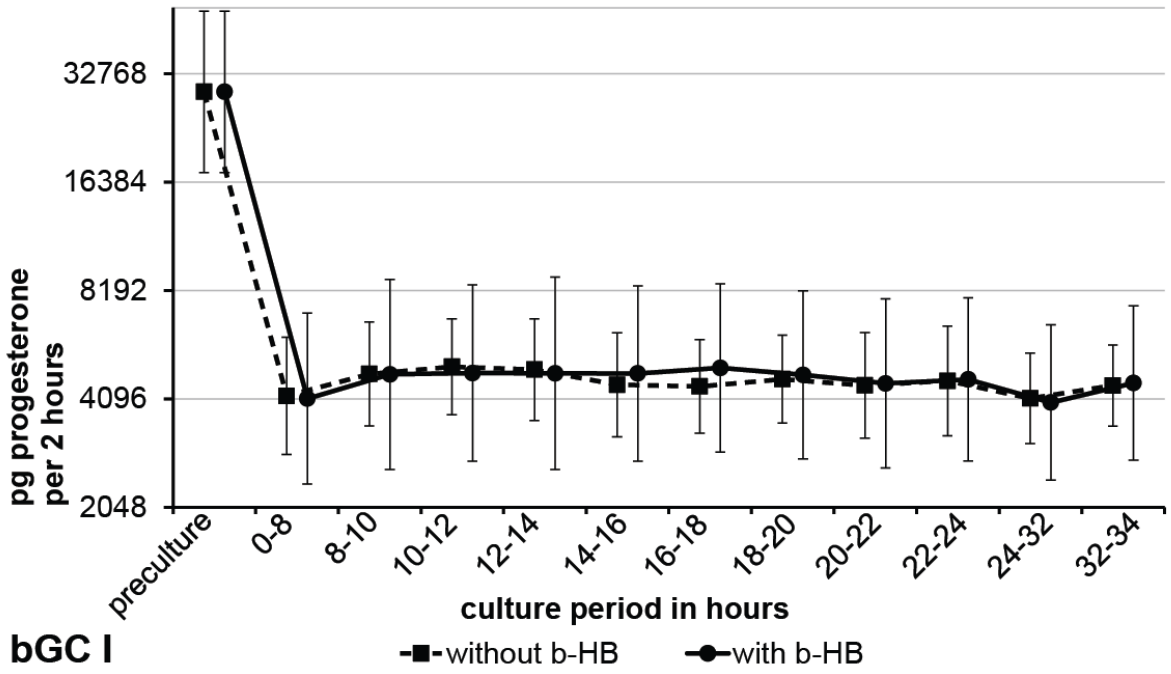

Figure 4-37 Geometric mean and deviation of the production of progesterone by bGC I in the culture with or without β-HB (b-HB).

In bGC III, the production of P4 during the preculture averaged per 2 hours was almost twice as high as in bGC I (55 337.79 pg, 1.17)(Figure 4-38). During the first interval in the perfused culture system, the amount of P4 produced in the culture without β-HB reached only about 15 % of the average amount produced during the preculture period (8 676.22 pg, 3.29), and the amount of P4 in the culture with β-HB was less than half as

high as in the culture without β-HB (3 740.67 pg, 1.83). During the whole culture from 8 hours on, the amount of P4 produced in the culture with β-HB was on average about 1 100 pg lower than in the culture without β-HB. In the culture without β-HB, the production of P4 declined about 35 % until the 10-12 hours culture period (5 479.58 pg, 1.20). After a short rise during 12-14 hours (5 704.35 pg, 1.17), it continued to decline about 20 % over the following five two-hour periods. During the over-night interval, the production of P4 rose again, resulting in an even higher amount of P4 during the last two-hour period the next day (5 162.89 pg, 1.20). In the culture with β-HB, the production of P4 rose about 20 % during the 4 hours after the low value during the first perfused culture interval (4 408.97 pg, 1.64), and decreased, except one slight rise at the culture period 16-18 hours (4 124.54 pg, 1.66), to values similar to the first perfused culture interval at 20-22 hours (3 764.08 pg, 1.71). In the interval between 22 and 24 hours, the production of P4 rose again, but was similarly low again during the over-night period (3 744.36 pg, 1.71).The production of P4 between 32 and 34 hours was higher than that overnight (4 010.16 pg, 1.63).

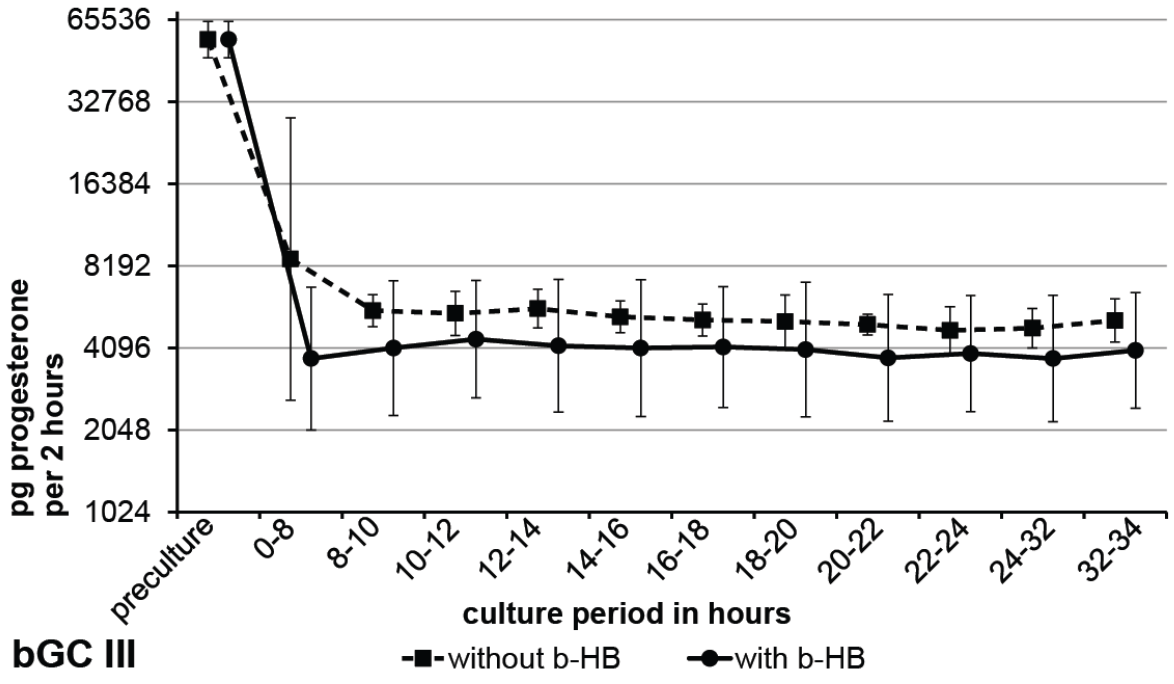

Figure 4-38 Geometric mean and deviation of the production of progesterone by bGC III in the culture with or without β-HB (b-HB).

Because of their right-skewed distribution, the data were $\log_{10}$ transformed for the statistical analysis. A significant influence was proven for the influence of the culture

time on the production of P4 (p = 0.0001). Also, the interaction between cell class and culture type was significant (p = 0.0304). The cell class and β-HB had no significant influence on the P4 production (p = 0.7632 and p = 0.5327, respectively). Also, the interactions between cell class and β-HB, between β-HB and culture time and between all three factors cell class, β-HB and culture time were not significant (p = 0.4877, p = 0.4957 and p = 0.6995).

As additional parameter, the production of ES in the last measured interval was normalised using the cell count obtained at the culture end. The production of P4 per 1 000 cells was calculated for each experimental repeat, and the arithmetic mean and the standard deviation calculated ($\bar{x}_a \pm \mathrm{SD}$). The relation between the culture without and with β-HB stayed the same as in the not for the cell count normalised data (Figure 4-39). In bGC I, the production of P4 per 1 000 cells was about 1.5 times higher in the culture with β-HB than in the culture without β-HB (16.79 ± 5.84 pg and 10.64 ± 1.19 pg, respectively). In bGC III, the production of P4 was slightly higher in the culture without than in the culture with β-HB (10.32 ±1.886 pg and 9.04 ± 3.07 pg, respectively).

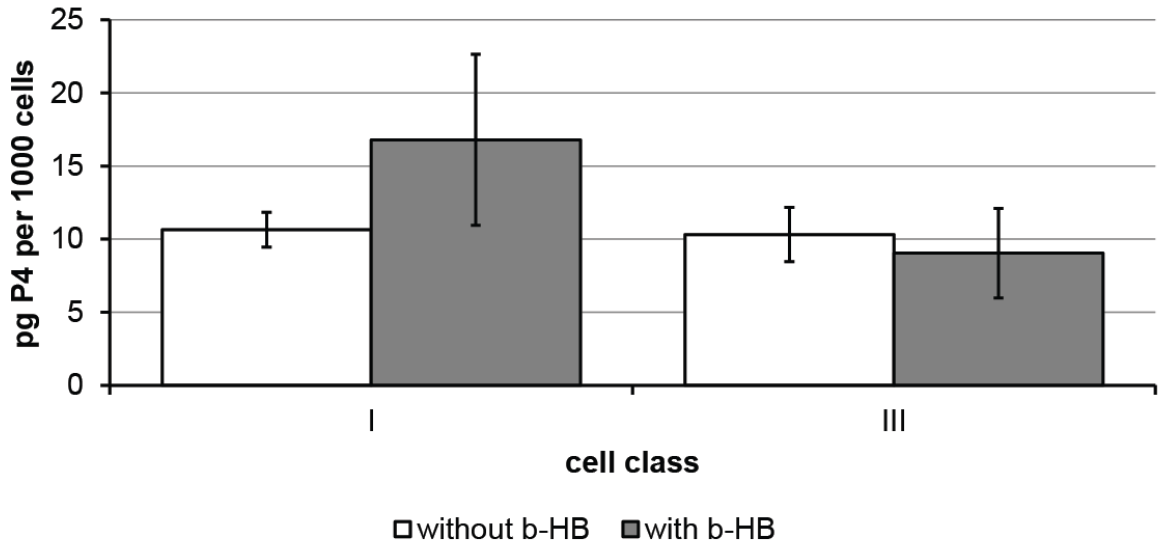

Figure 4-39 Arithmetic mean and standard deviation of the production of progesterone per 1 000 cells by bGC I and III after the culture with or without β-HB (b-HB).

4.2.2.2.3 Ratio of Total Estrogens to Progesterone

The ratio of ES to P4 was calculated by dividing the amount of ES with the amount of P4 produced during the last two-hour interval. The ratio of ES to P4 was higher in the

culture with β-HB than in the culture without β-HB (mean with β-HB: 0.06, mean without β-HB: 0.05). In the culture with β-HB, the ratio of ES to P4 was higher in bGC III (0.12) than in bGC I (0.002). In the culture without β-HB, this ratio was also higher in bGC III (0.10) than in bGC I (0.002).

4.2.2.3 Expression of Key Genes for Steroidogenesis

The variables of the gene expression showed a very high variability and were therefore not only $\log_{10}$ transformed for the statistical analysis, but the geometric mean and the dispersion factor ($\bar{x}_g$ (DF)) calculated for the data description are, for better clarity, also presented on a logarithmic scale.

4.2.2.3.1 Aromatase

The relative expression of P450arom was constantly higher in bGC III than in bGC I (Figure 4-40). In the pre-seeding samples, the relative expression of P450arom was more than 50 times higher in bGC III than in bGC I (38 821.47 (1.46) and774.54 (2.68), respectively). In bGC I, the relative expression was nearly 200 times lower after the culture than pre-seeding, whereby the relative expression in the culture with β-HB was only slightly higher than in the culture without β-HB (4.15 (2.83) and 3.87 (5.19), respectively). In bGC III, the difference was more than one order of magnitude higher between the pre-seeding and the post-culture samples than in bGC I. The post-culture relative expression was thereby nearly similar in the culture with and without β-HB (15.02 (1.60) and 15.20 (2.12), respectively).

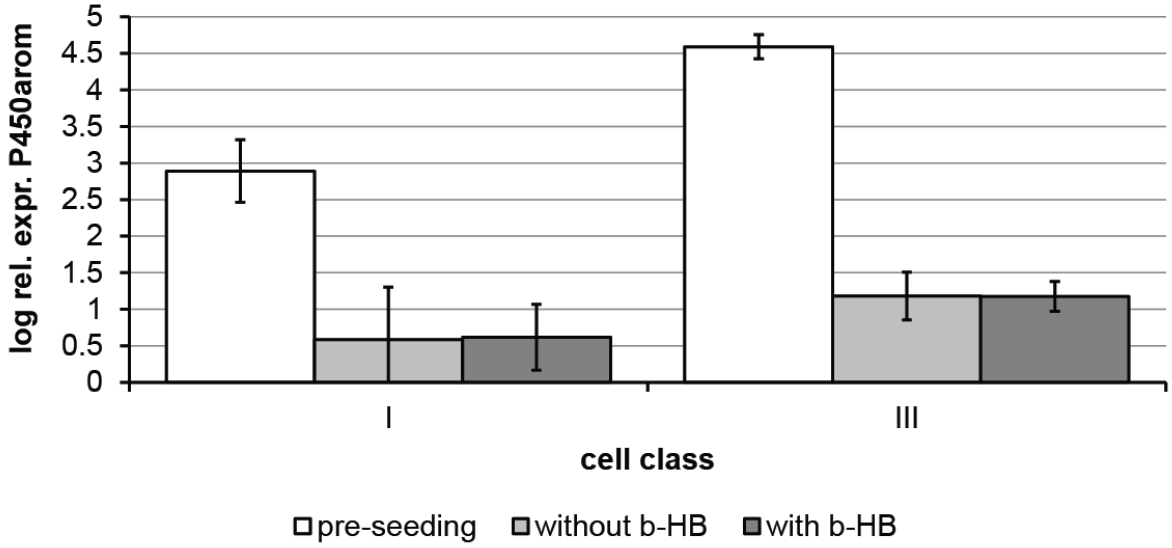

Figure 4-40 Logarithm of the relative expression of P450arom by bGC I and III before and after culture with or without β-HB (b-HB).

