THE POTENTIAL OF MELATONIN IN OVARIAN CANCER

A Thesis

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by

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ABSTRACT

Melatonin is an ancient molecule secreted at night by the pineal gland whose therapeutic properties have been increasingly investigated. Its beneficial effects have been studied in diverse malignancies, including ovarian cancer. Ovarian cancer (OC) is the leading gynecological cause of mortality in the United States. The chicken spontaneously develops OC with high incidence and mirrors the human disease. We evaluated the role of melatonin in OC using dietary melatonin supplementation and pinealectomy. With low dose dietary supplementation, we were able to increase nocturnal melatonin without altering its rhythm but failed to alter overall OC incidence. It is possible that the selected dosage may be insufficient or that malignant transformation occurred prior to enrollment date. Further studies using higher doses of melatonin or exploring chronic melatonin deficiency are underway and preliminary results are presented in Appendix I and II.

BIOGRAPHICAL SKETCH

Lucia Borlle graduated as a Doctor of Veterinary Medicine from the Universidad Nacional del Litoral, Argentina. She practiced veterinary medicine for two and a half years after graduation in her hometown before traveling the United States. At Cornell University, she worked with Dr. Kelly Hume in the College of Veterinary Medicine studying cancer biology and testing novel therapies for veterinary oncology. This experience was highly inspiring and motivated her to pursue further scientific training. In 2016, Lucia began her graduate studies with Dr. Johnson in the Department of Animal Science, where she investigated the role of melatonin in ovarian cancer using the domestic laying hen as an animal model. Her interests include animal health, cancer biology, immunology, and translational medicine, and she is committed to improve the wellbeing of all, animals and people.

To my inspiration, the sweet and “pícara” Libertad.

And to my rock and my model, Matías.

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TABLE OF CONTENTS

CHAPTER I

LITERATURE REVIEW

Introduction to the Literature Review

Ovarian cancer (OC) is the leading gynecologic cause of death. According to the American Cancer Society, more than 21,750 women are expected to be diagnosed in 2020 in the United States and over 13,900 are expected to die from the disease (American Cancer Society, 2020). The vast majority of cases are detected at late stages (Matulonis et al., 2016), when they are already metastatic, and even though most patients respond well to initial treatment, most relapse and die within five years. In contrast, cases of OC detected at early stages (stage I or II) have a better treatment outcome and longer overall survival, and 70-90% of them can be cured.

The term ovarian cancer refers to a highly heterogeneous group of diseases that have distinct origin, genomic profile, molecular features, clinical presentation, response to therapies, and prognosis. Epithelial ovarian cancer (EOC) is the most common histological type, accounting for approximately 90% of cases. Subtypes of EOC include serous, endometrioid, mucinous, and clear cell. Non-epithelial tumors are rare and include sex cord stromal and germ cell cancers (National Cancer Institute, 2016).

The first line of treatment involves surgical removal of the tumor, usually followed by chemotherapy, although recurrence and eventual resistance is common (Matulonis et al., 2016). Newly developed therapies, such as PARP inhibitors, checkpoint inhibitors, anti-angiogenic agents, and their combination with traditional chemotherapies are being tested in clinical settings (Matulonis et al., 2016). Despite the expansion of neoadjuvant therapy trials, the overall cure of ovarian cancer remains ~30% (Yang et al., 2017). The need for strategies for early diagnosis and more efficacious treatments is obvious.

Developing models to study ovarian carcinogenesis and to test novel therapies is imperative. In efforts to recapitulate the pathogenesis of ovarian cancer, three- and two-dimensional cell models, xenopatients (animals implanted with a tumor from another species, xenografted), organotypic models, cell lines, and engineered mouse models have been developed (Lengyel et al., 2014). Although these are useful for understanding the tumor microenvironment and OC molecular pathways, they fail to mimic the spontaneous development of OC and therefore are less suitable for studying OC origins. The laying hen is the only animal model that spontaneously develops ovarian cancer with an incidence as high as 35% by 3.5 years of age (Fredrickson, 1987, Urick et al., 2008, 2009), which allows its use in longitudinal studies using a relatively low number of animals. In this regard, the chicken provides a valuable tool for the investigation of OC pathogenesis, progression and response to therapies with relatively little cost (Lengyel et al., 2014). In addition to the high occurrence rates, this model has similar clinical presentation, disease progression, metastasis patterns, histological classification, and molecular alterations, such as expression of CA125, alterations in p53, Her/neu, Kras, upregulation of COX-1 (Hales 2013, Hakim 2013, Jackson 2007) as the disease in women (Fredrickson, 1987, Johnson and Giles, 2013, Hawkridge, 2014, Lengyel et al., 2014, Johnson et al., 2015).

It is a common practice in commercial poultry farms to adopt long lighting schedules, normally 16 hours of light and 8 hours of darkness, in efforts to enhance egg production. Consequently, hens ovulate repetitively and produce an egg almost daily. While this strategy has been successful to increase egg production, it has the associated effect of limiting melatonin, which is released during darkness (Bernard et al., 1997). Previous studies (Chuffa et al., 2015, Chuffa et al., 2016, Shen et al., 2016, Akbarzadeh et al., 2017, Chuffa et al., 2017, Zonta et al., 2017) have implicated melatonin in cancer development, progression, or treatment. These studies

have addressed the possible anti-proliferative (Reiter et al., 2010, Chuffa et al., 2017) and antioxidant (Reiter et al., 2016) properties of melatonin. For this reason, we have used the laying hen to study the role of melatonin in ovarian cancer development and its potential use as a therapeutic agent.

Ovarian Cancer

Ovarian cancer is many diseases (Teer et al., 2017). The term ovarian cancer (OC) refers not to a single entity, but to a group of very distinct diseases. This heterogeneous nature of OC prompted a classification into Type I and Type II based on molecular and clinical features (Kurman and Shih, 2016). According to this classification, **Type I** tumors include low grade serous, low grade endometrioid, clear cell, Brenner tumors, and mucinous histo-types. Tumors within this group commonly exhibit BRAF and KRAS mutations and wild type p53. They are less aggressive, more resistant and less refractory to chemotherapy, and usually confer a better prognosis. In contrast, **Type II** tumors are high-grade, mostly represented by high-grade serous subtype (HGSC), respond well to initial chemotherapy but relapse frequently and carry poor outcomes. Mutations within the p53 gene are present in over 96% of Type II OC. In addition, a fraction of Type II cases exhibit deficiencies in the homologous recombination DNA repair pathway. This latter feature may constitute the reason why they initially respond very well to chemotherapy (Kurman and Shih, 2016), since most chemotherapies induce DNA damage.

The majority of ovarian cancer cases are detected at late stages, when the prognosis is poor (Teer et al., 2017). The nonspecific nature of the symptoms and the lack of effective screening methods delay diagnosis and complicate treatment. First line treatment comprises surgery and platinum-based chemotherapy, and it initially yields good outcomes. Although

patients normally respond well to surgery and chemotherapy, most high-grade OC patients relapse within five years (National Cancer Institute, 2016). Consecutive relapses show incremental chemo-resistance and shorten treatment options. Conversely, survival and treatment outcome improve greatly when the disease is found at early stages (Matulonis et al., 2016). Naturally, efforts in the field have concentrated in early detection and novel therapies.

Classification of Ovarian Cancer in Women

Due to the high diversity of ovarian cancer (OC), a dualistic model has been proposed by Kurman and Shih (Kurman and Shih, 2010, 2016). In this model, the authors categorize OC subtypes according to tumor biology, molecular, histological, and clinical features. **Type I** tumors are mostly low grade, with the exception of clear cell OC. **Type II** are high grade and highly aggressive OCs. This classification emphasizes the differences between types and guides the development of targeted therapies based on a deeper understanding of molecular ovarian carcinogenesis.

Type I Ovarian Cancer

Type I OC includes low grade serous carcinoma (LGSC), endometrioid, and clear cell carcinoma, mucinous, mixed Müllerian carcinomas, and Brenner tumors. Altogether they account for ~10% of ovarian cancer deaths (Seidman et al., 2004, Kroeger Jr and Drapkin, 2017). They arise from benign lesions and very rarely progress to high grade tumors. Typical clinical presentation involves the finding of a unilateral, large cystic mass that is indolent and confined to the ovary. The presence of ascites, the pathological accumulation of liquid in the abdominal cavity, is rare. Type I tumors are mostly low grade, (except for clear cell ovarian carcinoma) and frequently diagnosed at early stages by pelvic palpation or intravaginal ultrasonography. In the absence of extra-ovarian disease, removal of the affected ovary is curative and the prognosis is

excellent, as reviewed by Kaldawy (Kaldawy et al., 2016). Dissemination to extra-ovarian tissues represents a therapeutic challenge. Because LGSC are intrinsically less responsive to chemotherapy, there are limited treatment options when the disease spreads beyond the ovaries (Kurman and Shih, 2016).

Low grade serous carcinomas (LGSC) represent around 10% of ovarian serous carcinomas (Kaldawy et al., 2016). In contrast to other type I OCs, these tumors are commonly bilateral and 30% present metastatic disease at diagnosis. The median age of diagnosis is 55.5 years (10 years younger than HGSC). Although the therapeutic approach is the same as for HGSC, the survival time is extended in LGSC (Kaldawy et al., 2016). LGCS develop from benign lesions in a stepwise fashion that involves papillary tubal hyperplasia of fallopian tube epithelium. Eventually, this lesion evolves to the immediate precursor of LGSC, the atypical proliferative serous tumor (APST) (Kurman and Shih, 2016). At the cellular level, mutations in either KRAS, BRAF, or ERBB2 activate the MAP kinase pathway and lead to neoplastic transformation (Singer et al., 2002, Singer et al., 2003). Carriers of BRAF mutations present better outcome than wild type (WT) BRAF or KRAS mutation carriers (Wong et al., 2010, Grisham et al., 2013, Tsang et al., 2013). Furthermore, KRAS has been associated with higher LGSC recurrence (Tsang, Deavers et al. 2013). In line with these data, BRAF mutations are uncommon in advanced low-grade OCs (Wong et al., 2010). It has been proposed that cells with BRAF alterations are more common in APST. Also, BRAF-carrying lesions show reduced estrogen receptor (ER), progesterone receptor (PR), Wilm's tumor 1 (WT1), and Ki-67 expression, but increased p16, and therefore, these cells display senescence features (Maniar et al., 2014, Zeppernick et al., 2014), which may explain the excellent outcome of LGSC.

Endometrioid and Ovarian Clear Cell Carcinomas (OCCC) develop from atypical proliferative tumors and are associated with endometriosis (Kurman and Shih, 2016). These two entities share a mutational profile that includes disruption of epigenetic regulation of gene expression, that is, mutations in the AT rich interactive domain 1A (ARID1A) (Jones et al., 2010, Wiegand et al., 2010). ARID1A is a known tumor suppressor and mutations within this gene can drive tumorigenesis of the ovary (Guan et al., 2014, Chandler et al., 2015). In wild type cells, ARID1A is part of the chromatin remodeling Switch/Sucrose Non-Fermentable (SWI/SNF) complex that regulates HDAC6, which deacetylates p53. This is the reason why ARID1A and p53 mutations are mutually exclusive in ovarian cancer (Bitler et al., 2017). When ARID1A-dependent inhibition of HDAC6 deacetylase is abolished, p53-dependent apoptosis is suppressed (Bitler et al., 2017). Other mutations seen in these subtypes include inactivation of PTEN, and activation of PIK3CA (in 20%) (Catasús et al., 2004, Nakayama et al., 2006, Jones et al., 2010). Interestingly, cancers that express PTEN and ARID1A mutations, are of endometrioid nature (Guan et al., 2014). Coexisting ARID1A and PIK3CA cause clear cell carcinomas (Chandler et al., 2015). Aberrant KRAS and BRAF genes are less common and affect less than 10% of endometrioid or OCCC cases (Mayr et al., 2006).