The influence of the cell class on the relative expression of aromatase was significant (p = 0.0048), as was the influence of culture time (p = 0.0001) and the interaction between cell class and culture time (p = 0.001). No significance was proven for the influence of β-HB on the relative expression of P450arom (p = 0.8778). No interaction was detectable between cell class and β-HB, between and culture time and between cell class, β-HB and culture time (p = 0.8446, p = 0.8778 and p = 0.8446, respectively).

4.2.2.3.2 3β-Hydroxysteroid Dehydrogenase

The relative expression of 3β-HSD was lower in the pre-seeding than in the post-culture samples. In bGC I, the pre-seeding relative expression of 3β-HSD was about 35 % of the pre-seeding relative expression in bGC III (2.30 (1.17) and 6.45 (1.14), respectively)(Figure 4-41). In bGC I, the relative expression in the post-culture samples rose to levels about 5 times higher than pre-seeding, whereby the relative expression was somewhat less in the culture with β-HB than without (10.75 (1.42) and 11.73 (1.32), respectively). In bGC III, the relative expression in the post-culture samples was almost twice as high as in the pre-seeding sample. In contrast to bGC I, the relative expression of 3β-HSD was slightly higher in the culture with than in the culture without β-HB (11.97 (1.53) and 10.75 (1.64), respectively). The post-culture expression of 3β-HSD had similar levels in bGC I and bGC III.

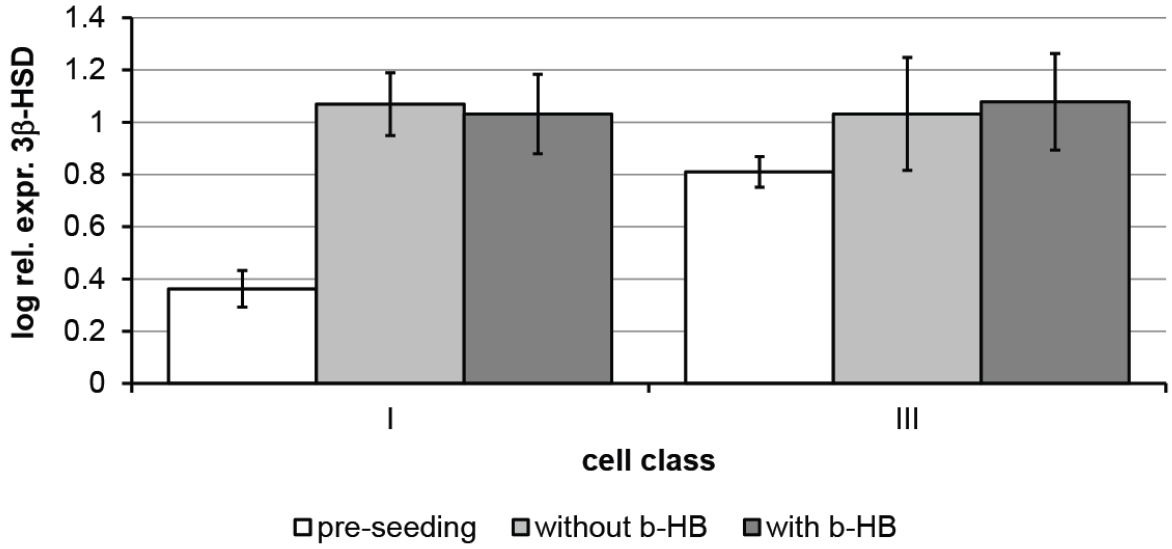

Figure 4-41 Logarithm of the relative expression of 3β-HSD by bGC I and III before and after culture with or without β-HB (b-HB).

The cell class and the culture time had a significant influence on the relative expression of 3β-HSD (p = 0.0078 and p = 0.0061, respectively). The influence of β-HB on the relative expression of 3β-HSD was not significant (p = 0.9258). No significant interactions were proven between cell class and 3β-HSD, between 3β-HSD and culture time and between all three factors cell class, β-HB and culture time (p = 0.0854, p = 0.9258 and p = 0.0854, respectively).

4.2.2.3.3 FSH-Receptor

The pre-seeding relative expression of the FSH-R was about 1.5 times higher in bGC III than in bGC I (1 336.94 (1.29) and 900.18 (1.55), respectively) (Figure 4-42). Post-culture, the relative expression was much lower in bGC I as well as in bGC III, whereby the post-culture relative expression with and without β-HB was higher in bGC I than in bGC III. In bGC I, the relative expression of the FSH-R was about 900 times lower after the culture without β-HB compared to the bGC I pre-seeding sample, whereby the relative expression after the culture with β-HB was about 35 % lower than after the culture without β-HB (8.57 (1.63) and 5.47 (1.49), respectively). In bGC III, the relative expression in the post-culture samples was about 500 times lower than pre-seeding, whereby the relative expression after the culture with β-HB was higher than after the culture without β-HB (3.30 (1.42) and 2.67 (1.88), respectively).

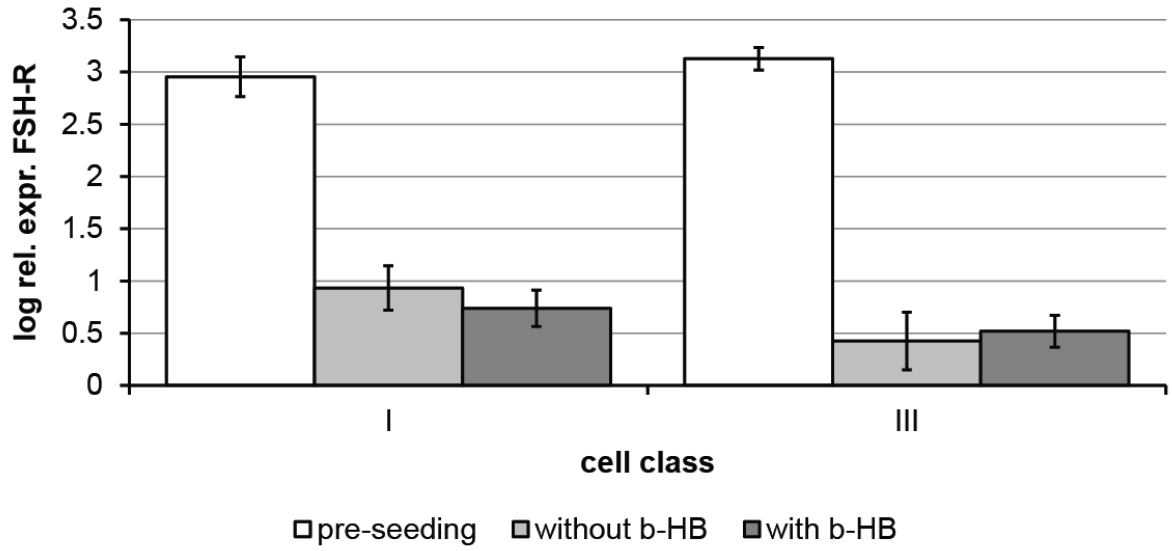

Figure 4-42 Logarithm of the relative expression of FSH-R by bGC I and III before and after culture with or without β-HB (b-HB).

The culture time had a significant effect on the relative expression of the FSH-R (p = 0.0004), as well as the interaction between cell class and culture time (p = 0.0089). The cell class and β-HB had no significant influence (p = 0.1691 and p = 0.5039). No significant interaction was found between the cell class and β-HB, between β-HB and the culture time and between the three factors cell class, β-HB and culture time (p = 0.1830, p = 0.5039 and p = 0.1830, respectively).

4.2.2.3.4 LH-Receptor

The relative expression of LH-R was only detectable in pre-seeding samples of bGC III and had a very high variability (2.58 (2.60))(Figure 4-43).

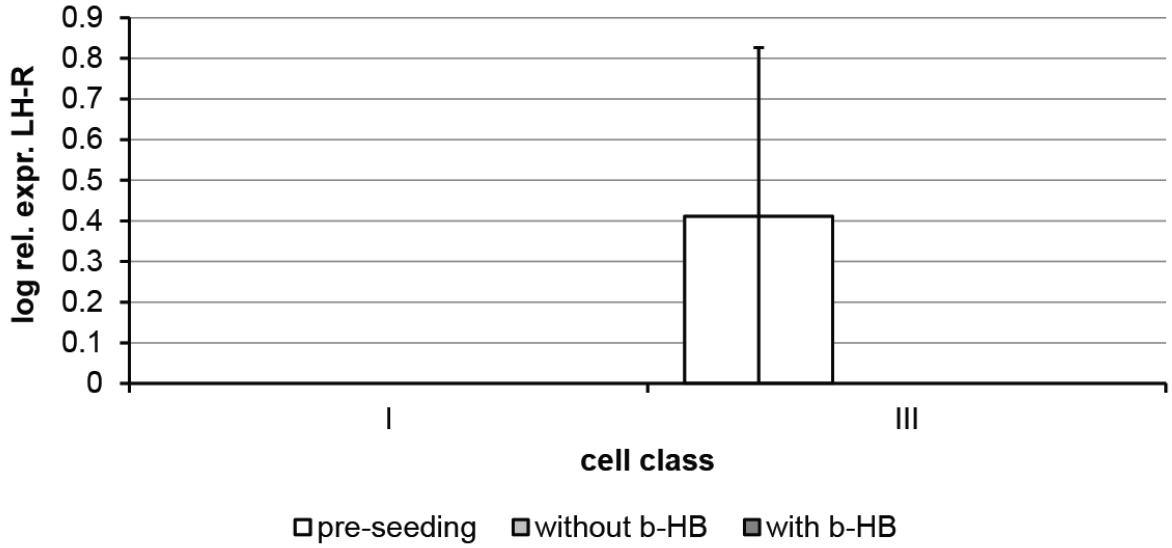

Figure 4-43 Logarithm of the relative expression of LH-R by bGC I and III before and after culture with or without β-HB (b-HB).

5 Discussion

5.1 Establishment of the Bovine Granulosa Cell Perfusion Culture

5.1.1 Medium Composition

The composition of the used standard medium was oriented at many recent publications concerning the culture of bGCs. A 1:1 mixture of DMEM/Ham's F-12 medium is a frequently used medium for the culture of bGCs [97,143,176–182]. The addition of a mixture of penicillin and streptomycin has an important antibiotic effect and is as well commonly used in bGC cultures [97,160,178,183,184]. Insulin, transferrin and selenium as well as bovine serum albumin are added to serum-free cultures of bGCs as serum replacers [97,143,160,180,183] and were in this study used to minimise the amount of added serum. Androstenedione is a substrate for estrogen synthesis commonly used in bGC culture [97,143,160,180,183–185], and androgens are added to bGC cultures, because bGCs cannot synthesise androgens themselves due to a lack of the necessary enzymes [39]. The follicular fluid of small antral follicles contains about 0.1 µM androgens, whereby the concentration of androstenedione and testosterone are similar [186–188]. What is different from most of the recent publications is the use of eCG as stimulant. eCG was chosen because it acts dually like FSH and LH in species other than the horse [189], whereby LH and FSH act on the same post-receptor second messenger system [34,51]. It also has similar effects on the differentiation and P4 production of bGCs as FSH and LH [190]. Furthermore, it is very stable due to its high glycosilation [189] and easily to obtain. Discrepancies between different preparations of FSH with different biopotencies are thus eliminated. eCG has been used before as a stimulant in a study with rat GCs and was found to stimulate these cells from small mitotically competent cells to large E2-secreting cells [191]. Kuran and co-workers compared the influence of different gonadotrophins on bGCs under static culture conditions [190]. After having tested the influence of FSH on bGCs, resulting in a dose-dependent stimulation towards bGC differentiation, they found similar cell reactions when using LH or eCG in comparison to FSH, whereas FSH was found to be 5.8 times more potent than eCG in terms of stimulating P4 production (referring to µu (1 µu = 1 mg NIH-FSH-S1) to iu levels) [190].

The use of serum in cell culture has both advantages and disadvantages. A disadvantage is its unknown composition. The higher the added amounts of serum are,

the greater is the influence of the unknown factors on the cell function. Serum can even have a more important effect on bGCs than a tested agonist [192]. On the other hand, serum contains an essential and up to date artificially not reproducible composition of factors essential for cell growth and survival. Without the use of serum, the normal function of GCs might be endangered and experiments must be limited in time [193,194]. *In vivo*, the interaction with a multitude of serum factors is normal and without using serum, the physiological situation is not properly mimicked [193]. Attachment factors necessary for GC adherence and viability, as for example fibronectin, are also serum components [195,196]. Cultured GCs also depend on adequate physiological levels of insulin [160,185,197]. An acceptable way for this work was to limit the amount of added serum to 0.5 % and to compensate this low amount by adding defined serum substitutes as for example the insulin-transferrin-selenium supplement. Furthermore, only serum from the same charge was used during all experiments.

Medium that is used without a CO_2 enriched atmosphere, as in the applied system, has to be stabilised by adding biological, CO_2 independent buffers such as HEPES [77]. The HEPES buffer concentration of 280 mM resulted in a medium pH of 7.41, which is closest to the favoured, cell physiological pH of 7.4 and was therefore used in all following experiments.

5.1.2 Retrieval of Bovine Granulosa Cells

Three different populations of granulosa cells have been suggested, based on the content of enzymes and the secretion of proteins, steroids and acids [20–22]. The mural population comprises the granulosa cells connected to the basement membrane, and the antral population comprises the cells closest to the antral cavity. The third population constitutes the cumulus oophorus, comprising the GC population adjacent to the oocyte and the corona radiata cells, which directly surround the oocyte. Through the isolation technique, the bGCs in this study mostly belong to the group of antral bGCs and also comprise some cumulus and corona radiata cells as well as oocytes. It was observed that oocytes and their directly surrounding cells do not attach to the culture substrate and are lost during the washing steps at the change from preculture to new wells or perfusion culture. Therefore, the cell population used for the perfusion culture comprises mainly antral bGCs and some cumulus cells. Using antral bGCs can optimize E2, T4 and P4 production in response to stimulation with FSH

compared to mural bGCs [198]. Also, aspirated bGCs secreted markedly more E2 than mural bGC [97] and secrete higher amounts of E2 under chronic exposure to FSH than mural GCs [198]. The measurement of the follicle diameter from the ovarian surface without previous isolation of the follicles from the ovarian stroma and the isolation technique using needle and syringe is time-saving and allows the processing of many follicles in an acceptable time, also concerning cell survival in the isolated ovaries. In a comparative study, the diameters of follicles measured from the ovarian surface and from dissected follicles had a good correlation (r = 0.83) [199]. For this study, as many as possible follicles from different animals should be isolated to have sufficient cell pools for the conducted experiments and to compensate interindividual differences.

5.1.3 Investigation of Different Materials as Support for Bovine Granulosa Cells

The significant influence of the different cell carrier materials on the cell numbers, their growth pattern and the production of E2 emphasizes the importance of choosing an appropriate material for the designated experiments [77].

The attachment of a sufficient number of cells is a sign for an adequate carrier material [200]. It was also important to have enough cells for later analysis. Concerning the aspect cell count, polycarbonate 0.2 was with the highest average number of attached cells the most favoured carrier material, followed in some distance by polycarbonate 0.4. The difference between these two materials concerning the cell count was not significant in the pairwise comparison after Student-Newman-Keuls, presumably due to the high variability of the values.

An equally distributed growth of the cells is also a sign for the suitability of a carrier material [200]. Polycarbonate 0.2 showed the most equal distribution (according to the lowest CVQ), followed by polycarbonate 0.4. In the pairwise comparison after Student-Newman-Keuls, polycarbonate 0.2 was the only material that was significantly different from another material, and that was collagen.

Also, the production of E2 had to be high enough to be measurable in the later experiments, and the E2 production, as a characteristic of intact bGCs, should be supported and maintained during the culture. Concerning the production of E2, polycarbonate 0.4 had the highest yield and thus is the most convenient material for this purpose, closely followed by polycarbonate 0.2. Because only two experimental repeats were accessible concerning the production of E2 by polycarbonate 0.4 and

also 0.2, they could not be included in the pairwise comparison after Student-Newman-Keuls.

Concluding, polycarbonate 0.2 had the best properties in the combination of the three tested parameters and showed the most significant differences in comparison to the other materials in the pairwise comparison after Student-Newman-Keuls concerning cell count and CVQ. It was therefore used as cell carrier material in all following experiments.

The more cells attached to the carrier material, the more equal was their distribution. Because both a high and an equal attachment are signs for an appropriate cell carrier material [200], it can be expected that both values improve with a better applicability of the tested material. The positive correlation of the cell count with the production of E2 is in contrast to the study of Portela and co-workers, who found a lower cell plating density to be supportive to an estrogenic bGC type, and thus E2 production, whereas a higher plating density promoted luteinisation [201]. Supporting to this theory is, that the equal distribution of the cells had a slightly positive influence on the E2 production, because cells that are more equally distributed use the capacity of the growth area better.

If the calculation of the production of E2 per 1 000 cells on the different material is considered, polycarbonate 0.4 and polycarbonate 0.2 resulted in the third and second lowest amount. This is due to their high cell numbers. The production of E2 per 1 000 cells was calculated, because the per cell hormone production is a parameter commonly used for the description of cultures of hormone producing cells. It was not considered in the choice of an appropriate cell carrier material for this study, because high cell numbers as well as high E2 production should be reached on the chosen material as described before. However, this parameter was calculated, because it might be of interest for other applications.

The lower cell count and E2 production and the less equal spread of the attached cells on the other materials indicate their inferior supporting properties for bGCs.

5.1.4 Comparison of Static and Perfusion Cell Culture

5.1.4.1 Cell Numbers

The cell number was obtained at the end of the culture period and represents the situation after preculture and culture in either the static or the perfused system, with a

total culture time of nearly 5 days. In all cell classes and both systems, less cells than the originally seeded 600 000 cells per culture system and cell class could be detected at the end of the experiment using fluorescence microscopy. This can be partly due to cell loss during seeding, because it was possible that, despite very careful pipetting, cell suspension inadvertently overflew the just with medium covered cell carrier during application of the cell suspension or later during the handling of the culture plates. The overflown cells attached to the bottom of the culture plate instead the surface of the cell carrier material and could be regularly detected there during the microscopic control of the preculture plates after the transfer of the cell carriers. Because only few cells could be seen during this control, the lower post-culture cell numbers must have had other reasons additionally.

Oocytes have a positive influence on GC proliferation [202], and a loss of association with the oocyte affects the ability of GCs to undergo proliferation [202–204]. Also, the viability of bGCs was found to be twice as high when cocultured with bTCs [205,206]. In the here described culture method, this lack of communication with the oocyte and bTCs probably had a negative influence on the proliferation ability of the cultured bGCs.

Additionally, differentiation and mitosis [207–211] as well as proliferation and steroidogenic capacity [212] were found to be inversely related in GCs. Under these aspects, the differentiation towards luteinisation and the considerable amount of steroid production of the bGCs in this study counteract bGC proliferation and might contribute to low cell numbers.

Also, androgens were shown to have an inhibitory effect on bGC proliferation [177], which were added as precursors for steroid production in the here described culture system. An antiproliferative effect of P4 on human GCs has also been reported by Chaffkin and co-workers [213], and the high amounts of P4 produced during the culture here in both systems can have a negative effect on the cell proliferation.

Although eCG was shown to inhibit porcine GC apoptosis in a dose-dependent manner [214] and FSH can suppress spontaneous apoptosis in bGC [215], the cell numbers did decrease during the culture despite addition of eCG to the culture medium.

Not only impaired proliferation, but also increased cell death, including apoptosis and necrosis, might have contributed to decreasing cell numbers during cultivation.

The differences between the cell numbers after the culture in the two different systems were not statistically significant, presumably due to the high variability of the data. Also, the cell numbers were not statistically significant influenced by the cell class, which is in accordance with Korenman and co-workers, who could not find a dependency of the growth rate on the size of the follicles, where the bGCs were obtained from [73].
Nonetheless, differences in the geometric mean of these data will be discussed, as they might indicate at least some possible influence of the culture system or the cell class on the cell numbers.
The difference between the mean cell numbers after the two different cultures was highest in bGC III, with more than twice the amount of cells counted in the perfused compared to the static system. In bGC II, the mean cell numbers were as well lower in the static than in the perfused system, but the difference was not as distinct as in bGC III. The lower amounts of cells found after culture in the static system can be influenced by the higher amount of P4 produced in this system in comparison to the perfused system. As described before, P4 was shown to have an antiproliferative effect on human GCs [213]. Furthermore, P4 can accumulate around the cells in the static culture, whereas it is constantly carried away in the flow of the perfused culture.
The difference between the two systems might be highest in bGC III, because these cells are in a more differentiated stadium and not able to adapt to new surroundings as well as cells from smaller follicles in a less differentiated stadium. They also have a lower proliferative capacity. This might also be the reason why mean cell numbers of bGC III were in general lower than of bGC II or bGC I after culture.
In contrast to bGC III and II, mean cell numbers of bGC I were a little higher after the static culture than after the perfused culture. bGC I produced the lowest amounts of P4 after the static culture, so P4 might not have a comparable negative influence on bGC I cell numbers in the static system compared to bGC II and III. bGC I also showed the highest production of E2 at the end of the static culture compared to bGC II and III, and therefore might have reduced the concentration of androgens - substrates for E2 synthesis - in the surrounding medium, which were shown to have an antiproliferative effect [177]. Also, E2 was shown to act autocrine on estrogen receptor β to stimulate GC proliferation [27], and autocrine signalling is prevented to some extend by the medium flow in the perfusion culture. The integrity of small follicles, where bGC I were isolated from, is more difficult to assess, due to their tiny size, than from bigger follicles,

where bGC II and III were isolated from. This might lead to a higher fraction of early apoptotic, but not yet trypan blue dye excludable bGC I and contribute to the low cell numbers compared to the other cell classes.

5.1.4.2 Production of Total Estrogens and Progesterone

The production of ES among the different cell classes during the preculture period very well resembled the different developmental stages of the follicles they were obtained from, with being much higher in bGC III and higher in bGC II than in bGC I. The influence of the cell class on the production of ES was statistically significant. These findings are in accordance with other surveys, who found the capacity of GCs to produce E2 greatly increasing with increasing size of the follicle they were obtained from [97,177,216,215]. Correspondingly, the relative expression of P450arom, the enzyme responsible for E2 synthesis [42,218] in the pre-seeding samples was much higher in bGC III than in bGC II and in bGC I. Healthy antral follicles are characterised by the steroidogenic capacity of their GCs [28] and the high amounts of steroids produced during the preculture indicate the integrity of the punctured follicles.

The significant decline in the bGCs' ES production after the preculture during the following culture period was frequently described before in other publications, whereby the influence of culture time was dependent on the cell class and the applied system. Often, a rapid decline occurred during the first few culture days and continued from then on [97,207,216,219–221]. This rapid decline also explains the big differences between the amounts of ES produced during the more than 2 days preculture and the following amounts in this study. It was also stated that FCS might contain factors interacting with insulin and FSH to inhibit E2 production [222], and even though the medium FCS concentration was with 0.5 % rather low, it might have had some influence on the ES production during culture. But also in the normal developmental course of a follicle, the production of ES is considerably decreasing as the follicle has reached its maximum diameter [40,223], and so the declining ES levels might be part of the physiological differentiation of the cultured bGCs, at least of bGCs obtained from larger follicles.

In contrast to the before mentioned publications, where E2 production continued to decline during culture, the production of ES did not decline over the whole culture period after the initial decline here, but also showed increases in ES production in the culture of bGC I and bGC II, not in bGC III. The different development of ES production

between the different cell classes in the course of the culture is in accordance with the significant interactions of cell class and culture time on ES production. The increase in ES production was only transient in the perfused culture, but went on until the culture end under static conditions, which is in line with the significant interactions between culture time and applied system. It is described that the proliferation and differentiation process of GCs obtained from small follicles takes about four days *in vitro*, which is corresponding to the time a recruited follicle needs to differentiate into a preovulatory follicle [224,225]. This differentiation is leading to a higher capacity of ES production. bGC I and also – but to a lower extent, because they are already in an advanced stadium of differentiation – bGC II have this potential for further differentiation towards higher ES production, whereas bGC III, being in the most advanced stage of differentiation of the three cell classes, have not. These possible differentiation processes might explain the increase in ES production in bGC I and II. That this increase was only temporal in the perfused culture and that the perfused culture often showed a lower production of ES in all cell classes than the static culture – especially in the second half of the culture period – is in accordance with Anderson and co-workers [79]. They found, as the only other survey directly comparing the steroidogenic potential of ovarian tissue between perfused and static or *in vivo* conditions, that steroidogenesis was 20 % lower under perfusion [79]. Anderson and co-workers, working with rat ovary slices, made the deorganisation of the tissue and the rapid medium flow, that carried away extracellular precursor pools, responsible for this difference [79]. Tissue deorganisation is no factor in the actual study, as isolated cells are cultured here, and also the loss of extracellular precursor pools does not come into account, because the medium was supplemented with substrates for steroidogenesis. The medium flow *per se*, as only difference between static and perfused culture, seems to have an influence on ES as well as P4 production of bGCs, even though no significance could be proven for the influence of the culture system on the production of ES, due to a high variability of the data.