The endometrioid subtype makes up ~10% of ovarian cancer cases, is associated with a favorable prognosis and is usually diagnosed at stages I or II (McCluggage, 2008, Hollis et al., 2019). Most of these cancers are low grade and low stage at diagnosis (McCluggage 2008). Some variants of endometrioid OCs can display similarities in morphology to those seen in serous subtypes. To aid the distinction from other entities, immune-histochemical markers can be of use, such as WT1 (negative in endometrioid), positive ER and/or positive PR (McCluggage, 2008, Hollis et al., 2019). Furthermore, it has been stated that PR positive cases carry better

prognosis (Hollis et al., 2019). Ovarian clear cell cancer (OCCC) accounts for 5-10% of OCs (Tan and Kaye, 2007, Shu et al., 2015). It frequently presents at an early stage and has a good prognosis. Conversely, advanced OCCC carries a poor prognosis, worse than the HGSC counterpart at an equivalent stage (Shu et al., 2015).

Mucinous carcinomas (MC) are relatively rare and exhibit high morphological heterogeneity, often showing benign, borderline and malignant lesions within the same neoplasia (Seidman et al., 2004). Mucinous tumors develop in an orderly sequence from benign cyst, borderline, and eventually invasive carcinomas (Mackenzie et al., 2015). These tumors are thought to arise from mucinous cystadenomas, some from teratomas, and others from Brenner tumors (Kurman and Shih, 2016). Controversy exists regarding the tissue of origin of mucinous tumors. The low expression of PR and ER (Chen et al., 2017) supports non-Müllerian origin, but PAX8 (a Müllerian marker) was found in 50% of confirmed MC (Kurman and Shih, 2016). The diagnosis usually involves the finding of borderline or early stage mucinous carcinoma. A challenge is posed to their differentiation from metastatic disease due to their similarity with histological and molecular markers with pancreatic and gastrointestinal carcinomas (Mackenzie et al., 2015). Frequently, they display alterations within the RAS/MEK pathway. Activation of KRAS is a common mutation (Mayr et al., 2006), found in over 60% of mucinous tumors (Mok et al., 1993, Mackenzie et al., 2015). BRAF, and/or ERRB2 are also present, although, less commonly (Mayr et al., 2006, Mackenzie et al., 2015). Mutations in p53 are also a habitual finding in mucinous ovarian carcinomas. Less commonly, CDKN2A, PIK3CA, PTEN, CTNNB1, SMAD4, FRFG2, and SRC were found in mucinous carcinomas (Mackenzie et al., 2015)

Type II Ovarian Cancer

Type II cancers are predominantly represented by HGSC, but also include undifferentiated carcinomas, primary peritoneal carcinomas, and malignant mixed Müllerian tumors. These neoplasms are small, usually bilateral, and diagnosed with evidence of abdominal implants. As stated above, they have an aggressive fallopian tube precursor, called serous tubal intraepithelial carcinoma (STIC), and early stages are difficult to discern (Köbel et al., 2014). Type II tumors are initially responsive to chemotherapies, but commonly relapse with incremental resistance to platinum-based treatments. Chemotherapy protocols include combinations of a platinum derivative (cisplatin or carboplatin) and a taxane (docetaxel or paclitaxel), and new combinations are being tested (Matulonis et al., 2016). The molecular alterations of these neoplasms represent an opportunity for development of new treatments. Poly ADP ribose polymerase (PARP) inhibitors were proven beneficial to patients with BRCA and BRCA-related aberrations or other alterations in the homologous recombination (HR) pathway (Pennington et al., 2014). PARP inhibitors have been tested in first-line chemotherapy and in recurrent disease with success (Audeh et al., 2010, Gelmon et al., 2011, Sandhu et al., 2013, Coleman et al., 2015, Franzese et al., 2018, Mittica et al., 2018, Taylor and Eskander, 2018).

High grade serous carcinoma (HGSC) is the most common subtype of ovarian cancer (OC), as it represents 70% of epithelial OCs (Teer et al., 2017). Frequently, this entity presents as an aggressive tumor and at advanced stages. Historically, it was believed that HGSC originates in the ovarian epithelium as a result of repetitive insults from the ovulatory process (Fathalla, 1971). Subsequent work has established a sequential organization of events that precedes the development of HGSC (Kroeger Jr and Drapkin, 2017). The first molecular change that occurs is a mutation on the p53 gene. This characteristic feature that precedes malignant

transformation on otherwise normal tubal cells is referred to as “p53 signature”. Subsequently, a lesion called serous tubal invasive carcinoma (STIC) develops that extends to the ovary (Kroeger Jr and Drapkin, 2017).

This subtype is marked by obligatory p53 mutations, uncommon recurring mutations in other genes (except for BRCA1 and BRCA2), high copy number aberrations, and common homologous recombination deficiency (HRD) (The Cancer Genome Atlas Research Network, 2011). According to The Cancer Genome Atlas (TCGA), 96% of HGSC harbor p53 mutations (The Cancer Genome Atlas Research Network, 2011). However, a recent analysis of the 4% p53-negative cases revealed that they were misclassified (Vang et al., 2016), indicating that 100% of HGSC have p53 mutations. Additionally, defects in homologous recombination occur in half of HGSC cases, BRCA1 and BRCA2 being the most common cause, and less frequently, mutations in Fanconi Anemia genes, RAD51, RB, ATR, ATM, and CHEK2 (The Cancer Genome Atlas Research Network, 2011). Another frequent finding is amplification of the CCNE1 that encodes cyclin E involved in cell cycle regulation (Patch et al., 2015).

The (controversial) origins of ovarian cancer

In the 70s, Dr. Fathalla (1971) postulated that “incessant ovulation” is causative of ovarian carcinogenesis (Fathalla, 1971). This hypothesis gave basis to the paradigm that chronic rupture and repair caused by the repetitive ovulatory process was eventually tumorigenic. He proposed that the ovarian surface epithelium undergoes malignant transformation as a result of the accumulation of DNA abnormalities. Almost 30 years later, in the early 2000s, this hypothesis was challenged by the recognition of pre-cancerous lesions in fallopian tubes of BRCA carriers undergoing risk reducing salpino-oophorectomy (RRSO) (Piek et al., 2001a).

Patients that were positive for deleterious BRCA1/2 mutations often chose prophylactic RRSO, and ovaries and fallopian tubes were then subject to cautious examination, following a WHO protocol called SEE/FIM (Sectioning and Extensively Examining the Frimbriated End). The systematic evaluation of the fallopian tube led to an unexpected finding of pre-tumorous lesions that were highly proliferative and strongly positive for p53 staining. These lesions displayed an identical mutational profile to HGSC in the absence of ovarian cancer, which led to subsequent studies that also found early ovarian cancers in the fallopian tube (Piek et al., 2001b, Piek et al., 2003, Finch et al., 2006).

It is now believed that ovulation plays a permissive role in creating an environment that benefits development of ovarian neoplasia, especially in the Type I tumors, although, it doesn't explain OC origin entirely because pathologies not related with ovulation are associated with OC (Kroeger Jr and Drapkin, 2017). Since the introduction of RRSO and SEE/FIM, there has been a shift of paradigm. It is now widely accepted that most HGSC originate in the uterine fimbria (Kurman and Shih, 2016).

Risk Factors for Ovarian Cancer

Several factors have been associated with increased risk for ovarian cancer, such as genetics, reproductive status and history, and lifestyle. BRCA1 and BRCA2 germline mutations are the most relevant genetic factors present in 17% of OCs (Zhang et al., 2011, Alsop et al., 2012). Nevertheless, among women with ovarian cancer, BRCA mutation is a predictor of increased survival (Zhang et al., 2011, Bolton et al., 2012, Zeppernick et al., 2014). Parity, use of contraceptives, tubal ligation, oophorectomy, and salpingectomy have been associated with a reduced risk of ovarian cancer (Moorman et al., 2013, Friebel et al., 2014, Rice et al., 2014, Bassuk and Manson, 2015, Gaitskell et al., 2016). Hormone replacement therapy in post-menopausal women is associated with a higher risk of developing OC (Mørch et al., 2009, Pearce et al., 2009, Hildebrand et al., 2010). Additional risk factors include age, race, use of talc powder, clinical depression, alcohol consumption, tobacco use, overweight, and obesity (Matulonis et al., 2016, Sengupta and Honey, 2019).

In the wild, most animals keep the lifetime number of ovulatory cycles (LOC) to a minimum as they alternate periods of gestation, lactation, and seasonal anestrus, but LOC in a modern day woman's lifetime are substantially higher, ranging from 300-500 (Moorman et al., 2002, Murdoch et al., 2010, Peres et al., 2017). This may explain why animals rarely develop the neoplasia as LOC have been associated with elevated risk of ovarian cancer (OC) (Casagrande et al., 1979, Schildkraut et al., 1997, Webb et al., 1998, Moorman et al., 2002, Purdie et al., 2003, Tung et al., 2003, Tung et al., 2005, Pelucchi et al., 2007, Terry et al., 2007, Schildkraut et al., 2008, Gates et al., 2009, Robbins et al., 2009, Peres et al., 2017). Suppressing ovulation through oral contraceptive use, parity, and breastfeeding, reduce OC incidence (Whittmore et al., 1992). Indeed, epidemiological studies have found a decrease between 30% and 50% of cancer risk with

the use of birth control pills (Whittmore et al., 1992, National Cancer Institute, 2018). This protective effect extends proportionally to the duration of use and lasts for 30 years (Havrilesky et al., 2013, Wentzensen et al., 2016, Michels et al., 2018).

The ovulation-related mechanism that predisposes to carcinogenesis is unknown, yet a number of theories that involve damage of the ovarian epithelium have been postulated. Repetitive cycles of trauma and repair may cause oxidative DNA damage that provides the opportunity for genetic aberrations (Burdette et al., 2006, Murdoch et al., 2010). In addition, the surge of gonadotropins and prostaglandins causes weakness and rupture of the follicle wall resulting in ovulation with elevated blood flow and breakdown of connective tissue. This creates a cytokine- and inflammatory factor-enriched environment that can contribute to mutagenesis (Fathalla, 2013). Estradiol and testosterone may influence ovarian malignant transformation, as they induce proliferation of ovarian epithelium in *in vitro* conditions, although progesterone inhibited cellular growth (Syed et al., 2001, Hollis et al., 2019). Likely, ovulation is not the determining factor that leads to carcinogenesis, but it creates an environment propitious for malignant transformation, and growth, as suggested in several reports (Fathalla, 2013, Lengyel et al., 2014).

Environmental factors are known to influence tumor development. In fact, circadian rhythm disruption was regarded as a "potential carcinogen" by the International Agency for Research on Cancer (IARC, 2010). In line with this, several studies postulated that night or rotating shift workers have an elevated risk for hormone-dependent cancers (Hamilton, 1969, Schernhammer et al., 2001, Weiderpass et al., 2012, Bhatti et al., 2013, Schwarz et al., 2018). In particular, Carter and collaborators (Carter et al., 2014) reported that working at a rotating shift increases the risk of fatal ovarian cancer. Batthi et al. (2013) also found an increase in OC risk in

night or rotating work shift (Bhatti et al., 2013). Further studies are warranted to investigate the relation between shift work and ovarian cancer.