The ES production at the culture end calculated per cell count showed the same relation between the perfusion and the static system as the data not corrected for the cell count. Accordingly, these values can be similarly discussed concerning the relation between static and perfused system. A difference was that the production of ES per cell count was highest in the static culture of bGC III and not of bGC I, as without

correction for cell numbers. It is not clear, why bGC III had a higher per cell ES production after the culture than bGC I, because with further differentiation of cells of already differentiated follicles, the production of ES is normally decreasing.

To calculate and finally publish both the absolute and the per cell hormone production seems to be advisable, because it makes the obtained results comparable to studies using normalised as well as not normalised hormone values.

The production of P4 during the preculture was highest in bGC III, followed by bGC II and bGC I, whereby the differences between the three cell classes were not as big as concerning the production of ES and furthermore not significant. These different potencies of P4 production at the begin of the culture are in accordance with the findings of Spicer and co-workers concerning the *in vitro* capacity of GCs to produce steroids being dependent on the size and so the developmental stage of the follicle they were obtained from [177]. GCs begin to synthesise P4 a few hours prior to ovulation [226], which explains, together with the duration of the preculture time, the production of P4 even of bGCs obtained from small follicles in the first measured interval.

After the preculture, the amounts of P4 were similar between the three cell classes in the same system and relatively stable, congruent with the not significant influence of the culture time on the production of P4. This is in accordance with Korenman and co-workers, who found the P4 production of bGCs after some days of culture not being influenced by the size or hormone content of the follicle the cells were isolated from [73]. When the same cell class is compared in the two different systems, the drop of P4 production is much greater in the perfused than in the static system, according to the findings that the significant influence of the culture system was time-dependent. During the whole culture period, the mean production of P4 remained several times lower in the perfused than in the static system, and this difference was significant. This is, as it was for the production of ES, in accordance with the survey of Anderson and co-workers, where superfused rat ovary slices produced less steroids compared to before reported amounts synthesised in static culture *in vitro* or *in vivo* [79]. After the initial decline, the amount of P4 stayed relatively stable in the perfused culture and slightly increased in the static culture during the rest of the culture period. The increase of P4 secretion during static culture was also found by Luck and co-workers, where the secretion of P4 increased rapidly because of presumably luteinising GCs during

culture [221]. Also, in a stable porcine GC line, P4 had a role as autocrine regulator of its own synthesis [227], whereby autocrine as well as paracrine signalling is prevented to some extent in the perfused system by the medium flow, which might also contribute to the differences between the two systems. It was also stated that GCs from no matter what follicle size predominantly produce progesterone when cultured long-term [160,183], as it was found here. Spicer and co-workers did not find a positive effect of androgens on bGCs P4 production [177], whereas Henderson and co-workers saw a −positive effect of androgens on bGC P4 production [228]. As presumably more androgens are present around the cells in the perfused system because of the continuous medium turnover and the lower production of P4 by bGCs in the perfused compared to the static system, no positive effect of androgens on bGCs P4 production could be confirmed in this study.

Lower amounts of ES and P4 in the perfused system in comparison to the static system might also be a result of the prevention of paracrine and autocrine signalling by the constant medium flow. An influence of paracrine factors on steroid synthesis was stated before [183].

As the normalised ES production, the P4 production normalised for the cell count showed the same relation between the perfusion and the static system as the data not corrected for the cell count, so these values can be similarly discussed concerning the relation between static and perfused system. However, a difference in comparison to the not normalised data was, that the P4 production was lower in bGC II than in bGC I. Normally, from bGC II a higher amount of P4 as from bGC I would be expected, because they were already isolated in a more differentiated state.

The results obtained in the perfused system as well as in the static system indicate that the cultured bGCs undergo a differentiation towards luteinisation during both culture types and in all cell classes, despite they retain the ability to produce ES until the culture end. When GCs are isolated from the follicular environment before the LH-surge, they spontaneously develop characteristics of LH-differentiated cells, because factors from the follicular environment, that seem to prevent luteinisation, are lost [27]. It is typical for luteinising bGCs that their secretion of P4 is increasing over the culture period, and that their E2 production is declining [221,229], as it is occurring in this study. Serum, even though it was only present in a small amount in the used medium, can promote this luteinisation with its various growth factors [230], and

cholesterol present in serum can be a precursor for P4 synthesis [231] and so enhance P4 production. Higher doses of FSH and LH enforce the natural tendency of GCs to luteinise *in vitro* [222], and eCG, used as stimulant in this study, has a similar effect than FSH and LH [189]. The maintenance of ES production that was also found in this study is a desirable feature in modern bGC cultures in order to investigate steroid production. It was achieved before in different bGC culture experiments [160,183,185]. But even in these serum-free long-term bGC cultures, who aim to resemble the non-luteinised stage with preserved E2 production in response to gonadotrophin stimulation, the ratio of E2:P4 is mostly lower than 0.1 [232], and so comparable to this work. In the static system, the mean ratio of ES to P4 was 0.02 ± 0.01, but in the perfused system this ratio was 0.09 ± 0.06, and so in average nearly as high as in the culture systems who aim to resemble the non-luteinised stage. The mean ratio of ES to P4 was higher in GC III (0.09 ± 0.05) than in GC I (0.05 ± 0.01) and GC II (0.03 ± 0.01), though it was stated that bGCs from bigger follicles might reach a more luteinised stage earlier than bGCs isolated from small follicles [215].

5.1.4.3 Expression of Key Enzymes and Receptors for Steroidogenesis

5.1.4.3.1 Aromatase

Expression of P450arom was detected in all cell classes in pre-seeding as well as in post-culture samples. It was reported before that follicles with a diameter smaller than 4 mm do not express P450arom mRNA [52], but this discrepancy might be explained with the technique of measuring the follicular diameter as it was visible on the ovaries' surface in this study or by the method detecting the expression. Spicer and co-workers investigated the comparison between surface diameters and diameters of dissected bovine ovarian follicles and found on the one hand a good correlation between the two parameters (r = 0.83), but also that the surface measurement tends to underestimate the real diameter [199]. It might therefore have happened that in bGC I, isolated from follicles with a surface diameter smaller than 4 mm, also bGCs from slightly bigger follicles were present, which already acquired P450arom expression. Also, the here used real-time RT-PCR is a very sensitive method and may detect expression at lower levels as the *in situ* hybridisation used in [52,233].

The expression of P450arom in bGC II and III in the pre-seeding samples is in accordance with the work of Bao and co-workers, who found P450arom expression in

nearly all recruited follicles with a diameter of 4 - 6 mm and in all follicles from 6 - 9 mm in diameter [52].

The relative expression of P450arom in the pre-seeding samples was higher the bigger the follicle was the bGCs were isolated from, even though no significant influence could be proven of the cell class on the P450arom expression. Despite clear differences in the means of their data, Lenz and co-workers did also not find a significant correlation between follicular size and expression of P450arom due to extreme interindividual variations [233], and the data here also showed a very high variability. In other publications, the expression of P450arom was found to increase with follicle size and to be highest in the large dominant follicle [42,187].

After the culture, the relative expression was much lower than in the pre-seeding samples, with the greatest difference in the bGC III samples. Accordingly, the influence of the culture time on the expression of P450arom was statistically significant and also cell class-dependent. As it has been discussed concerning the steroid production before, the bGCs seem to undergo a differentiation towards luteinisation, despite they keep their ability to produce ES and accordingly some P450arom expression and activity. It is normal during luteinisation, that the expression of P450arom decreases, and with it the production of E2 [233,234]. But the experiments of Lenz and co-workers also showed, that P450arom expression is at low levels still present in the CL [233], strengthening the findings of others that estrogens are produced in the bovine CL, maybe having autocrine or paracrine effects [235]. In culture, reduced activity of P450arom and consequently decreasing E2 production together with a definite increase in P4 production indicate luteinisation of GCs [221,229].

In another publication, the expression of P450arom seems to increase during culture under stimulation of FSH [183], but the values were calculated in comparison to the expression after two days in culture, where already significant decreases in the expression might have happened [183]. The expression of P450arom after two days in culture was already only barely detectable in bGCs of follicles with a diameter smaller than 4 mm in that survey [183]. According to the results here, Berndtson and co-workers found the expression of P450arom to decrease rapidly during the first 24 hours of culture using a ribonuclease protection assay [236].

The use of eCG as stimulant might in some way also contribute to the downregulation of the P450arom expression, because it has affinity to the FSH as well as the LH

receptor. FSH stimulated, but LH inhibited the expression of P450arom in rats [237]. Fitzpatrick and co-workers proposed, that a relative small increase in intracellular cAMP is evoked by FSH, stimulating steady-state P450arom expression, whereas LH evokes higher intracellular cAMP levels, which induce luteinisation and a decreased expression of P450arom [237]. They also suggested an activation of protein kinase-C second messenger pathways by high LH levels, leading to a decreased expression of P450arom [45]. eCG, having effects on both the FSH- and LH-R, might therefore contribute to the downregulation of P450arom expression found in this study.

That the expression of P450arom was not detectable in few single post-culture samples might indicate a stronger luteinisation with loss of P450arom expression or that the cells start to undergo atresia in these samples, because in early atretic follicles, also only low or no activity of P450arom can be detected [187].

If the differences in the relative expression of P450arom between the static and the perfused system are considered, the geometric means indicate that the expression is higher in the static than in the perfused system in bGC I and II, and higher in the perfused than in the static system in bGC III. Statistically, the culture system had no influence on the relative expression of P450arom, maybe due to the high variability of the data. Possible reasons for a lower expression in the perfused system in bGC I and II might be, as argued in the section about hormone production (chapter 5.1.4.2), a dilution of auto- and paracrine factors. In bGC III, which accordingly also reached the highest ratio of ES to P4 in the perfused system, the higher expression of P450arom might be a result of the more physiological conditions and the constant supply with fresh medium.

5.1.4.3.2 3β-HSD

In contrast to some studies, which reported 3β-HSD to be only present in bGC from follicles with a diameter larger than 8 mm using *in situ* hybridisation [34,38] or in preovulatory follicles using immunohistochemistry [33], but according to the findings of Lenz and co-workers, who found 3β-HSD transcripts in bGC of all follicular sizes using real-time PCR, 3β-HSD expression was found in all bGC classes in the present study. Lenz and co-workers explain this discrepancy by the greater sensitivity of real-time PCR compared to *in situ* hybridisation [233].

No significant differences could be confirmed in the statistical analysis concerning the expression of 3β-HSD, whereby the influencing factor time was just below the

significant level. This might be due to the high variability of the data. A high variability of the expression of 3β-HSD, even in GCs from follicles with similar sizes, was also found by Lenz and co-workers [233]. Nonetheless, differences in the means will be discussed in the following.

The relative expression of 3β-HSD in the preculture samples was higher in bGC III than in bGC I and II, what is in accordance with other surveys, which found the expression of 3β-HSD to be increasing with increasing follicle size [34,38,233]. Why the relative expression was slightly higher in bGC I than in bGC II is not clear and might be due to the high variability of the data. The post-culture expression was much higher than the pre-culture expression in all cell classes. This rise can be explained with the previous mentioned differentiation towards luteinisation of the cells in the here described culture, because the expression of 3β-HSD increases in bGCs during luteinisation [33,233,238]. This is in contrast to the findings of Voss and co-workers, who found the expression of 3β-HSD to decrease in preovulatory follicles [239,240]. In the perfused system, the expression of 3β-HSD was lower than in the static culture in all cell classes. This indicates a prolonged luteinisation process in the cells in the perfused system and a longer preservation of the non-luteinised phenotype in the perfused culture, as it is desired in modern GC culture. In a stable porcine GC line, P4 increased the expression of 3β-HSD [227], and the higher presence of P4 in the static system than in the perfused system might also contribute to the higher expression of 3β-HSD in the static system. Healthy follicles had a higher expression of 3β-HSD than atretic ones [38], so a lower expression of 3β-HSD could also be a sign for early atretic processes.

5.1.4.3.3 FSH-R

The expression of FSH-R is exclusive to the gonads and there to GCs [241]. Concerning the expression of FSH-R, the culture time was the only statistically significant influencing factor, whereby the interaction of culture time with the cell class was statistically just below the significant level. This might be due to the high variability of the relative expression. Differences in the means will be discussed nonetheless. In the pre-culture samples, the expression of FSH-R was detectable in all cell classes, according to other surveys, which detected FSH-R in bGCs and cumulus cells from a one- to two-layer stage on [34,38,42,52,242]. The expression increased with the size of the follicle the bGCs were obtained from, as it was reported before [38]. The

post-culture expression was much lower than the pre-culture expression, and loss of FSH-R expression is a sign for luteinisation or atresia of bGC [186,243,244]. Also, a continuous stimulation of rat or bovine GCs with FSH resulted in a downregulation of the expression of FSHr mRNA [245,246], and the constant presence of eCG, having FSH as well as LH capacity, in the culture medium might have led to some kind of FSH-R downregulation here.