Ovarian Cancer in the Laying Hen

The laying hen model for ovarian cancer was first proposed in 1971 by Dr. Fathalla (Fathalla, 1971). Previously, the high incidence of ovarian adenocarcinomas was observed in poultry (Campbell, 1951, Wilson, 1958), but data were scarce regarding the disease because commercial farms generally replace their hens before the age of onset of ovarian cancer. In 1987, Dr. Fredickson (Fredrickson, 1987) conducted an epidemiological study that served as landmark for the field with observations that still hold true: high rate and the spontaneous nature of OC, its morphological heterogeneity, and the OC occurrence between 2 – 4 years of age (but not earlier than 2 years) of age in the chicken. This was the first large-scale epidemiological study of ovarian cancer in the laying hen, and served as basis for the establishment of the animal model.

The chicken reproductive anatomy and physiology presents some particularities that are relevant for the study of ovarian cancer. The laying hen, as most birds, has only a left ovary and oviduct. The single ovary contains follicles organized hierarchically. Small primordial and primary follicles are completely enclosed within the ovarian cortex and contain the oocytes arrested at the first meiosis. As they grow, they erupt from the ovarian surface and develop into small white (less than 5 mm in diameter), and to small yellow (between 5 and 9 in diameter) follicles (Apperson et al., 2017). There is rapid growth that leads to ovulation after this phase. The pre-ovulatory hierarchy involves the orderly ovulation of F1, the largest follicle, followed by F2, the second largest, in the next ~24 hours, and so forth. Sexual maturity is reached at about 20 weeks, with peak egg production at approximately 30 weeks, when hens produce almost an egg daily (one egg every 24 – 26 hours). The interval between ovulatory cycles increases with age,

and younger birds generally lay more often. Most hens maintain consistent laying rates for one year, when production decreases and then, often after a molt, returns to a slightly lower level of production than during the first year. Ovarian tumorigenesis occurs after 2 years of age and causes partial or complete cessation of egg production as some follicles are replaced by hemorrhage, cysts, and/or cauliflower-like nodules (Fredrickson, 1987, Barua et al., 2009, Johnson and Giles, 2013, Johnson et al., 2015).

The domestic laying hen is the only animal that spontaneously develops ovarian cancer at a very high rate. Between 2.5 and 3.5 years of age, ~35% (~50% in the present work, unpublished) of these animals will present signs of the disease (Fredrickson, 1987, Urick et al., 2009, Johnson and Giles, 2013). By three years of age, a laying hen has ovulated over 500 times (Apperson et al., 2017), which may be equivalent to women undergoing menopause. At that point in life, the risk for ovarian cancer increases greatly for both species (Moorman et al., 2002, Lengyel et al., 2014, Matulonis et al., 2016, Peres et al., 2017). This provides support to the link between life-time ovulations and OC risk.

Similar to humans, there is an association between ovulation and ovarian cancer incidence in poultry. Laying hens that were manipulated to produce fewer eggs, and consequently have fewer ovulations, presented a reduced incidence of ovarian adenocarcinoma (Barnes et al., 2002, Giles et al., 2010, Carver et al., 2011). Hens that fail to ovulate due to a genetic mutation, called “restricted ovulators (RO)” were used to compare OC incidence to wild type (WT) siblings. As expected, a reduction of OC incidence from 27% to 3% was observed in 3 year-old WT versus RO hens, respectively (Giles et al., 2010). Also, the hens’ nutrition can be manipulated to provide a sufficient level to maintain body weight and health but nor for producing eggs. Caloric restriction reduced laying rates and, consequently, OC incidence

Figure 1.1. Examples of normal on the right and cancerous ovaries from White Leghorn laying hens.

(Carver et al., 2011). Regarding OC prevention by oral contraceptives, a study in laying hens has demonstrated that progestin alone or in combination with estrogen causes a decrease of ovarian cancer risk of 91% and 81%, respectively (Treviño et al., 2012). Rodriguez and collaborators also found a protective effect of progestins against reproductive cancers in laying hens (Rodriguez et al., 2013). Factors that reduce the number of ovulations, such as the use of oral contraceptives, parity, and breastfeeding also decreases OC in women (Whittmore et al., 1992). It is believed that the series of events that lead to ovarian cancer after repetitive ovulatory cycles may involve accumulation of DNA damage over time contributing to neoplastic transformation (Fathalla, 2013, Lengyel et al., 2014) although it is uncertain whether these mutations arise in the oviduct or ovary.

Based on growing evidence, it is being accepted that many high-grade ovarian tumors originate in oviductal tissues. Interestingly, several reports provide evidence to support oviductal origin of laying hens' ovarian tumors as well (Giles et al., 2004, Treviño et al., 2010). Support for the oviductal origins of ovarian cancer in the laying hen was provided by transcriptomic analyses. Gene expression studies revealed that ten of the twenty-five top up-regulated genes expressed in ovarian cancer but not in normal ovary were oviduct-related (Treviño et al., 2010). Early evidence regarding the oviduct involvement in OC development was initially suggested by Haritani in 1984 (Haritani et al., 1984). In this paper, the presence of ovalbumin, a protein mostly secreted by the oviduct, was detected in hens' adenocarcinomas. Importantly, these animals presented concomitant oviductal lesions. Giles and collaborators also demonstrated ovalbumin expression in all of ovarian tumors of laying hens (Giles et al., 2004). In contrast to Haritani's publication, the later study did not find concomitant ovarian and oviductal lesions, which led the authors to assume that the oviduct was not always involved. However, it is

possible that microscopic neoplasias were not detected in tubal tissues. Alternatively, the ovarian tumors may have originated in the oviduct. A more recent investigation proposed HER-2/neu as a potential reporter of ovarian origin, as 10/19 ovarian cancers, but only 1/17 of oviductal cancer expressed it (Hakim et al., 2009). Further research is needed to confirm the relevance of such markers.

As in women, avian ovarian cancer expresses CA-125, but normal epithelium does not (Jackson et al., 2007). This high molecular weight glycoprotein, also called mucin 16, is expressed on the cell membrane of epithelial ovarian neoplasias, and it is routinely used in clinical settings for screening and monitoring of ovarian cancer. Jackson and collaborators (Jackson et al., 2007) were able to optimize the immunohistochemistry (IHC). The authors demonstrated that this tumor marker is suitable for use in avian ovarian tumors and that chicken and human similarly express CA125.

Detailed histological characteristics and classification of ovarian cancer in the laying hen was published by Barua and collaborators (Barua et al., 2009). Like in humans, the chicken displays all four major types of epithelial ovarian cancer: serous, endometrioid, mucinous and clear cell carcinomas as well as their putative precursor lesions (Barua et al., 2009). This study included 155 laying hens ranging from 1 to 5 years of age and found striking similarities in the avian specimens to the human counterparts. As in women, the serous type displays papillary structures with slit-like spaces and marked nuclear atypia; endometrioid subtype shows complex glandular architecture; mucinous specimens present multiple glandular structures crowded with occasional ciliated goblet cells, and eosinophilic foci; and clear cell cases displayed vacuolated cells with abundant clear cell cytoplasm invading the theca layer of stromal follicles (Barua et al., 2009). Furthermore, putative pre-malignant lesions were identified in the laying hen that, as

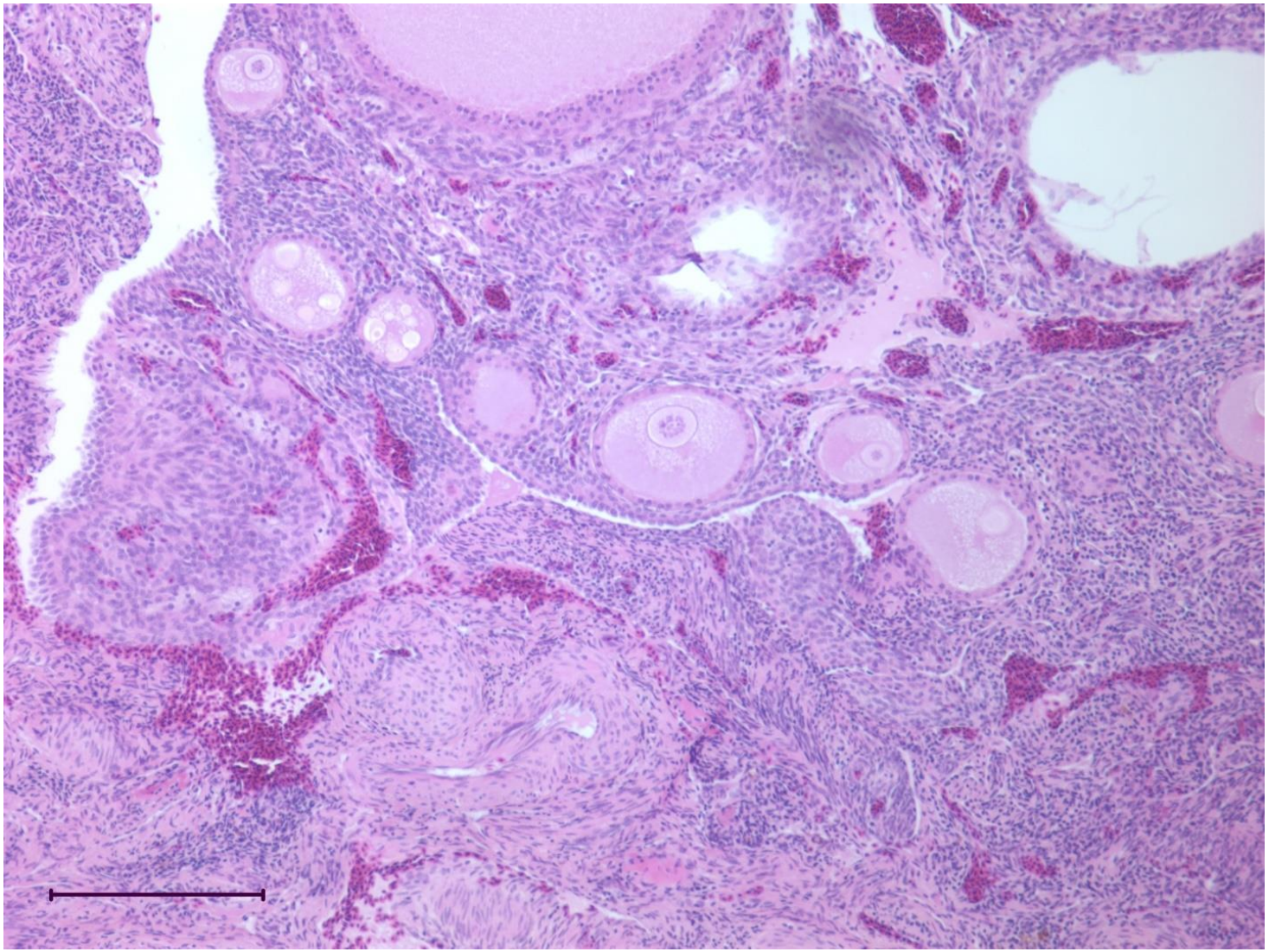

Figure 1.2. Histological slide from a normal ovary from a 3-year-old laying hen. Hematoxylin and eosin stain. Scale bar represents 100 µm. A section of ovarian tissue from the cortex region with several follicles, a possible cyst, vascular vessels and normal appearing stromal tissue.

In women, include epithelium dysplasia, inclusion cysts, and surface epithelium transformation. Although this was not an epidemiological study focused on determining the incidence of each subtype, mucinous OC was the most commonly diagnosed, as opposed to serous OC in women (McCluggage, 2008, Matulonis et al., 2016).

Importantly, many of the symptoms observed in women also present in the chicken. A laying hen that suffers from OC will appear bloated, lethargic, and standing in an abnormal posture (Johnson and Giles, 2013, Johnson et al., 2015). The presence of ascites, abnormal accumulation of fluid in the abdomen, is a frequent finding. At necropsy, disseminated metastatic nodules affecting intestines, peritoneum, mesenterium, oviduct, and less frequently liver, are usually noted (Fredrickson, 1987). The ovary morphology varies from large cystic masses to nodular cauliflower-like ovaries (Johnson et al., 2015). Ovarian cancer stages in the laying hen are equivalent to the human's counterpart. Stage I involves absence of macroscopic disease, but noticeable neoplasia at histological examination. Stage II involves ovarian cancer confined to the ovary. Stage III includes dissemination to abdominal organs and Stage IV involves ascites and further metastatic progression (Treviño et al., 2010). Similarly, in human ovarian cancer, the International Federation of Gynecology and Obstetrics (FIGO) stage I comprises only involvement of one or two ovaries, Stage II includes pelvic metastasis, Stage III involves implants extending beyond the pelvic cavity, and Stage IV includes distant metastasis (Prat, 2015). Diagnosis of ovarian cancer relies on imaging, particularly transvaginal ultrasound. As in women, laying hen tumors can also be detected through this technique with high efficiency (Barua et al., 2007). Furthermore, OC can be detected prior to the development of clinical signs via ultrasonography (Barua et al., 2007).

The mutational profile of laying hen ovarian cancer exhibits similarities to humans. As stated above, a comparable pattern of mutations was observed in ovarian carcinogenesis relevant genes, such as p53, RAS, Her2/neu (Hakim et al., 2009, Bosquet et al., 2011, Seo et al., 2011). A salient commonality between the two species is that p53 appears mutated in tumors of high grade, whereas *ras* mutations are a finding on low grade adenocarcinomas (Hakim et al., 2009). In addition, hens' tumors, as humans', also express CA-125 (Jackson et al., 2007), COX1 (Urick and Johnson, 2006), mesothelin (Yu et al., 2011), E-Cadherin (Ansenberger et al., 2009), and selenium binding protein (Stammer et al., 2008). Moreover, vascular endothelial growth factor (VEGF) expression in ascites cells and fluid, as in women, was correlated to ascites volume in the chicken counterpart (Urick et al., 2008, Bekes et al., 2016).

Regarding the environmental factors that may influence ovarian cancer, light exposure may play a relevant role. Birds exhibit strong photoperiodism that controls seasonal reproduction. In response to changes in day length, hypothalamic-hypophyseal-gonadal axis activation triggers the release of gonadotropins and results in gonadal stimulation. Indeed, gonadal size of migratory birds during breeding season increases a hundred-fold (Nakane and Yoshimura, 2019). In efforts to increase egg production, many commercial poultry farms use prolonged light exposure on a year-long schedule of 16L:8D light schedule (i.e.16 hours of light and 8 hours of dark). This strategy, along with appropriate nutrition and management, results in highly efficient follicular development and therefore high egg production (Johnson et al., 2015).

The potent sensitivity to photoperiod can be manipulated to induce ovarian cyclicity and to investigate ovarian cancer. Moore and collaborators (Moore and Siopes, 2004) were able to promote ovarian adenocarcinomas in 2-year-old turkeys by exposing them to 16 hours of light daily. The animals with palpable ovarian cancer (n = 15) were then subjected to the influence of

short photoperiod (8 hours of light and 16 hours of darkness, 8:16 L:D) for 8 weeks. This lighting schedule caused a regression of tumors in all individuals by the end of the 8 weeks. The mean time to complete regression was 4.4 weeks. In the second stage of this experiment, hens were exposed to a long photoperiod (16:8 L:D), which resulted in regeneration of tumors with a mean time of 5.4 weeks. Based on this evidence, it was concluded that ovarian carcinomas in the turkey can be regulated by photoperiod (Moore and Siopes, 2004). Although these results show promise, caution should be employed in the interpretation as no control group was present to rule out random chance. Also, tumor growth was subjectively measured, and operator bias was not considered in the data interpretation. In a second experiment (Moore and Siopes, 2004), a flock (n = 22) of turkeys with OC induced by a long photoperiod (16L:8D) were transferred to a short photoperiod for 8 weeks. At this point, the birds were selected for treatment (n = 11 control, n = 11 melatonin) and exposed to 15 weeks of long photoperiod. The melatonin group received daily an intramuscular injection of 50 ug/ml melatonin (control group received diluent) one hour before lights were turned off. Melatonin administration resulted in a significant delay in tumor re-occurrence; control hens had palpable masses at 4.5 weeks and melatonin hens had them at 8.5 weeks. Melatonin did not prevent the re-growth of tumors, however. The results presented in this article highlight the relevance of photoperiod and the potential role of melatonin in ovarian cancer (Moore and Siopes, 2004).

Melatonin

Melatonin, N-acetyl-5-methoxytryptamine, is a highly conserved molecule that has been identified in every studied organism, including bacteria, fish, reptiles, mollusks, yeast, and plants. Its most ancient function is free radical scavenging and antioxidant activity, therefore maintaining cellular homeostasis and cell integrity. Melatonin also is involved in other important processes, such as immunomodulation, regulation of seasonal reproduction, circadian entrainment, and anti-cancer properties. Importantly, melatonin acts as a chronobiotic by synchronizing environmental cues of daily and seasonal light and the internal circadian rhythm as reviewed by Armstrong (Armstrong and Redman, 1991).

Although melatonin acts in an autocrine or paracrine manner in some organisms and tissues, it is mostly regarded as a hormone produced and released during darkness. In vertebrates, the pineal gland synthesizes and secretes it in response to darkness (Quay, 1964, Binkley et al., 1975, Lewy et al., 1980). During the day, environmental light initiates a signaling cascade of events that results in the inhibition of melatonin synthesis from the pineal gland. Photoreceptors located in the eyes sense the light and send a neural signal to inhibit melatonin production, reviewed in Claustrat (Claustrat et al., 2005). Avian photoreception, as in other non-mammalian vertebrates, utilizes extraocular photoreceptors (Okano et al., 1994). The pineal gland itself was shown to sense light, and the photopigment pinospin was isolated from chick pineal gland (Okano et al., 1994). Furthermore, birds have additional deep brain photosensitive cells called "OPN5-positive cerebrospinal fluid contacting-neurons" that sense light (Guh et al., 2019). These photoreceptors convey the luminous message to the master clock, the suprachiasmatic nucleus (SCN), which suppresses pineal melatonin production through inhibition of the paraventricular nucleus (PVN) (Pandi-Perumal, 2005). During darkness, the SCN constraint is

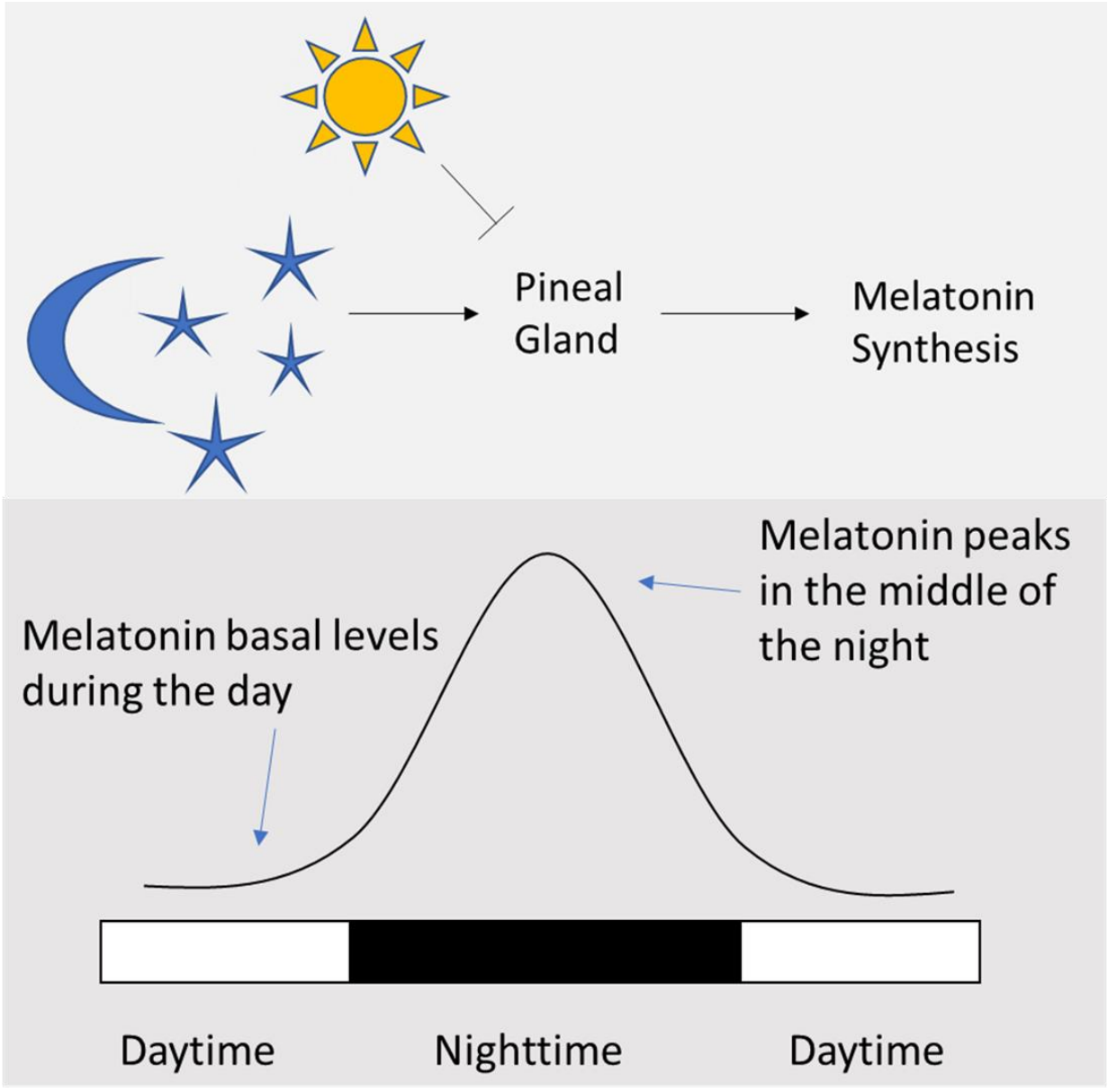

Figure 1.3. Schematic of daily melatonin secretion.

interrupted. A noradrenergic stimulus promotes the synthesis of the critical arylalkylamine-*N*-acetyltransferase (AA-NAT) enzyme, whose activity drives the melatonin peak at night (Zawilska et al., 2009). Melatonin production involves a series of enzymatic modifications of the essential amino-acid tryptophan in the pineal gland. AA-NAT is responsible for the acetylation of serotonin that then is further modified to melatonin, and circulating melatonin reflects AANAT levels. Pinealocytes cannot store this hormone, and it is immediately released to the circulation after production, where its half-life is approximately 20 - 40 minutes, depending on the species (Cipolla-Neto and Amaral, 2018). Once in circulation, melatonin can diffuse through the cerebrospinal fluid and go to target tissues. In the bloodstream, melatonin circulates bound to albumin, it is metabolized in the liver and excreted in the urine (Cipolla-Neto and Amaral, 2018).