The expression of FSH-R was higher in the cells after the perfused culture than after the static culture, which supports the hypothesis of a prolonged luteinisation process in the perfused system. eCG was able to stimulate FSH-R expression in rat GCs [247] and might as a component of the culture medium also contribute to the maintenance of FSH-R⁻ expression in the present study. The post-culture relative expression was lowest in bGC III, which showed the highest relative expression in the pre-culture samples, and both can be explained by the advanced differentiation of bGC obtained from large follicles. After the static culture of bGC III, no FSH-R expression was detectable at all. It was proposed, that if FSH, and so presumably also eCG, binds to its receptor, it induces FSH-R internalisation and suppresses FSH-R expression [241,243,248]. Maybe the post-culture expression of FSH-R is lowest in bGC III, because they showed the highest pre-culture expression and therefore downregulation processes were triggered. Also, GCs from larger follicles might undergo luteinisation, and therefore loss of FSH-R expression, earlier than GCs from smaller follicles, as they are in a more differentiated state at the point of isolation.

FSH-R expression is also regulated by paracrine factors, whereby some decrease and some increase its expression [241]. Because paracrine signalling is prevented to some extent in the perfused system, but FSH-R expression was higher in this system, paracrine factors suppressing FSH-R expression seem to be more important than paracrine factors increasing its expression.

5.1.4.3.4 LH-R

LH-R mRNA was only detectable in pre-culture samples of bGC III, and also not in all tested samples. This is in accordance with the finding, that LH-R is predominantly expressed in one healthy, the future dominant, follicle with a diameter larger than 8 mm [8,34,38,51,169,186,249,250], at the time when the theca interna is forming [52]. Presumably not only future dominant follicles were punctured in the present study. FSH-R expression is increasing with follicular size [34,38,250] and if the follicle enters

regression, LH-R expression is not detectable any more [51]. In contrast, Roberts and co-workers were the only ones who detected LH-R mRNA in bGCs of small (< 4 mm) and large (> 5 mm) follicles [169]. eCG stimulated LH-R expression in rat GCs [247], even though it was not able to support LH-R expression in this study on bGCs. In mice, mural GCs express larger amounts of LH-R than antral GCs [251], and in the present study predominantly antral bGCs were cultured. A requirement of both FSH and E2 for stimulating the expression of LH-R has been stated [49,249,252,253], and though both were present – the function of FSH represented by eCG – no relative expression of LH-R was found after the culture.

5.1.4.4 Conclusions

The only statistically significant difference between the static and the continuous medium supply was the production of P4, which was lower in the perfused than in the static system. This effect was also influenced by the culture time. That bGCs produce less P4 in the perfused system might indicate a prolonged luteinisation process, with a longer sustained non-luteinised phenotype, as it is preferred in modern bGC cultures. The expression of the tested genes also indicates a slower luteinisation in the perfused system. In the perfused culture, the ratio of ES to P4 was higher than in the static culture, indicating that the cells keep the non-luteinised phenotype longer. Furthermore, high levels of P4 production in the static culture might indicate some kind of stress luteinisation due to the accumulation of cell products.

The production of ES was present during the whole culture time in both systems, and though the means of the amounts of ES produced appear to be lower in the perfused system, as it is discussed in the previous sections, this effect was statistically not significant. Differences in the gene expression between the two systems, though tendencies of differences in the means were also discussed in the previous sections, were not significant, too.

Allegrucci and co-workers suggested, that they could avoid detrimental effects of metabolic by-product accumulation in bGC cultures when using higher volumes of medium [206], and metabolic by-product accumulation is also prevented in the perfusion system. A perfused culture was praised before for human GCs, because a physiological milieu is maintained, hormones, metabolic and waste products are immediately carried away, and non-physiological effects are minimised [96]. Also, human GC perfusion culture in combination with RIA for hormone analysis was shown

to be a sensitive method to detect the GCs' delicate hormone responses after stimulation with low concentrations of different substances [95].

Concluding, the here established perfusion system for bGCs has no disadvantages compared to the static culture, and may even be more suitable for a long time culture of bGCs due to a prolonged luteinisation process. It has the advantage of imitating physiological conditions with its continuous medium supply and allows exact tuning of the culture conditions and permanent monitoring via used medium without direct manipulation of the culture. From the three used cell classes, bGC III showed the best performance in the perfusion culture and also the highest ES to P4 ratio.

5.2 Influence of Metabolites on Bovine Granulosa Cells

5.2.1 Cell Numbers

The cell numbers were determined at the end of the experiment, after pre-culture and perfusion culture with a total culture time of slightly more than 3.5 days, using fluorescence microscopy after nuclear staining with DAPI. After all cultures, less than the originally seeded 600 000 cells were detected. General reasons for the decline of the cell count in the perfused system, irrespective of the presence of a metabolite, were discussed in chapter 5.1.4.1 and are applicable for the culture here, as well. Also, metabolite independent differences between the means of the cell count of the different cell classes can be adopted from chapter 5.1.4.1. That is because, as expected, the overall cell numbers were lower in bGC I than in bGC III in the perfused system here as well as in the perfused system that was compared to the static system. The influence of the cell class on the cell numbers was statistically not significant, and there was also no significant interaction between the cell class and the presence of a metabolite. Also, the presence of urea or β-HB in the culture medium had no significant influence on the cell numbers obtained at the end of the culture, although there were noticeable differences in the means of these values. This might indicate that there was in fact no influence of these parameters, or be due to the high variability of the data.

The time-related increase and decline of the metabolite concentration, that resulted – if an ideal medium flow and intermixture were postulated – in an equal rise of the urea concentration from 0 to 5 mM and from β-HB from 0 to 2 mM over 8 hours, followed by a plateau phase of 5, respectively 2 mM for 2 hours and then by an equal decline over the next 8 hours, until no more metabolite was present in the medium (Figure 3-3), and

then was followed by another 10 hours in culture medium without metabolite, might have been too short to have a significant influence on the culture end cell numbers. Also, the cell numbers might already have recovered from the metabolic stimulus in the metabolite free phase until the culture end. The time-related variation of metabolite concentration was applied to study possible time-related effects during culture on the monitored variables ES and P4, and might not have been ideal to influence parameters only obtained at the culture end. Furthermore, the maximum concentrations of urea and β-HB were both in a moderate range, occurring during average negative energy balance in high yield cows. Higher concentrations in combination with a longer challenge would maybe have resulted in a stronger influence.

That β-HB had no statistically confirmable influence on bGC cell numbers, even though ketones can be used as alternative energy source at low glucose levels [254], supports the theory that ovarian cells normally do not use ketones as energy source *in vivo* [255]. Merely, it has to be seen under the aspect, that sufficient glucose levels were present in the basis culture medium used here (DMEM/Ham's F-12 medium with 3.151 g glucose per litre). The present findings are contrary to the study of Cheng and co-workers, who found a positive effect of β-HB in a concentration of 0.02 g/l on cell proliferation and survival in L929 cells, a murine aneuploid fibrosarcoma cell line [256], but this might be due to the cell type.

According to the results presented here, other surveys did also not find a negative influence of urea on follicular parameters. Moallem and co-workers found no influence of higher follicular fluid urea levels on follicle diameters examining non lactating heifers [148]. Also, feeding plasma, and with that follicular fluid [139,149], urea levels elevating diets to non-lactating cows did not impair the numbers of follicles in previous studies [147,257] and had furthermore no [257] or even a positive influence on follicle size and growth rate, even though the enhanced growth was associated with cystic aberrations [147]. Accordingly, the size of Graafian follicles was also found to be bigger in cows than in heifers [110].

On the contrary, feeding plasma urea levels enhancing diets to lactating cows resulted in reduced follicular size and growth rate and led to a reduced size of the corpus luteum [258]. A generally slower growth rate of dominant follicles was found during negative energy balance, resulting in prolonged acquisition of ovulatory size [259]. Accordingly, dominant follicles of cows that have overcome the negative energy balance nadir seem

to grow faster and to have a larger diameter than dominant follicles developing during earlier phases [259]. Non esterified fatty acids, also important indicators of negative energy balance, had a negative influence on bGC viability [143]. Short term changes in nutrition were shown to influence the follicular development without influencing FSH levels [260].

Under these aspects it is noticeable, that the means of the cell numbers were in both cell classes cultured with one of the two metabolites lower as if cultured without metabolites. Thereby, urea seemed to have a greater impact on bGC III than on bGC I, and β-HB *vice versa* on bGC I, indicating that urea might influence follicular development on the level of bGCs in earlier and β-HB in later stages.

5.2.2 Hormone Production and Gene Expression

5.2.2.1 Production of Total Estrogens and Progesterone

Metabolite independent changes in the hormone production of ES and P4 were similar to the results obtained in the perfused culture of the experiment comparing the static and the perfused system for bGCs and the discussion of these influences can be adapted from chapter 5.1.4.2. The production of ES was here and there significantly influenced by the cell class and the culture time. In contrast, the culture time also significantly influenced the production of P4 here, and this difference can be explained with the shorter culture time of this experiment, giving the time-dependent drop of P4 production between preculture and the following values a greater impact.

Because of the time-related increase and decline in the concentration of the metabolites, the production curves of ES and P4 have to be discussed with respect to the changing metabolite concentration in the cultures. Despite a lack of statistical significance of the metabolites' influence on bGCs' hormone production – that is presumably due to the high variability of the data – the production curves of ES and P4 from bGC I and III cultured with or without metabolite will be discussed separately and tendencies of a negative or positive influence of the metabolites on the mean hormone production compiled. These tendencies will then be discussed together. The discussion of metabolite independent changes in the overall hormone production, as the decline in ES and P4 production after pre-culture, the general development of steroidogenesis during perfusion culture with the significant influence of the culture

time and the here also significant influence of the cell class on the production of ES can be adapted from chapter 5.1.4.2.

Because the amounts of ES and P4 produced during the culture period 0 - 8 hours were already different between the culture containers with and without later exposure to metabolites, even though both were supplied with the same medium without metabolites at that time, these differences have to be taken into account if discussing later measurements.

To an interim normalisation of the following amounts of ES and P4 to the amount produced in the same culture between 0 and 8 hours was not kept up with, because significance levels could not be improved by this method and because the high variability of the 0 - 8 hour data made their adequacy as normalisation parameter questionable.

5.2.2.1.1 Influence of Urea

In the culture testing the influence of urea on ES production by bGC I, the average amounts of ES produced in the culture without urea were already lower compared to the amounts in the culture with urea in the period between 0-8 hours, a period where the exposure to urea had not yet started (Figure 4-21, page 68). The curves stayed parallel, and so presumably undisturbed, by increasing urea levels in the culture with urea, until the time period 12-14 hours. In the interval 14-16 hours, just before maximum levels of urea were reached, the production of ES was not any more parallel in both cultures, but started to be on an equal level. Because an increase in ES production happened at that time in the culture without urea, it seems as if the cells cultured with urea were not able to perform the same increase, and so maybe started to be influenced by the rising urea levels in that kind. Between 16 and 18 hours, where the maximum concentration of urea was reached, the production of ES was similar in both cultures, and one two-hour interval later, the production of ES in the culture system with urea was in average lower than in the culture without urea. From that time on, the production of ES was only once higher in the culture with urea, but in all other intervals and also at the culture end lower than in the culture without urea, and that in respect to primarily higher production levels during the unexposed period of the perfusion culture. This curve progression indicates, that urea concentrations from 4 to 5 mM negatively influence the production of ES by bGC I, and that the cells stay

affected by this negative influence even if urea levels are decreasing and disappearing again.

The mean production of ES by bGC III showed only negligible differences between the culture with and without urea. At the beginning, before urea levels were increasing, and until 12-14 hours culture time, the production of ES was slightly lower and once slightly higher in the culture without urea than in the culture with urea (Figure 4-23, page 70). From then on, at a time where urea levels reached their maximum concentration and started decreasing again, the curves were nearly similar. Why the production in the over-night interval 24-32 hours is only half as high in the culture with than in the culture without urea, is not clear. It might be a hint to a late-onset negative influence of the higher urea concentrations before, but this measurement has a multiply higher variability than the other data in this experiment. Thus, it seems more sensible to classify this measurement as an outlier, also under the aspect that values in the following, last time interval are similar between the culture with and without urea again. Concluding, these particular production curves indicate no influence of transient urea concentrations of 5 mM on the ES production by bGC III.

The production of P4 by bGC I was lower in the unexposed pre-culture period in the culture with than in the culture without urea (Figure 4-25, page 72). One time interval later, where the urea concentration began to rise, the difference in the means was higher, because the production of P4 rose in the culture without urea, but declined in the culture with urea. If this difference can be accredited to the just rising levels of urea in the one system is questionable. The abnormally high variability of the mean value in the culture without urea rather indicates an outlier. In all following measurements, the production of P4 was lower in the culture with than in the culture without urea, the curves are parallel and the proportion is similar to the 0-8 hour values. Together, this indicates no influence of the presence of urea in the culture medium on the production of P4 by bGC I.

In bGC III, the production of P4 was lower in the culture with urea than in the culture without urea in the unexposed 0-8 hour culture period (Figure 4-26, page 72). Until 16 hours culture time, just before urea levels reached their maximum, the production curves of P4 were parallel in the culture with and without urea, but the difference was slightly higher than in the 0-8 hour period. In the culture period 16-18 hours, were the levels of urea reached their maximum, the production of P4 increased in the culture

with urea to levels equal to the culture without urea and afterwards stayed equal for 14 more hours, even though urea levels were decreasing from then on again. In the last measured interval, the production of P4 increased to amounts nearly twice as high as in the culture without urea. This implies, that urea concentrations of about 5 mM enhance P4 production in bGC III, and also have a late effect on their P4 production, even though not present any more at that time.

If the hormone production per cell count is considered, the production of ES is also slightly lower in bGC I cultured with urea compared to the culture without urea, and thus the two values are consistent with each other. But the production of ES in bGC III corrected for the cell numbers is, in contrast to the not corrected values, where no difference could be seen, higher after the culture with than after the culture without urea. The low cell numbers obtained after the culture with urea compared to the culture without urea are in line with these findings. Concluding, it seems as if urea has a negative influence on the cell numbers, but also a positive influence on the ES production per cell. The cell number corrected values of P4 production by bGC I indicate a lower per cell production of P4 after the culture with urea. Because cell numbers were not much influenced by the presence of urea, this is consistent with the lower amounts of P4 produced in the culture with compared to the culture without urea, even though this difference in hormone production was not considered to be influenced by the presence of urea as explained above. In bGC III, the cell corrected as well as the normal amount of P4 produced at the end of the culture was higher in the culture with than in the culture without urea, implicating an enhancing effect of urea on the total as well as the cell count normalised production of P4.

5.2.2.1.2 Influence of β-HB

The production of ES by bGC I was higher in the culture with β-HB in the unexposed culture period between 0-8 hours than in the culture without β-HB (Figure 4-33, page 80). This relation stayed similar until the culture interval 12-14 hours, where the production of ES reached similar levels in both cultures and also stayed similar for the following 2 hours. Whether this transient decline in ES production in the culture with β-HB is related to the rising β-HB levels at that time remains elusive, because from the culture interval 16-18 hours on, where maximum levels of β-HB are reached, the same proportion between the two cultures as at the beginning of the culture can be observed again. There is only one further interval until the culture end, where the production of

ES is higher in the culture without than in the culture with urea, and at the end of the culture, the production in both cultures is nearly the same. Concluding, no considerable influence of β-HB could be found on the ES production by bGC I.

The production of ES by bGC III is similar in the 0-8 hours period in the culture with and without β-HB (Figure 4-35 page 82). It is in the following 14 hours, where β-HB-levels rise to maximum and fall again, lower in the culture without β-HB than in the culture with β-HB. This might be due to the presence of β-HB, but if it is so, the effect is not dependent on the amount of β-HB. Until the culture end, the amounts of ES produced in both cultures were nearly the same again. This might indicate a positive influence of the presence of β-HB on the ES production of bGC III, whereby this effect is not concentration related and also only present in the period with direct exposure to β-HB.

The amounts of P4 produced by bGC I were similar in the culture without β-HB and with β-HB in the metabolite free culture period 0-8 hours (Figure 4-37, page 84). The P4 production curves stayed parallel until the culture period 12-14 hours, where levels of 1.5 mM β-HB were reached in the culture with β-HB, and the P4 production rose slightly in these cells, whereby the P4 production in the culture without β-HB slightly declined. The higher P4 production in the culture with β-HB was present until the culture period 20-22 hours, where β-HB levels declined again to levels of about 1 mM. In the remaining culture time, the production of P4 was similar in both cultures. Concluding, a concentration higher than 1 mM β-HB was able to enhance the production of P4 by bGC I transiently.

In bGC III, the production of P4 was from begin on, even if the exposure to β-HB had not yet started, lower in the culture with β-HB than in the culture without β-HB (Figure 4-38, page 85). This pattern stayed the same during the whole culture period, indicating that β-HB had no influence on the production of P4 by bGC III.

The ES production by bGC I normalised for the cell count was minimally higher after the culture with β-HB than after the culture without, compared to equal production levels without cell count correction, and this difference might be negligible. In bGC III, where the production of ES was also similar at the culture end without correction for cell numbers, the production of ES per cell count was higher after the culture with β-HB than without. This indicates a positive influence of β-HB on the ES production per cell of bGC III, as it was also discussed in the previous section. The cell corrected production of P4 by bGC I was higher after the culture with β-HB, although P4

production levels were similar at the culture end without correction for the cell numbers. This is in accordance with the positive influence concluded from the according discussion of the hormone production curves. Even though no difference of β-HB could be detected on the P4 production by bGC III, the cell number corrected values indicate negative influence on the P4 production. In combination with the lower, but stable hormone values during the whole culture period in the system containing β-HB, the per cell hormone production might be influenced somehow independent of the presence of the metabolite.

Summarised from the discussion of the hormone production curves and the interpretation of the cell count corrected hormone production, urea had

- a negative influence on the production of ES by bGC I,
- no influence on the production of ES by bGC III,
- no influence on the production of P4 by bGC I,
- a positive effect on the production of P4 by bGC III,
- no influence on the ES to P4 ratio in bGC I,
- a negative influence on the ES to P4 ration in bGC III,

whereby β-HB had

- no influence on the production of ES by bGC I,
- a positive influence on the production of ES by bGC III,
- a positive influence on the production of P4 by bGC I,
- no influence on the production of P4 by bGC III,
- no influence on the ES to P4 ratio in bGC I,
- a positive influence on the ES to P4 ratio in bGC III.

Excursion: Oscillations in the Basal ES Production of bGCs

It was noticed that the production of ES from bGC I, if monitored in two-hour intervals, showed an oscillating pattern. This oscillation was most clearly to see when the ES production was very low, as in experimental repeat 1, 2 and 4 of the investigation of the influence of urea and in experimental repeat 1, 2 and 3 of the investigation of the influence of β-HB (Figure 4-22, page 69 and Figure 4-34, page 81), respectively. Concomitant, a striking parallelism was detected between the oscillating production of ES of cells from the same cell pool, but cultured in separate culture containers. This parallelism was accentuated by including a curve describing the sum of the ES values

measured in the different cultures from the same cell pool. In this additional curve, the oscillation is amplified, which emphasises the parallel curve progression. There were no other possible factors which could have influenced the separate cultures from the same cell pool in this way as the cell pool itself.

Two-hour intervals were only monitored over 16 hours, resulting in 8 comparable curve points. From the measurements between 0-8 and 24-43 hours, the hormone production per 2 hours was a calculated value. Pulsatile hormone secretion profiles are normally attributed to the pulsatile secretion of higher-ranking pituary hormones. The findings in this study indicate that at least basal/minimal ES secretion by bGC from small follicles seems to be regulated by intrinsic factors of the bGCs themselves. The data obtained here were not sufficient to prove this hypothesis, but should motivate to further investigate this phenomenon.

5.2.2.2 Expression of Key Enzymes and Receptors for Steroidogenesis

The changes from pre-culture to post-culture relative expression were similar to the according changes in the perfusion culture from the experiment comparing the static with the perfusion culture, with decreasing values in P450arom, FSH-R and LH-R and increasing values in 3β-HSD relative expression. The discussion of these general metabolite independent changes can be adapted from chapter 5.1.4.3. It is noticeable that in this experiment, in contrast to the perfusion culture from the experiment comparing the static with the perfusion culture, the factors cell class and culture time and the interaction between cell class and culture reached more often a significant level in influencing the gene expression. This might presumably be due to the shorter over-all culture time in the experiments here, because this was the only difference between the experiments apart from the additional presence of a metabolite in half of the cultures, and a significant influence of the metabolite on the gene expression could not be proven. But even though not significant, differences in the mean gene expression can be seen between the post-culture samples cultured with and without metabolite, and will be discussed in the following section. Discrepancies between the influence of urea or 3β-HSD on the production of ES and P4 and the according relative expression of genes linked to this hormone production might be due to discrepancies between the bare expression of a gene and the effective translation of this gene into a functional protein. Also, the absolute number of cells present in the culture must be considered as a factor influencing total hormone production, whereby the relative

expression is normalised with the relative expression of a housekeeping-gene, and so ideally corrected for the cell number. Furthermore, the gene expression was a variable obtained at the end of the culture, and influences of the metabolites on the hormone production were most relevant in the middle of the culture, where the metabolite was actually present. As discussed in connection with the cell numbers (chapter 5.2.1, page 107), the study design was optimised to detect time-related changes in the hormone production by analysing medium samples taken during culture.

5.2.2.2.1 Aromatase

The relative expression of P450arom was somewhat lower in bGC I after culture with urea than after culture without urea. Even though this difference is small, it is in line with the finding that urea had a negative influence on the production of ES by bGC I. Accordingly, urea had also a negative effect on bGC I cell numbers. In bGC III, the relative expression of P450arom was higher after the culture with urea than after the culture without urea, and this emphasises the positive influence of urea on the production of ES by bGC III. Additionally, cell numbers of bGC III were also higher after the culture with then after the culture without urea.

P450arom was somewhat higher expressed in bGC I after the culture with β-HB compared to the culture without β-HB. In combination with its negative influence on the mean cell number of bGC I, this is in line with the results, that β-HB had no influence on the production of ES by bGC I. Even though a positive influence of β-HB was found on the production of ES in bGC III, this could not be confirmed with the corresponding gene expression, because virtually no difference was found between the relative expression of P450arom after the culture with and without β-HB. This can be explained with the lower cell numbers after the culture with β-HB.

Concluding, the relative expression of P450arom, a key enzyme in the production of ES, was according to the hormone production, if influences of the metabolite on the cell numbers were taken into account.

5.2.2.2.2 3β-Hydroxysteroid Dehydrogenase

The relative expression of 3β-HSD, the enzyme catalysing the production of P4, was higher in bGC I after the culture with urea than after the culture without urea. However, urea had no positive influence on the P4 production of bGC I, and this is in accordance with lower cell numbers after the culture with urea. In bGC III, the relative expression of

3β-HSD was lower after the culture with urea than after the culture without urea. But connected with a clear positive influence of urea on mean bGC III cell numbers, this is not opposed to the positive influence of urea on the production of P4 by bGC III.
The relative expression of 3β-HSD in bGC I was slightly higher in the culture without than in the culture with β-HB. This does not reflect the situation in the hormone production, because β-HB had a positive influence on the production ofP4 by bGC I. This discrepancy cannot be explained with the influence of the metabolite on the cell numbers, because β-HB reduced bGC I cell numbers. Although a positive influence was found on the relative expression of 3β-HSD by the presence of β-HB, no influence was found on the production of P4 by bGC III, presumably due to the lower cell numbers in the culture with 3β-HSD. Concluding, the relative expression of 3β-HSD as obtained at the culture end could be related to the influence of urea or β-HB on the hormone production during culture, if the cell numbers were considered.

5.2.2.2.3 FSH-R

The relative expression of FSH-R was augmented by the presence of urea in bGC I and enhanced by presence of urea in bGC III. As FSH-R expression in lost with further differentiation of bGCs towards luteinisation [186,243,244], and luteinisation is combined with an decrease in ES and an increase in P4 production, this is in accordance with the findings that urea had a negative influence on bGC I ES production and a positive influence on bGC III ES production, but also contrary to no influence of urea on bGC I P4 production and a positive influence on bGC III P4 production. These antithetic findings cannot be explained with influences of the cell numbers, because changes in the cell numbers have a similar effect on both ES and P4 production.
β-HB had a negative influence on the relative expression of FSH-R in bGC I and a positive influence on the relative FSH-R expression in bGC III. For bGC I, this is in accordance with a higher production of P4 in the presence of β-HB, but also lower cell numbers cannot explain why β-HB had no influence on the production of ES by bGC I. If the data were statistically analysed for bGC I and bGC III separately – an evaluation that did not improve other significance levels apart from this and was therefore not included in the data description before – the influence of β-HB was improved to levels just below significance on the expression of FSH-R by bGC I ($p = 0.0570$). The higher relative expression of FSH-R in bGC III after the culture with β-HB is in line with its

positive influence on bGC III ES production, but no influence on the P4 production cannot be explained with lower cell numbers after the culture with β-HB.
Summarised, the relative expression of FSH-R was in accordance with most of, but not all influences of the metabolites on the hormone production, even if influences of the metabolite on the cell numbers were taken into account.

5.2.2.2.4 LH-R

Expression of LH-R was, as in the previous experiment comparing perfusion to static culture, lost during culture and accordingly not suitable to examine the influence of the metabolites on the post-culture relative expression. Reasons for the loss of LH-R expression were discussed in chapter 11.4.3.