Melatonin can target most tissues and act in a receptor-dependent or receptor-independent manner (Reiter et al., 2010). Because it is soluble in lipids and water, melatonin can transverse the cytoplasmic membrane and act directly on the cytoplasm (Zawilska et al., 2009). Melatonin's robust free radical scavenger properties and antioxidant properties are examples of receptor-independent functions. The mechanistic pathway involves the donation of an electron or a hydrogen atom to inactivate oxygen radicals (hydroxyl, peroxynitrite anion, singlet oxygen, superoxide anion, hydrogen peroxide, nitric oxide, and hypochlorous acid). Importantly, metabolites generated during melatonin interaction with ROS (reactive oxygen species) are strong scavengers for toxic oxidizing agents as well (Reiter et al., 2016). As a consequence, melatonin provides protection against a major cause of DNA damage, the ROS produced during physiological cellular processes (Reiter et al., 2016).

Another example of a non-receptor mediated function of melatonin entails calmodulin regulation. Calmodulin is a ubiquitous protein that controls a broad range of cellular functions,

such as protein balance, gene transcription, cytoskeletal organization, cell proliferation, endo- and exocytosis, gene transcription, among others. Melatonin binds to calmodulin with a high affinity and constitutes an endogenous antagonist, even at nanomolar concentrations (Soto-Vega et al., 2004). Some have proposed that the photoperiodic message of melatonin may be translated by calmodulin at a subcellular level (Soto-Vega et al., 2004). Data regarding the role of calmodulin on melatonin synchronization of cellular physiology remain scarce.

Receptor-mediated actions involve G protein-coupled transmembrane receptors (called MT1 and MT2) and a retinoic related orphan nuclear hormone receptor (RZR/RORα) (Cipolla-Neto and Amaral, 2018). Birds, fish, and amphibians have a third G-coupled protein receptor that has evolved to GPR50 in mammals (Gautier et al., 2018). GPR50 is a membrane protein that can dimerize with MT1 or MT2 but that cannot interact directly with melatonin. It acts as an indirect melatonin antagonist as it reduces binding to the membrane receptors (Cipolla-Neto and Amaral, 2018). In mammals, there is a third binding site located in the cytosol, a detoxifying enzyme referred to as MT3. This quinone reductase (quinone reductase 2) enzyme confers protection against cellular oxidative damage by suppressing electron transfer (Jung and Ahmad, 2006). Membrane receptors MT1 and MT2 homo- or hetero-dimerize upon melatonin binding and interact with several downstream messengers, such as adenylyl cyclase, phospholipase C, phospholipase A, and calcium and potassium channels (Cipolla-Neto and Amaral, 2018). Orphan receptors, as the retinoic acid-related orphan receptor/retinoid Z melatonin nuclear receptor (ROR/RZR), reduce gene transcription of the 5-lipoxygenase pathway (Reiter et al., 2016), and stimulate antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, and glutathione reductase (Antolín et al., 1996, Rodriguez et al., 2004, Tomás-Zapico and Coto-Montes, 2005).

Pertinent to our research is the role of melatonin in modulating reproduction. By acting as an internal zeitgeber (from the German zeit = time and geber = giver, a cue that can entrain biological rhythms to dark and light environmental cycles), it conveys environmental photoperiod information to the hypothalamus-hypophysis-gonadal axis. Receptors for melatonin have been found in all three components of the axis (Reiter et al., 2010), and the chicken ovary presents all three membrane receptor types, MT1, MT2, and Mel1c. The proposed mechanism by which melatonin transmits environmental information to reproductive organs involves signaling to *pars tuberalis* (PT)-specific thyrotrophs that respond to melatonin in a receptor-mediated fashion (Kameda et al., 2002, Ikegami and Yoshimura, 2013, Ubuka et al., 2013). This interaction results in the production and dimerization of CRY1 and PER1 (clock genes) and consequent expression of thyroid stimulating hormone (TSH). This hormone reaches the neighboring tanycytes in the PT vicinity and induces the production of DIO2 while reducing DIO3. DIO2 is an enzyme that converts T4 (inactive) to T3 (active), the active form of thyroid hormone. As a consequence, gonadotropin releasing hormone (GnRH) induces the synthesis of gonadotropin and seasonal reproduction, reviewed by Cipolla (Cipolla-Neto and Amaral, 2018). An additional player in birds is represented by GnIH neurons that are stimulated by melatonin and therefore send an inhibitory signal to the reproductive axis (Ubuka et al., 2013). The overall melatonin message is conveyed through similar pathways to both long day breeders (those who reproduce on long days) and short-day breeder (those reproductively active in short days, such as sheep). However, how this information is translated to effector organs is species-specific.

Melatonin in Ovarian Cancer

Circadian rhythm disruption is associated with increased risk for various diseases, including cancer. Large epidemiological studies have provided robust evidence regarding such association (Schernhammer et al., 2001, Weiderpass et al., 2012, Bhatti et al., 2013, Carter et al., 2014, Liu et al., 2018). Aspects of modern life, such as light pollution and light at night cause profound disruption of physiological circadian rhythms. Examples of chronic disruption are rotating or night shift work. Data collected from the American Cancer Society's Cancer Prevention Study-II supports the link between rotating shifts and fatal ovarian cancer (Carter et al., 2014). However, there are contradictions in the literature (Poole et al., 2015). Nightshift was associated with higher risk of invasive and borderline ovarian tumors in an approximately 3000-participant study (Bhatti et al., 2013). Melatonin is suppressed by situations of light at night in a wavelength-dependent manner (Thapan et al., 2001). Given the wide range of functions of melatonin that protect cellular integrity, it has been postulated to be responsible for the association between circadian disruption and cancer risk (Cohen et al., 1978, Stevens et al., 2007).

In the context of ovarian cancer, melatonin exhibits anti-cancer properties in *in vitro* and *in vivo* settings. Ovarian cancer cell lines exposed to increasing doses of melatonin showed reduction in cell viability in a dose- and time-dependent response manner (Chuffa et al., 2013a, Chuffa et al., 2016). Melatonin was able to also reduce the cell viability of the stem cell subpopulation of SKOV3 cells, proliferation, and invasiveness in a receptor-dependent manner (Akbarzadeh et al., 2017). In addition, melatonin reduced the expression of genes related to epithelial-to-mesenchymal transition and to the migration of cancer stem cells (Akbarzadeh et al., 2017). Other factors involved in the invasiveness of cancer cells, the matrix

metalloproteinases (MMPs), were associated with poor prognosis for their extracellular matrix remodeling capacity (Plaks et al., 2015). Isoforms MMP-2 and MMP-9 were downregulated by melatonin in ovarian cancer cell lines (Akbarzadeh et al., 2017). Other reports demonstrated that cell proliferation of PA-1 and OVCAR cell lines was inhibited by melatonin through a blockage in the cell cycle (Shen et al., 2016). In addition, melatonin was reported to increase the therapeutic effect and to reduce the toxicity of cisplatin (a common chemotherapeutic agent used as first line treatment against OC) in ovarian cancer cells (Kim et al., 2012). In agreement with the *in vitro* findings, *in vivo* models developed by Chuffa and colleagues (2013) revealed similar results. Melatonin treatment reduced the frequency and tumor size of DMBA-induced ovarian cancer in rats (Chuffa et al., 2013a). This effect was mediated by a melatonin-induced reduction in Her-2, p38, pAKT, and mTOR levels (Ferreira et al., 2014).

How melatonin hampers ovarian carcinogenesis is a matter of active investigation and it may involve some or multiple mechanisms related to angiogenesis, immunomodulation, cellular proliferation, oxidative processes, apoptosis, and inflammation (Kim et al., 2012, Chuffa et al., 2013a, Chuffa et al., 2016, Shen et al., 2016, Akbarzadeh et al., 2017, Chuffa et al., 2017, Li et al., 2017). Regarding proliferation, melatonin causes cell cycle arrest in ovarian cancer cell lines through downregulation of cyclin-dependent kinases 2 and 4 (CDK2, CDK4) (Shen et al., 2016), and stimulates the CDK inhibitor p21 transcription (Carlberg, 2000). Apoptosis was favored after melatonin treatment via upregulation of p53, BAX and downregulation of survivin (Chuffa et al., 2016, Shen et al., 2016). Tumor progression and metastasis rely not only on the invasive capacity of tumor cells, but also on the blood supply that is available. Melatonin was shown capable of blocking OC angiogenesis via the inhibition of key factors, such as the vascular endothelial growth factor (VEGF), VEGF receptor 2 (VEGFR2), hypoxia-inducible factor 1 alpha (HIF-1α),

and TGFβ-1 (Chuffa et al., 2017, Zonta et al., 2017). In the context of hormone-dependent cancers, such as breast, prostate and ovarian cancer, melatonin additionally affects the synthesis and action of estradiol (Chuffa et al., 2013b) and can negatively impact some components of the HPG axis (Menéndez-Menéndez and Martínez-Campa, 2018). However, this aspect of melatonin's anti-tumor properties remains mostly unexplored in ovarian cancer.

The virtues of this small molecule are promising and suggest the development of chemotherapy protocols that include melatonin as an adjuvant.

Concluding Remarks

Despite the advances achieved during the last decades in the understanding of pathogenesis of ovarian cancer, the overall survival has not changed in 30 years. Only around 47% of patients will survive 5 years (National Cancer Institute, 2016). Ovarian cancer is not one but many diseases with different histological and molecular features, origins, clinical presentations, and treatments (Matulonis et al., 2016). The development of therapeutic strategies that account for this diversity has increased drug efficacy, yet, cure rates remain around 30% (Yang et al., 2017). Melatonin's role as an anti-tumor agent suggests promise (Cohen et al., 1978, Jung and Ahmad, 2006, Kim et al., 2012, Schernhammer et al., 2012, Wang et al., 2012, Chuffa et al., 2013b, Jablonska et al., 2014, Shen et al., 2016, Zhao et al., 2016, Akbarzadeh et al., 2017, Chuffa et al., 2017, Li et al., 2017, ZEMŁA et al., 2017, Zonta et al., 2017, Menéndez-Menéndez and Martínez-Campa, 2018, Yuan et al., 2018). Evidence points toward its benefits as a prophylactic, a treatment, and as a co-adjuvant for traditional chemotherapies. Importantly, melatonin is an affordable, over-the-counter compound with no toxicity other than isolated cases of fatigue, headache, and dizziness to date (Dawson and Encel, 1993). This research aims to

study the role of MEL on OC development and its potential therapeutic value using the laying hen as a model for ovarian cancer.

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CHAPTER II

Melatonin Supplementation to Hens at Risk for Ovarian Cancer

Introduction

Ovarian cancer (OC) is the deadliest gynecologic malignancy. Estimations from the American Cancer Society anticipate over 21,750 new cases and 13,940 ovarian cancer deaths in 2020 in the United States (American Cancer Society, 2020). The vast majority of cases belong to the epithelial high-grade serous subtype (HGSC) that accounts for 70 to 75% (Kurman and Shih, 2016, Teer et al., 2017). Frequently, these tumors are highly aggressive and advanced at the time of diagnosis. First-line surgery and platinum-based chemotherapy usually yield good responses, but recurrence with increasing chemoresistance is a common finding. Therapeutic options for recurrent disease are expanding, yet cure rates remain unchanged for the last few decades (Yang et al., 2017). Therefore, there is an urgent need for rapid improvement in the prophylaxis and treatment of patients suffering from this deadly disease.

Several models have been developed to study ovarian cancer biology and to explore novel treatments. Among them, *in vitro* models comprise cell lines, organoids, and 3D culture models; *in vivo* models include Drosophila model, chemically induced rat OC and genetically induced mouse OC, as reviewed by Lengyel et al. (Lengyel et al., 2014). Although all these models have some advantages and have contributed to the understanding of the disease, most fail to reproduce the cascade of events that leads to carcinogenesis and eventually results in progression and metastasis. The laying hen is the only animal that spontaneously develops ovarian cancer at a high rate. As many as 35% of the laying hens of a flock will present signs of advanced ovarian cancer by 3 years of age (Giles et al., 2010, Treviño et al., 2012). It is hypothesized that the high OC incidence in the chicken relates to egg production, which reflects

the number of ovulations. A similar association is observed in humans. In women, the number of lifetime ovulations is a factor that increases OC risk (Bassuk and Manson, 2015, Yang et al., 2016, Peres et al., 2017). Interestingly, by three years of age, a laying hen has ovulated approximately as many times as a woman that undergoes menopause, that is about 300-500 ovulations. Repeated ovulations create an ovarian environment rich in cytokines, reactive oxygen species (ROS), growth factors, and other inflammatory molecules that may facilitate carcinogenesis (Fathalla, 2013). Although ovulation is not the sole cause of ovarian cancer, it is thought to play a permissive role for carcinogenesis. Consistently, practices that block ovulation also reduce ovarian cancer incidence in both the laying hen and women (Treviño et al., 2012, Havrilesky et al., 2013, Bassuk and Manson, 2015). The laying hen is unique in that it provides a model that mimics clinical presentation, histological types, molecular markers, and progression patterns of human disease (Barua et al., 2007, Johnson and Giles, 2013, Johnson et al., 2015). Importantly, the chicken presents the opportunity for performing large-scale and long-term ovarian cancer biology studies with relatively low cost.

In order to drive high egg production in commercial hens, the birds are exposed to a long photoperiod. That is, their day lengths are typically 15-16 hours of light with 8-9 hours of darkness. The artificially reduced dark cycle causes a chronic scarcity of melatonin, a hormone which is predominantly released during the night or dark phase (Taylor et al., 2013). Indeed, a growing body of evidence has prompted the International Agency for Research on Cancer (IARC) to classify rotating and night shift work schedules as “probable” carcinogens for humans (IARC, 2010). Melatonin has been proposed as the mediator between cancer risk and shift work (Stevens et al., 2007), and research that corroborates the anti-tumor effects of melatonin is expanding. In human ovarian cancer, epidemiological data have associated lower circulating

melatonin levels and ovarian cancer (Weiderpass et al., 2012, Bhatti et al., 2013, Carter et al., 2014). Fatal ovarian cancer was significantly increased in women with lower plasma melatonin (Carter et al., 2014). These observations suggest that a deficiency of the darkness hormone could have a role in the events leading to malignant transformation and/or progression.

Melatonin is normally secreted by the pineal gland during hours of darkness (Pelham, 1975). During the light phase, its production and secretion are inhibited via a mechanism that involves an initial signal from photoreceptors to the suprachiasmatic nucleus and results in a blockage of the "default" neural stimulus exerted on the pineal gland (Pandi-Perumal, 2005). At night, melatonin production and secretion are no longer constrained, and it is actively synthetized from the essential amino acid tryptophan and released to the circulation. Melatonin is crucial for synchronizing the body's circadian rhythms to environmental photoperiods (Cipolla-Neto and Amaral, 2018). Chronic disruption of circadian rhythms is known to increase the risk for several pathologies including cancer (Stevens et al., 2007). Not only does melatonin act as a chronobiotic, but it also exerts protective effects against diverse biological insults, including cancer (Reiter et al., 2010). Its anti-tumor properties rely mostly on its function as a robust antioxidant, and to a lesser extent on its anti-proliferative, anti-angiogenic, pro-apoptotic and immunomodulatory functions (Chuffa et al., 2015). In the context of ovarian cancer, melatonin was used to treat human OC cell lines, and was shown to inhibit cell proliferation (Petranka et al., 1999) and to increase cell death via p53, p27 and pRb phosphorylation (Kim et al., 2012, Shen et al., 2016) and through inhibition of telomerase activity (Futagami et al., 2001). Furthermore, key factors of invasiveness and metastasis, such as E-cadherin and VEGF, are inhibited as a result of melatonin treatment (Akbarzadeh et al., 2017). In the context of ovulation, melatonin was referred to as a "potent preventative" of ovarian damage as it was capable of

neutralizing the potentially carcinogenic reactive oxygen species (ROS) associated with ovulation (Huang et al., 2015). *In vivo* experiments have further confirmed the anti-ovarian cancer properties of melatonin. Chuffa et al. (2013) found that melatonin reduced tumor size and number of nodules in rats with chemically induced OC. Furthermore, Moore and Siopes demonstrated that that melatonin can delay ovarian adenocarcinoma re-growth in poultry (Moore and Siopes, 2004). These studies indicate that melatonin has potential for the prevention or treatment of ovarian cancer and further trials should be pursued.

Melatonin is an affordable and over-the-counter compound widely available for purchase. It may have the potential to be re-purposed against ovarian cancer without the regulatory hurdles of approval for human consumption. Based on the promise, affordability, and safety of melatonin, we designed a trial to evaluate the prophylactic and potential therapeutic effects of oral melatonin supplementation against OC in the laying hen.

Materials and Methods

Animals and Husbandry

One hundred and nineteen 2-year-old Single-comb White Leghorn hens in their second cycle of laying were randomly allocated to either a control (Ctrl, n = 59) or a melatonin (Mel, n = 60) group. Animals were individually caged and maintained on a 16L:8D (16 hours of light and 8 hours of darkness) schedule. Animals were randomly caged at two height levels in the room and top cages received an average of 51 lux and bottom cages received an average of 40 lux during the light phase. Monthly body weights were taken during the length of the experiment and egg production was monitored daily. All procedures were reviewed and approved by Cornell University Institutional Animal Care and Use Committee (IACUC). Hens in the Ctrl group were

fed with standard commercial laying hen feed (Agway). Melatonin-treated hens were supplemented with 17.36 ug/kg of body weight of melatonin (N-acetyl-5-methoxytryptamine, melatonin, 99% powder, Alfa Aesa. Tewksbury, MA, USA). The dosage was selected to approximate a previously used low dose that effectively raised plasma melatonin in laying hens without altering the daily rhythm (Taylor et al., 2013). The supplemented diet was prepared weekly to ensure freshness of the diet. Both groups had *ad libitum* access to water and food. Hens were kept on the supplemented and regular diet for 14 months. Several hens were lost due to leg problems and other unrelated diseases, during the trial so that analysis regarding cancer incidence and time to cancer included 57 hens per group.

Melatonin RIA assay

Circulating plasma levels of melatonin were assayed using a commercial non-extraction direct melatonin RIA kit, as in Steele et al., 2003 (Steele et al., 2003), following the manufacturer's recommendations (Direct Melatonin RIA, 79-MELHU-R100, Alpco. Salem, New Hampshire, USA). The standard curve ranges from 2.5 pg/ml to 750 pg/ml. Based on an early pilot trial, we selected dilution factors to ensure that values fell within the curve. All daytime samples were diluted 1:2 (equal parts of plasma and buffer supplied with the kit) and nighttime samples were diluted in 1:5. High- (73-120 pg/ml) and low-concentration (7-14 pg/ml) melatonin control samples were provided with the kit and were used to assess precision of the assay. Parallelism analysis was performed using the experimental samples and the recovery average was 75% with a standard error of 3.2, as described previously (Smolec et al., 2005). The intraassay coefficient of variation for the low sample was 9% and was 5.5% for the high sample. The inter-assay CV for the low sample was 9.2% and 8% for the high sample.

Tissues and Blood Collection

Blood samples were obtained at zeitgeber time 1 (lights are turned on at ZT 0), 7, 13, and 19 (ZT 1, ZT 7, ZT 13, ZT 19) from a subset of birds (Ctrl, n = 6; Mel, n = 6 at each time) to measure the melatonin levels. Blood collection was done about midway through the experiment, after the animals had been on the melatonin-supplemented diet for 8 months. Blood samples (1.5 – 3 ml) were collected by brachial vein venipuncture in heparinized syringes and then centrifuged at 1500 revolutions per minute (464 xg) for 10 minutes. Plasma was removed and stored at -80º Celsius with 30 ul sodium citrate for subsequent assay. Throughout the study, any hen exhibiting cancer signs (lethargy, distended abdomen, pale comb, abnormal posture) was humanely euthanized and necropsied to confirm diagnosis and to collect tissue samples. At the end point of the experiment, all remaining hens were euthanized and necropsied. Ovary, oviduct, intestine, and liver specimens were collected and stored at -80º Celsius and ovarian tissue was collected in formalin for histopathological analysis.

Histological analysis

Representative samples of the ovary and oviduct were fixed overnight in 10% neutral buffered formalin and then transferred to 70% ethanol. Fixed tissues were submitted to the Cornell Histology Laboratory for routine slide preparation and staining. Standard hematoxylin and eosin (H&E) staining was used to assess tissue morphology. All samples of ovary were evaluated for signs of neoplasia and confirmed ovarian cancer cases were evaluated to discriminate between mucinous, serous, endometrioid and clear cell tumor types.

Statistical Analysis

Sample size was calculated using WinPepi (version 11.65. Abramson, 2011). We hypothesized that we would achieve a possible reduction of 35% to 20% ovarian cancer incidence, with a one-sided test and 5% type I error (alpha) and 20% type II error (beta). To detect a significant difference between groups, 58 hens were needed per group.

Reported proportions, as binary outcomes, were analyzed by logistic regression using the GLIMMIX procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC).

Outcomes of quantitative nature that were measured several times on the same animal (egg production, body weight) were analyzed by ANOVA with repeated measures, using MIXED procedure in SAS.

To determine the time to cancer and time to death by cancer and time to death, data were analyzed with Cox's proportional hazards regression using PHREG procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC, USA). Kaplan-Meier survival curves were obtained by analysis using MedCalc (version 12.5.0.0; MedCalc Software, Ostend, Belgium) and were utilized to illustrate results from statistical analysis.

Plasma melatonin was analyzed by ANOVA with the MIXED procedure of SAS. Treatment, location, and cancer effect were analyzed in different models.

Proportions were generated with FREQ procedure of SAS, and quantitative outcomes were reported as LSM ± SEM. Significance is considered as a P value < 0.05 and a tendency is considered as a P value between 0.05 – 0.10.

Results

Melatonin Levels

To assess the efficacy of dietary melatonin treatment, plasma melatonin was measured in a group of representative hens. The overall effect of diet presented a *P* value of 0.05 but there was a group by time interaction ($P < 0.05$) with a difference only at nighttime. Melatonin levels were significantly different near the middle of the dark phase at ZT 19. There was no significant difference between groups during daytime measurements. As expected, melatonin levels were significantly higher during nighttime, (ZT 19) in both groups (Fig. 2.1).