5.2.3 Summarised Discussion of the Influence of Urea and β-HB on bGC Function and the Link to Possible Influences on Fertility

The level of energy intake, and concomitant changes in plasma metabolites, can stimulate the production of E2 from bGCs [136,141], which supports possible influences of changes in urea and β-HB concentrations on ES production by bGCs. Accordingly, dominant follicles of cows that have overcome the negative energy balance, and with that lower plasma levels of urea and β-HB, produce higher amounts of E2 than dominant follicles developing during earlier phases [259]. Elevated β-HB levels (> 0.1 mM as measured in milk) were found to prolong the post partum anovulatory period [261], and this might be mediated by inadequate bGC hormone production not leading to a sufficient LH surge to trigger ovulation. Accordingly, in a study investigating in the influence of β-HB on bGC function, Vanholder and co-workers found a significant influence of β-HB on E2 and P4 production per cell [157]. Also, dietary restriction over a longer period of time leads to limited E2 concentrations at the follicular level [262], and also short term energy deficits reduce the E2 content of follicles [141]. These effects might be mediated by increased levels of urea and β-HB. Furthermore, oestrus behaviour must be triggered by high systemic concentrations of E2 produced by the GCs of the preovulatory follicle. Insufficient follicular E2 production, which might in addition be triggered by higher metabolic clearance rates, contributes to a masked oestrus behaviour, and this suboestrus makes the detection of oestrus in high yielding animals even more difficult [111]. The occurrence of anoestrus in dairy cows has been rising from 7 % in 1986 [263] to 11-22

% in the UK and the Netherlands [112,130]. In Spain, the number of suboestric cows has also been rising over the last years, whereby an- or suboestrus was with 41.5 % the most abundant reproductive disorder in high producing dairy cows [264]. Furthermore, estrogens are important regulators of ovarian function [265], and E2 was recognized as follicle survival factor [266].

In contrast, a supplementation of dietary fat and with that elevating the presence of lipogenic C2 compounds, what can – in the course of an generated imbalance of C2 to glucogenic C3 compounds – also lead to increased presence of ketone bodies as β-HB, was found to enhance the E2 production of preovulatory follicles [259,267,268]. Accordingly, non-esterified fatty acids, also enriched by surplus C2 compounds, were found to enhance the production of E2 by bGCs, although they had a negative influence on bGC viability [143]. In non-lactating heifers, high crude protein diet levels, which resulted in elevated urea concentration in the follicular fluid, enhanced follicular fluid E2 and P4 content in comparison to low or intermediate crude protein diets, without influencing the E2 to P4 ratio [148]. β-HB in a concentration of 0.02 g/l had a positive effect on cell proliferation and survival in L929 cells [256], which might be explained with increasing intracellular calcium concentrations and activation of signal transduction pathways or by increasing mitochondrial respiration [269,270]. A possible activation of intracellular signal transduction pathways in bGCs by β-HB might also lead to an enhanced steroidogenesis in these cells. Coyral-Castel and co-workers found by experiments in goats that some fatty acids could improve ovarian steroidogenesis and therefore have beneficial effects on goat fertility [271]. But metabolites may transiently improve steroidogenesis in bGCs despite of negative influences on cell viability, because Keren-Tal and co-workers found that GC can perform more efficient and increased steroidogenesis during apoptosis because of the clustering of the steroidogenic organelles [272].

Concerning the production of P4, cows fed on a diet elevating plasma urea levels had lower plasma P4 levels compared to animals fed on normal diet, and the supplementation of dietary fat, that can balance the C2 to C3 compound ratio, but may – if exaggerated – also lead to increased ketone levels, was able to compensate this effect [258]. In contrast, a diet elevating plasma urea enhanced plasma P4 levels in the study of Sinclair and co-workers, but was associated with elevated cyst formation [147]. It is interesting under this aspect, that a lack of an increase in progesterone

concentration after the GnRH/LH surge renders the hypothalamus insensitive to further E2 stimulation, and cyst-like follicles can develop [273]. These cysts can in turn lead to elevated plasma P4 levels later in the cycle. Lucy and co-workers found, that cows in lactation have larger Graafian follicles than heifers, but lower blood concentrations of P4 [110], indicating a lower steroidogenic capacity of the bGCs, possibly mediated by metabolites. Accordingly, high urea diets negatively influence the weight of corpora lutea and their ability to synthesise P4 [274] as well as blood P4 concentrations [275]. Impaired luteal activity was also found in cows with elevated plasma β-HB levels [276]. A late onset or an inadequate production of P4 may be involved in low pregnancy rates in dairy cows [56] and atypical milk P4 profiles have been associated with declining fertility [105].

Non esterified fatty acids in concentrations as they occur in the follicular fluid of high yielding dairy cows have a negative influence on oocyte maturation [139], and ammonia has a negative effect on bGCs *in vitro*, whereby they lose their ability to support *in vitro* oocyte maturation [277]. Also, impaired steroidogenesis due to high urea levels can influence the maturation of the oocyte [147] and elevated levels of β-HB were found to have a negative influence on conception [261]. This is in contrast to Jorritsma and co-workers, who concluded in their review, that the negative influence of urea on fertility takes place during cleavage and blastocyst formation at the stage of the fertilised embryo [140]. In the present study, it was shown that urea has also a potential to affect fertility on the level of the bGCs, and thus in processes before ovulation. A positive correlation between the concentration of P4 and interferon-τ has been reported [278], implicating that inadequate P4 levels may affect embryo survival additionally over alternative pathways. In cows that were fed with a diet increasing circulating insulin and insulin-like growth factor I, isolated bGC I, but not bGC II, secreted significantly higher amounts of E2 compared to cows fed on a normal diet, whereby no difference in the production of P4 was detected [136]. These findings support the results that bGC from different cell classes can differently react to the same metabolic stimulus.

Concluding, there are many possible ways in which a disturbed function of bGCs can influence fertility in dairy cows, and urea and β-HB are potential mediators between the deficient metabolic situation and impaired fertility.

5.2.4 Conclusions

“In order to achieve long-term success in controlling bovine fertility, a concerted effort involving both basic research and its application on the dairy farm will be required [108].”

In the discussion of the data means there are many indications for a disturbance of bGC function mediated by urea and β-HB, and with that finally a disturbance of fertility. However, the influence of the metabolites on bGC function was not statistically provable, maybe due to reasons like high data variability or experimental design, as discussed previously.

Also other mechanisms might lead to the negative influence of the deficient metabolic situation on fertility in high yielding dairy cows, but most probably it is a combination of many factors contributing to the ubiquitous subfertility syndrome. In any case, it is desirable to avoid severe negative energy balance status in order to improve fertility.

It is possible, with a careful and precise management, to obtain high milk yields and satisfying reproductive performance in modern dairy cows at the same time [279,280]. For example, in Scandinavia, traits for reproduction and health were included in a special selection system, and milk production could be improved without negative effects on fertility [281]. Genetic improvements are desirable, but their establishment takes long. In the meantime, one cornerstone for the improvement of milk cow fertility is the prevention of metabolic disorders via good nutritional management [111]. Adequate nutrition during the transition period is of particular importance, because cows that are over conditioned (more than 3.75 on a 1-5 scale [282]) at calving have a higher decline in feed-intake before calving and a delayed increase in dry matter intake combined with a higher loss of body condition and more severe negative energy balance during early lactation [283,284].

For controlling plasma β-HB concentrations, the balance of lipogenic C2 to glucogenic C3 dietary compounds is crucial [285]. Supplementation of propionate, which is antiketogenic and supports the liver glycogen stores, can decrease plasma β-HB levels [286]. Furthermore, cows feeding from glucogenic diets have lower milk fat yield, but higher calculated energy balance, lower plasma non esterified fatty acids and β-HB and liver triacylglyceride concentrations as well as an earlier resumption of post partum ovarian activity including ovulation [287,288]. High protein levels can have a positive

impact on milk production, but also a negative influence on fertility, possibly mediated through increasing plasma urea levels [203,289–291]. Under the aspect, that it is the first aim of a dairy farmer to produce large amounts of high quality milk and that the number of calves is of secondary importance [292], one interesting proposal is to drive genetic selection towards persistent lactations and hence to stretch calving intervals to minimize pregnancy as well as post partum stress with all its negative influences [292]. Anyway, a trend of an increasing calving interval has been recognised [293], which thus can have economic advantages for high yielding dairy cows. Another idea has been put forward by Patton and co-workers, who tested the effect of milking high yielding dairy cows only once a day in the early post partum period, and which resulted in a less negative energy balance and an earlier resumption of the ovarian cycle [294], only combined with a moderate loss in milk production.

Amongst genetics, endocrine therapy and nutritional approaches, management of dairy cows and cow welfare have to be improved to diminish stress factors, because any stress factor additional to the burden of high milk yield can comprise the benefits of increasing production [107].

6 Summary

Fertility in dairy cattle is ubiquitously declining over the last decades. The situation is apparently linked to rising milk production and thus to the deficient metabolic situation in high yielding cows. Investigations on cellular level can elucidate fundamental underlying processes, in particular if the applied system allows simulating (patho-) physiological conditions. Therefore, a perfusion cell culture system was established for bovine granulosa cells (bGCs) and compared to a conventional, static culture system. Subsequently, the perfusion system was used to investigate the influence of negative energy balance-related metabolites on bGC function.

For the establishment of the perfusion culture system, culture conditions were adapted from actual publications concerning conventional bGC culture. DMEM/Ham's F12 medium as basal component, minimal serum content in combination with the use of serum replacers, the use of eCG as stimulant and androstenedione as substrate and CO_2-independency by the use of HEPES-buffer were the main characteristics of the established medium. Ovaries of cows from mixed breeds were collected at the local abattoir. bGCs were isolated by repeated aspiration of the follicular fluid of antral follicles, whereby follicles were classified according their surface diameter as small (< 4 mm), medium (4-8 mm) and large (> 8 mm). Accordingly, it was separated between bGC classes I, II and III, isolated from small, medium and large follicles.

To find a suitable carrier material for bGCs, equal parts of all three bGC classes were cultured for 24 hours under static conditions on 12 different carrier materials. Polycarbonate with a pore size of 0.2 µm showed the best performance in the three tested variables cell attachment, cell distribution (obtained by nuclear staining with DAPI and fluorescence microscopy, because most of the materials were not accessible using conventional light microscopy) and production of 17β-estradiol (E2, measured by radioimmunoassay, RIA), whereby the carrier material had a significant influence on the number of cells, their growth pattern and their production of 17β-estradiol. Polycarbonate with a pore size of 0.2 µm was therefore used in all following experiments.

For the comparison of the perfusion with the static culture, the three bGC classes were cultured separately. RNA-samples were extracted from the freshly isolated cells. After 56 ± 1 hours static preculture, the cell carriers were transferred into new 24-well plates or the perfusion system, whereby the only difference between the two systems was the

way of medium supply. During 56 hours culture in the new setting, medium samples were taken, respectively medium collection tubes changed, every 8 hours for later analysis for total estrogens (ES, comprising E2 and estrone) and progesterone (P4) using RIA. At the culture end, cell numbers were counted using fluorescence microscopy, and RNA was extracted. RNA-samples were analysed for the relative expression of key factors regulating steroidogenesis, which comprised the enzymes P450aromatase (P450arom) and 3β-hydroxysteroid dehydrogenase (3β-HSD) and the receptors for FSH (FSH-R) and LH (LH-R).

As expected due to the different developmental states, the cell class had a significant influence on the production of ES, as well as the culture time. The culture system did not significantly influence the production of ES. According to previous studies the production of P4 was not influenced by the cell class and also not by the culture time, but it was significantly lower in the perfused than in the static system. The relative expression of P450arom and FSH-R was significantly influenced by the culture time, whereby the influence of culture time on the relative expression of 3β-HSD was just below the significant level. Expression of LH-R was only detectable in pre-culture samples from bGC III. The culture system had no significant influence on the gene expression, whereby differences in the relative expression of the P450arom, FSH-R and LH-R could be interpreted as a slower luteinisation process in the perfused culture. In comparison between the two systems, the lower production of P4 in the perfused system as well as a higher ratio of ES to P4 indicate a prolonged luteinisation process in this culture system, with sustained ability to produce ES. No negative effect of the perfused system on the tested parameters was found.

Concluding, the established perfusion system for bGCs has no disadvantages compared to the static culture, and may even be more suitable for a long time culture of bGCs due to a prolonged luteinisation process. It has the advantage of imitating physiological conditions with its continuous medium supply and allows exact tuning of the culture conditions and frequent monitoring via used medium without direct manipulation of the culture. From the three used cell classes, bGC III showed the best performance in the perfusion culture and also the highest ES to P4 ratio.

To investigate the influence of negative energy balance-related metabolites on bGC function, bGC I and bGC III were cultured in the perfused system with and – as negative control – without a time-dependent exposure to urea and β-hydroxybutyric

acid (β-HB). After 55 ± 1 hours static preculture, the cell carriers were transferred to the perfusion system and kept there under equal conditions for 8 hours until the exposure to the metabolite started. If an ideal medium intermixture is supposed, the concentration of the metabolite increased over 8 hours then, reached maximum concentrations of 5 mM urea and 2 mM β-HB for 2 hours, and then decreased over the following 8 hours, until no more metabolite was present in the medium. The culture continued another 10 hours under metabolite-free conditions. During the period with exposure to the metabolite and in the last culture interval, medium samples were taken every two hours and in the remaining culture time every eight hours. Medium samples were analysed for their E2 and P4 content using RIA. Pre- and post-culture RNA samples were analysed for the relative expression of for P450arom, 3β-HSD, FSH-R and LH-R using real-time RT-PCR. Cell numbers were determined at the culture end using nuclear staining with DAPI and fluorescence microscopy.