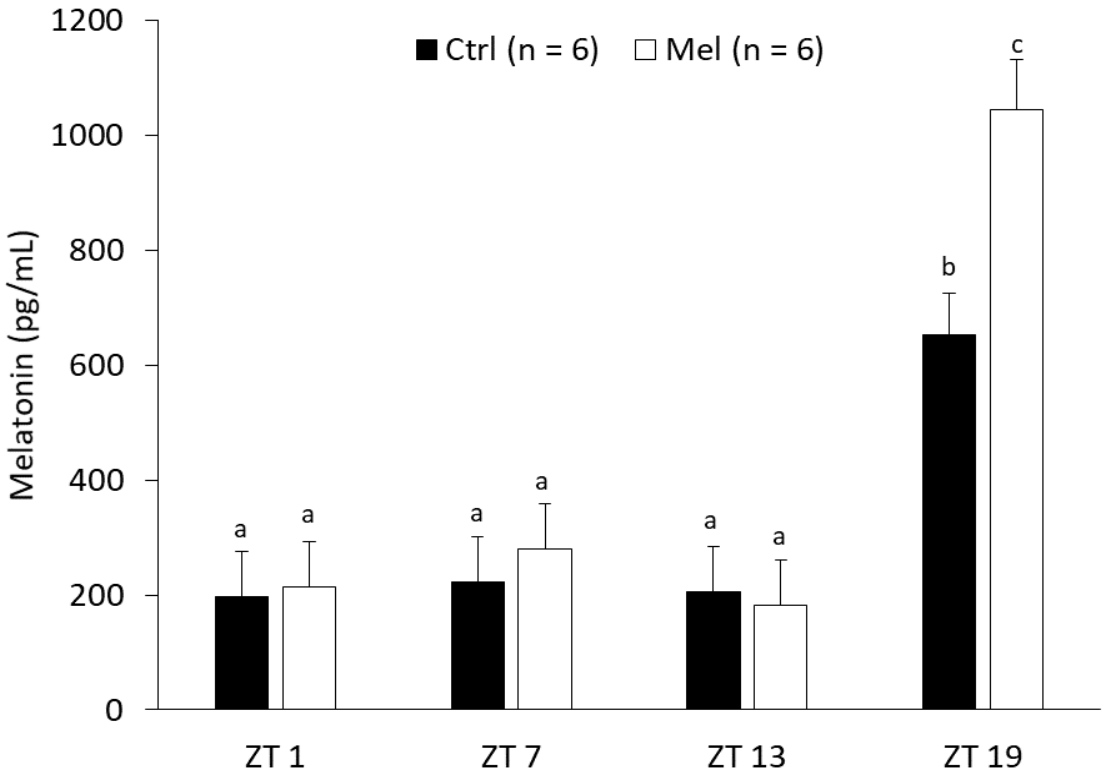

Figure 2.1. Melatonin levels in plasma. Plasma melatonin levels in selected hens at four different times (ZT 1 = 7 am, ZT 7 = 1 pm, ZT 13 = 7 pm, ZT 19 = 1 am). Hens were fed melatonin-supplemented diet for approximately 12 months prior to blood sampling. Six different hens were sampled per time point. Different letters above the bars indicate significant ($P < 0.05$) difference between times and treatment groups.

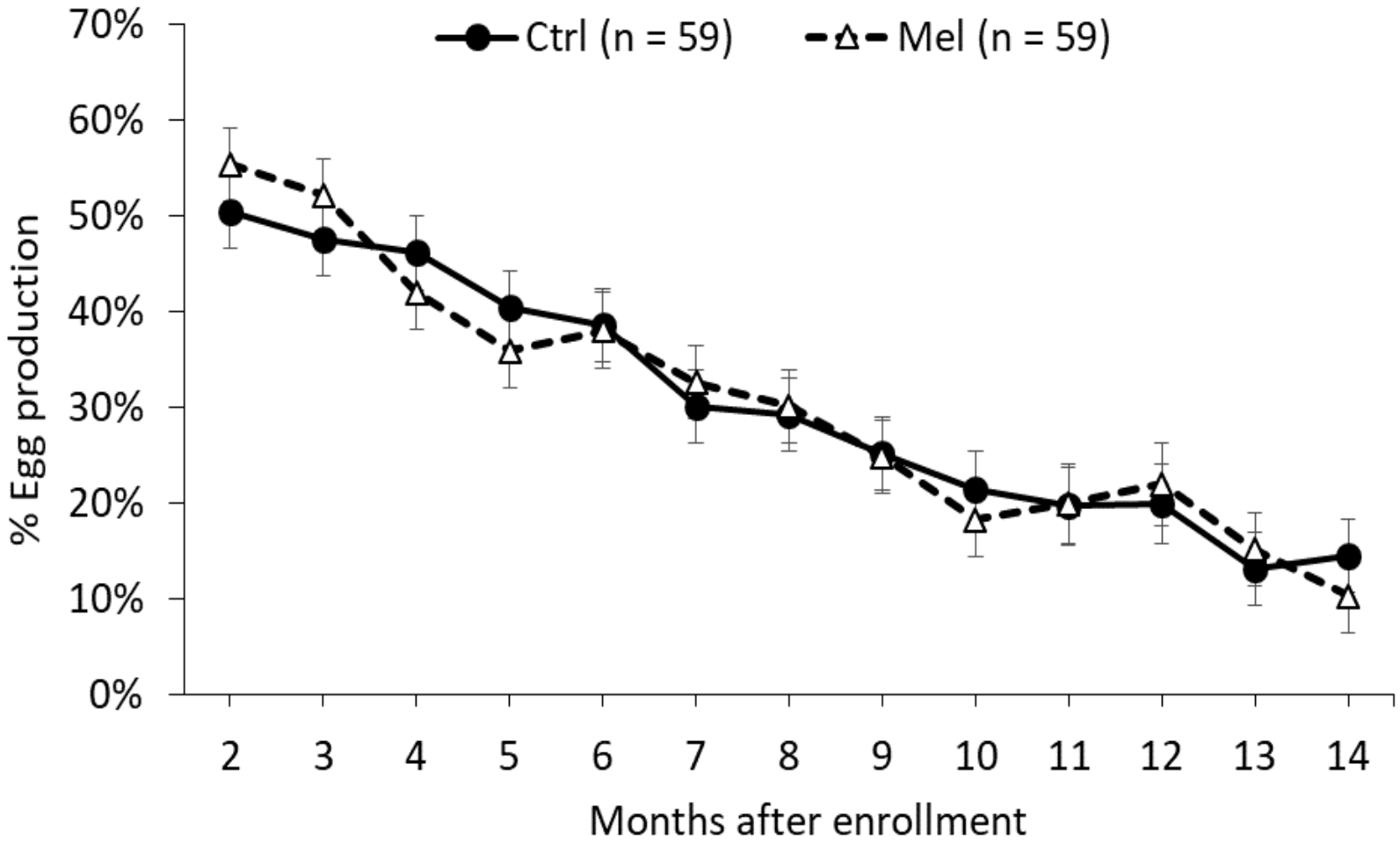

Figure 2.2 Percent egg production. Individual egg production was recorded daily for all hens (Ctrl = control; Mel = melatonin-treated) throughout the duration of the experiment (14 months). Percent egg production represents the number of eggs produced in a month divided by the number of days in that month, 100% egg production is one egg per day.

Egg production and melatonin levels

There was no effect of treatment on egg production ($P = 0.99$; Fig. 2.2). The egg production decreased over time in both groups ($P < 0.0001$). Hens located in bottom cages produced significantly fewer eggs than the hens in the upper cages ($P = 0.004$; Fig. 2.3). There was no significant difference in plasma melatonin by location ($P = 0.30$; Fig. 2.4).

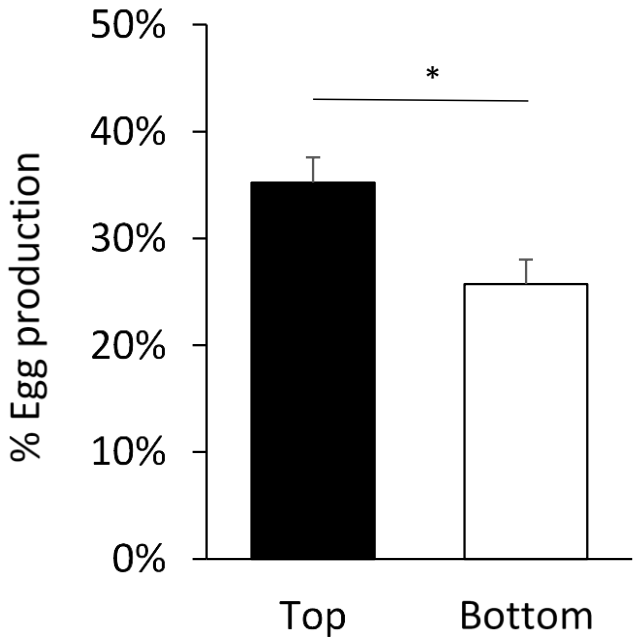

Figure 2.3. Percentage egg production relative to cage position. Top represents cages located at 1.82 m from the floor (average ~51 lux) and bottom represents cages which were at 1.34 m from the floor (average ~40 lux). Asterisk indicates $P < 0.05$. Egg production of all hens (melatonin-treated and control) was averaged for the experimental period.