No significant influence of the presence of a metabolite on the tested parameters was found. It has to be taken into account that the high variability of the parameters can mask statistical relevance of influencing factors, in particular if the discussion of the obtained means of the values clearly indicates differences. Also, variables obtained at the end of the culture, like the cell count and the relative gene expression, might already have recovered from the exposure to the metabolite in former culture periods. The following assumptions were made on basis of the discussion of the data means (↓= negative influence, ↑= positive influence, -- = no influence):

		urea	β-hydroxybutyric acid
bGC I	cell numbers	↓	↓
	production of ES	↓	--
	production of P4	--	↑
	ratio of ES to P4	--	--
bGC III	cell numbers	↓	↓
	production of ES	--	↑
	production of P4	↑	--
	ratio of ES to P4	↓	↑

The influence of the metabolites on the relative expression of the genes relevant for steroidogenesis was according to their presumed effect on the production of ES and P4.

The present study provided for the first time a comparison of a conventional static with a modern perfusion culture system for bGC, based on the same cell pool. The establishment of this perfusion cell culture system is the basis for further investigations in bGCs and GCs of other species under organotypic conditions with the great advantage of workgroup independent reproducibility.

7 Zusammenfassung

Die Fruchtbarkeitsleistung von Milchkühen sinkt seit Jahrzehnten. Dieses Geschehen ist offensichtlich mit der steigenden Milchproduktion und folglich mit der defizienten metabolischen Situation der Hochleistungstiere verbunden. Untersuchungen auf zellulärer Ebene können die grundlegenden Prozesse dieses Zusammenhangs aufklären, vor allem, wenn das verwendete System eine Nachbildung entsprechender (patho-)physiologischer Bedingungen erlaubt. Aus diesem Grund wurde ein Perfusionszellkultursystem für bovine Granulosazellen (bGZ) etabliert und mit einem konventionellen, statischen Kultursystem verglichen. Im Anschluss daran wurde das Perfusionssystem benutzt, um den Einfluss von Metaboliten, die bei negativer Energiebilanz auftreten, auf bGZ zu untersuchen.

Zur Etablierung des Perfusionszellkultursystems wurden Erkenntnisse aus aktuellen Publikationen über bGZ Kulturen adaptiert. Die Hauptcharakteristiken des etablierten Mediums waren die Verwendung von DMEM/Ham's F-12 Medium als Basis, ein reduzierter Serumgehalt ausgeglichen durch Serumersatzstoffe, die Verwendung von eCG als Stimulanz und Androstendion als Substrat, sowie der Einsatz eines CO_2 unabhängigen HEPES-Puffers. Am örtlichen Schlachthof wurden Ovarien von Kühen unterschiedlichen Alters und unterschiedlicher Rasse gesammelt. bGZ wurden durch wiederholte Aspiration der Follikelflüssigkeit antraler Follikel isoliert, wobei die Follikel nach ihrem oberflächlichen Durchmesser als klein (< 4 mm), mittel (4 - 8 mm) oder groß (> 8 mm) klassifiziert wurden. Entsprechend wurde zwischen den bGZ Klassen I, II oder III, jeweils isoliert aus kleinen, mittleren oder großen Follikeln, unterschieden.

Um ein geeignetes Zellträgermaterial für bGZ zu finden, wurden gleiche Teile aller drei Zellklassen für 24 Stunden unter konventionellen Kulturbedingungen auf zwölf verschiedenen Materialien kultiviert. Polycarbonat mit einer Porengröße von 0.2 µm zeigte die besten Ergebnisse bezüglich der drei getesteten Größen Zellzahl, Zellverteilung (gemessen mittels Fluoreszenzmikroskopie nach Kernfärbung mit DAPI) und Produktion von Östradiol-17β (E2, gemessen mittels Radioimmunoassay, RIA). Dabei hatte das Zellträgermaterial einen signifikanten Einfluss auf die Zellzahl, die Zellverteilung und die Hormonproduktion. Polycarbonat mit einer Porengröße von 0.2 µm wurde folglich in allen darauf folgenden Experimenten verwendet.

Für den Vergleich zwischen statischem und Perfusionszellkultursystem wurden die drei bGZ Klassen getrennt kultiviert. Aus den frisch isolierten Zellen wurde RNA

isoliert. Nach 56 ± 1 Stunden statischer Vorkultur wurden die Zellträger entweder in neue 24-well Kulturplatten oder in das Perfusionssystem transferiert, wobei der einzige Unterschied zwischen den Kulturen die Art der Medienversorgung war. Während der folgenden 56 Stunden Kultur unter den neuen Bedingungen wurden alle 8 Stunden Medienproben zur späteren Hormonanalyse auf Gesamtöstrogen (Estron und E2 umfassend) und Progesteron (P4) mittels RIA genommen, beziehungsweise entsprechend die Medienauffangbehälter ausgetauscht. Am Ende der Kulturzeit wurde die Zellzahl mittels Fluoreszenzmikroskopie bestimmt und RNA extrahiert. Die RNA-Proben wurden mittels real-time RT-PCR auf die Expression von Schlüsselenzymen und -rezeptoren der Steroidogenese, 3β-Hydroxysteroid Dehydrogenase (3β-HSD), P450-Aromatase (P450arom), FSH-Rezeptor (FSH-R) und LH-Rezeptor (LH-R), untersucht.

Die Zellklasse hatte, wie durch den unterschiedlichen Differenzierungszustand zu erwarten, einen signifikanten Einfluss auf die Produktion von ES, sowie auch die Kulturzeit. Das Kultursystem beeinflusste die Produktion von ES nicht signifikant. Wie in früheren Publikationen bereits festgestellt wurde, hatten die Zellklasse und die Kulturzeit keinen signifikanten Einfluss auf die Produktion von P4, wohl aber das Kultursystem. Im Perfusionssystem war die Produktion von P4 signifikant niedriger als im statischen System. Die relative Expression von P450arom und FSH-R wurde signifikant von der Kulturzeit beeinflusst, wobei der Einfluss der Kulturzeit auf die relative Expression von 3β-HSD nur knapp nicht signifikant war. Die Expression von LH-R konnte nur in Vorkulturproben von bGZ III nachgewiesen werden. Das Kultursystem hatte keinen signifikanten Einfluss auf die Genexpression, wobei Unterschiede in den Mittelwerten der Ergebnisse auf einen verzögerten Luteinisierungsprozess im Perfusionssystem schließen lassen. Die signifikant niedrigere Produktion von P4 in Kombination mit einem höheren Quotienten von ES zu P4 sprechen zusätzlich für eine verlangsamte Luteinisierung der Zellen im Perfusionssystem im Vergleich zum statischen System, wobei auch die Produktion von ES aufrechterhalten wurde. Kein negativer Einfluss des Perfusionszellkultursystems auf die getesteten Parameter konnte nachgewiesen werden.

Es kann daraus geschlossen werden, dass das etablierte Perfusionszellkultursystem für bGZ keine Nachteile im Vergleich zum statischen System aufweist, und durch

einen verzögerten Luteinisierungsprozess sogar geeigneter für eine Langzeitkultur von bGZ sein kann. Das Perfusionszellkultursystem hat außerdem den Vorteil, mit seinem kontinuierlichen Medienfluss physiologische Bedingungen zu imitieren. Damit erlaubt es die exakte Einstellung der Kulturbedingungen sowie ein andauerndes Monitoring der Zellen über verbrauchtes Medium, ohne direkt an der Kultur zu manipulieren. Von den drei verwendeten Zellklassen zeigten bGZ III die besten Ergebnisse im Perfusionszellkultursystem und auch den höchsten Quotienten zwischen ES und P4.

Um den Einfluss von Metaboliten, die bei negativer Energiebilanz auftreten, auf bGZ zu untersuchen, wurden bGZ I und bGZ III mit oder – als Negativkontrolle – ohne eine zeitgesteuerte Exposition mit Harnstoff oder β-Hydroxybutyrat (β-HB) im Perfusionssystem kultiviert. Nach 55 ± 1 Stunden statischer Vorkultur wurden die Zellträger in das Perfusionssystem transferiert und dort die ersten 8 Stunden unter Metabolit-freien Umständen kultiviert. Eine ideale Mediendurchmischung vorausgesetzt, stieg die Metabolitenkonzentration in der exponierten Kultur dann über die folgenden 8 Stunden an, blieb 2 Stunden bei maximaler Konzentration konstant (5 mM Harnstoff und 2 mM β-HB) und sank dann über die folgenden 8 Stunden wieder ab, bis kein Metabolit mehr im Medium war. Die Metabolit-freie Phase wurde für weitere 10 Stunden aufrechterhalten. Während der Expositionsphase und in den letzten 2 Kulturstunden wurden jeweils alle 2 Stunden Medienproben genommen, in den übrigen Phasen alle 8 Stunden. Die Medienproben wurden mittels RIA auf den Gehalt an ES und P4 und Prä- und Postkultur RNA Proben mittels real-time RT-PCR auf ihre relative Expression von P450arom, 3β-HSD, FSH-R und LH-R untersucht. Die Zellzahl wurde am Ende der Kultur nach DAPI-Kernfärbung mittels Fluoreszenzmikroskopie bestimmt. Es konnte kein signifikanter Einfluss der Metaboliten auf die getesteten Parameter aufgezeigt werden. Dabei muss berücksichtigt werden, dass die hohe Variabilität der Ergebnisse die statistische Relevanz der beeinflussenden Faktoren beeinträchtigt, besonders unter dem Gesichtspunkt, dass die Mittelwerte der Ergebnisse sehr wohl einen Einfluss vermuten lassen. Zudem können sich die Zellen zum Ende der Kultur schon wieder von der temporären Exposition mit den Metaboliten regeneriert haben, was einen Einfluss der Metaboliten während der Kultur auf die Variablen, die am Ende der Kultur bestimmt wurden, wie z.B. Zellzahl und Genexpression, verschleiert.

Folgende Hypothesen konnten auf Basis der Diskussion der Datenmittelwerte gestellt werden (↓ = negativer Einfluss, ↑ = positiver Einfluss, -- = kein Einfluss):

		Harnstoff	β-Hydroxybutyrat
bGZ I	Zellzahl	↓	↓
	Produktion von ES	↓	--
	Produktion von P4	--	↑
	Quotient von ES zu P4	--	--
bGZ III	Zellzahl	↓	↓
	Produktion von ES	--	↑
	Produktion von P4	↑	--
	Quotient von ES zu P4	↓	↑

Entsprechend ihrem Einfluss auf die Produktion von ES und P4 war der Einfluss der Metaboliten auf die Expression der Schlüsselgene der Steroidsynthese.

In dieser Studie wurde zum ersten Mal ein konventionelles, statisches Kultursystem mit einem modernen Perfusionszellkultursystem für bGZ aus demselben Zellpool verglichen. Die Etablierung dieses Perfusionszellkultursystems legt den Grundstein für die weitere Erforschung von bGZ und Granulosazellen weiterer Spezies unter organotypischen Bedingungen mit dem großen Vorteil der forschungsgruppenübergreifenden Reproduzierbarkeit.

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Appendix

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Abbreviations

arithm. mean	arithmetic mean
bGC(s)	bovine granulosa cell(s)
bGC I	bGCs obtained from follicles with a surface diameter < 4 mm
bGC II	bGCs obtained from follicles with a surface diameter of 4 - 8 mm
bGC III	bGCs obtained from follicles with a surface diameter > 8 mm
bTC(s)	bovine theca cell(s)
CVQ	coefficient of variation of the distribution of the stained nuclei on the four circle quarters
eCG	equine chorionic gonadotrophin = PMSG, pregnant mare serum gonadotrophin
ES	total estrogens, comprising 17β-estradiol and estrone
E2	17β-estradiol
FCS	fetal calf serum
FSH	follicle stimulating hormone
FSHr	follicle stimulating hormone receptor
GC(s)	granulosa cell(s)
geom. mean	geometric mean
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ITS	insulin-transferrin-selenium supplement
LH	luteinising hormone
LHr	luteinising hormone receptor
$\log_{10}$	logarithmus to the basis 10
PBS	phosphate buffered saline
PET	polyethylene terephthalate
P450arom	P450 aromatase
P450scc	P450 cholesterol side-chain cleavage enzyme
P450c17α	17α-hydroxylase-C17,20-lyase
real-time RT-PCR	real-time reverse transcription polymerase chain reaction
TC(s)	theca cell(s)
3β-HSD	3β-hydoxysteroddehydrogenase-Δ5/4-Isomerase
17β-HSD	17-β-hydroxysteroiddehydrogenase

Eidestattliche Erklärung

Ich habe die vorgelegte Dissertation selbstständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

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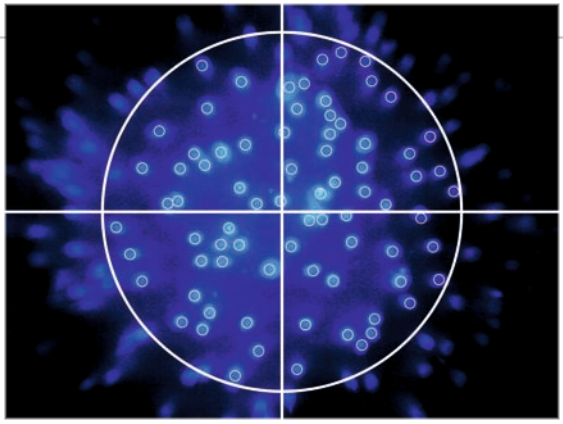

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