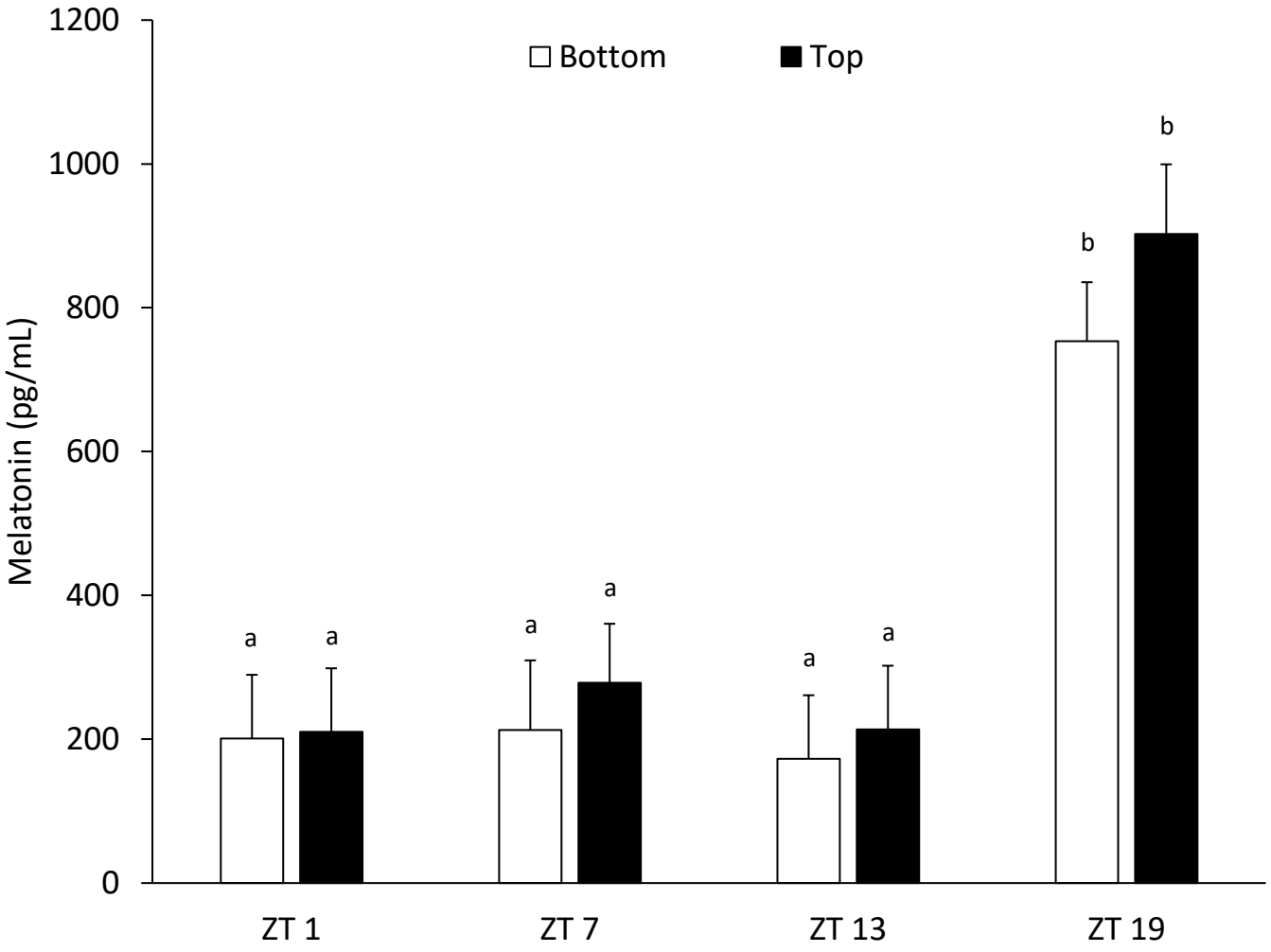

Figure 2.4. Melatonin values by location. Different hens were sampled at each time point (n = 5 – 7 hens per group). Top indicates cages receiving an average of 51 lux and bottom indicates those receiving 40 lux during the light phase. Different letters above the bars indicate statistical differences ($P < 0.05$) between times and/or groups. Bars represent similar (n = 2 - 4) numbers of hens with and without treatment per location.

Body Weight

Body weight was not different between groups nor over time ($P = 0.74$) and presented no substantial fluctuations in either group throughout the study period. Yearly average body weight in the Mel group was 1.86 kg and 1.87 kg in the Ctrl group (Fig. 2.5).

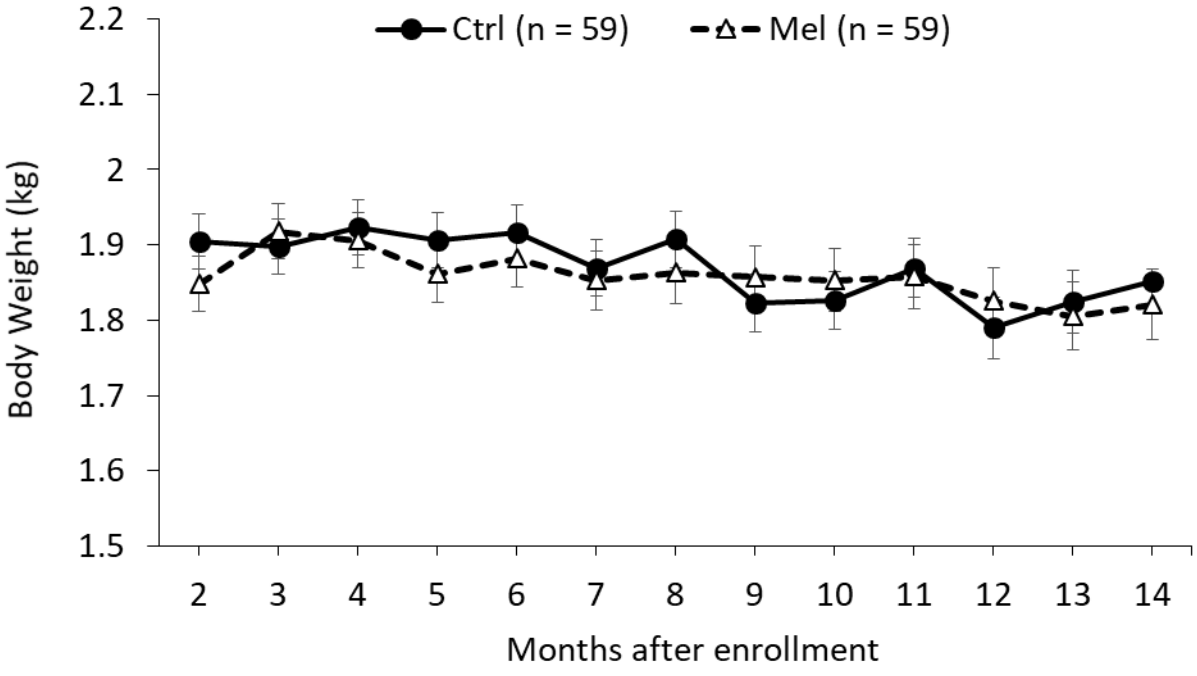

Figure 2.5. Body weight in hens from the control (Ctrl) and from the melatonin (Mel) group. Depicted are body weight averages of all hens of each group. No statistical ($P > 0.05$) difference was detected between groups.

Cancer Incidence and plasma melatonin

A normal ovary is presented in Figure 2.6 and an example of an advanced (stage IV) hen ovarian cancer is presented in Figure 2.7. Melatonin supplementation did not cause a significant reduction of ovarian cancer (OC) incidence, as shown by the proportion of hens that developed OC before the end of the experimental period, hens that developed OC at the end of the experimental period, and total hens that developed OC in Table 2.1. There was also no difference ($P > 0.10$) in the proportion of hens that died and the proportion that died because of ovarian cancer between groups (Table 2.1). A trend ($P = 0.10$) towards reduction of OC is observed in top caged hens, in which there was a numerical decrease in cancer and cancer deaths as compared to bottom caged hens. Regarding ovarian cancer stages, melatonin (Mel) and control (Ctrl) groups exhibited similar proportions of all stages, except for stage II and stage III that showed higher incidence in hens located in bottom cages (Table 2.2). Nocturnal plasma melatonin levels were lower ($P < 0.05$) in the subset of hens eventually presenting with OC compared to normal hens (Fig. 2.8).

We detected serous (Fig. 2.10, 2.11; 39.6%), endometrioid (Fig. 2.12, 2.13; 56.2%) and clear cell (Fig. 2.14, 2.15; 4.2%) ovarian cancer histotypes with no differences between treatment groups nor location ($P > 0.10$), although we did not observe any case of mucinous OC. In addition, we observed two cases of oviductal cancer with no ovarian involvement in the melatonin group (Table 2.3). A normal ovary is showed in figure 2.9 for comparison.

Table 2.1. Cancer incidence and cancer deaths

	Treatment group			Location			*P*-value	
	Ctrl % (n/n)	Mel % (n/n)	Adj. OR (95%CI)	Bottom % (n/n)	Top % (n/n)	Adj. OR (95%CI)	Trt	Loc
Proportion of hens that developed cancer before the end of experimental period	42.1 (24/57)	38.6 (22/55)	1.15 (0.54-2.47)	45.8 (27/59)	34.6 (19/55)	1.60 (0.74-3.43)	0.72	0.23
Proportion of hens diagnosed with cancer at the end of the experiment by necropsy	24.6 (14/57)	21.1 (12/57)	1.25 (0.50-2.96)	25.4 (15/59)	20.0 (11/55)	1.36 (0.56-3.33)	0.66	0.50
Total proportion of hens that developed cancer	66.7 (38/57)	59.7 (34/57)	1.36 (0.58-3.21)	71.2 (42/59)	54.6 (30/55)	2.01 (0.88-4.87)	0.47	0.10
Proportion of hens that died before the end of experimental period	43.9 (25/57)	45.6 (26/57)	0.92 (0.43-1.96)	50.9 (30/59)	38.2 (21/55)	1.68 (0.79-3.57)	0.83	0.18
Proportion of hens that died because of cancer out of the total death	80.0 (20/25)	65.4 (17/26)	3.04 (0.40-23.30)	70.0 (21/30)	76.2 (16/21)	0.71 (0.09-5.41)	0.28	0.74

The effect of the interaction between treatment and location was not significant for any of the outcomes analyzed ($P > 0.10$).

Table 2.2. Cancer stages by treatment and by location.

	Treatment group			Location			*P*-value	
	Ctrl % (n/n)	Mel % (n/n)	Adj. OR (95%CI)	Bottom % (n/n)	Top % (n/n)	Adj. OR (95%CI)	Treatment	Location
Stage 1	14.0 (8/57)	14.0 (8/57)	0.96 (0.26-3.54)	13.6 (8/59)	14.6 (8/55)	0.88 (0.24-3.25)	0.94	0.85
Stage 2	1.8 (1/57)	1.8 (1/57)	1.00 (0.06-17.37)	3.4 (2/59)	0 (0/55)	-	0.99	0.50
Stage 3	17.5 (10/57)	8.8 (5/57)	2.22 (0.69-7.18)	18.6 (11/59)	7.3 (4/55)	2.93 (0.85-10.07)	0.18	0.09
Stage 4	33.3 (19/57)	35.1 (20/57)	0.91 (0.29-2.83)	35.6 (21/59)	32.7 (18/55)	1.21 (0.39-3.77)	0.87	0.74
Stage 1 & 2	15.8 (9/57)	15.8 (9/57)	0.99 (0.33-3.00)	17.0 (10/59)	14.6 (8/55)	1.19 (0.39-3.61)	0.99	0.76
Stage 2 & 3	19.3 (11/57)	10.5 (6/57)	2.05 (0.68-6.21)	22.0 (13/59)	7.3 (4/55)	3.63 (1.08-12.17)	0.20	0.04
Stage 3 & 4	50.9 (29/57)	43.9 (25/57)	1.34 (0.47-3.80)	54.2 (32/59)	40.0 (22/55)	1.84 (0.65-5.20)	0.58	0.25

The effect of the interaction between treatment and location was not significant for any of the outcomes analyzed ($P > 0.1$).

Table 2.3. Cancer histotypes and proportions.

	Treatment group			Location			*P*-value	
	Ctrl % (n/n)	Mel % (n/n)	Adj. OR (95%CI)	Bottom % (n/n)	Top % (n/n)	Adj. OR (95%CI)	Treatment	Location
Clear cell	3.5 (2/57)	0.0 (0/57)	-	3.4 (2/59)	0.0 (0/55)	-	0.50	0.50
Endometrioid	21.1 (12/57)	26.3 (15/57)	0.75 (0.27-2.07)	23.7 (14/59)	23.6 (13/55)	1.02 (0.37-2.83)	0.57	0.97
Serous	15.8 (9/57)	17.5 (10/57)	0.88 (0.24-3.28)	15.3 (9/59)	18.2 (10/55)	0.82 (0.22-3.07)	0.85	0.78
Oviductal cancer	0.0 (0/57)	3.5 (2/57)	-	0.0 (0/55)	3.4 (2/59)	-	0.5	0.23

The effect of the interaction between treatment and location was not significant for any of the outcomes analyzed ($P > 0.1$).

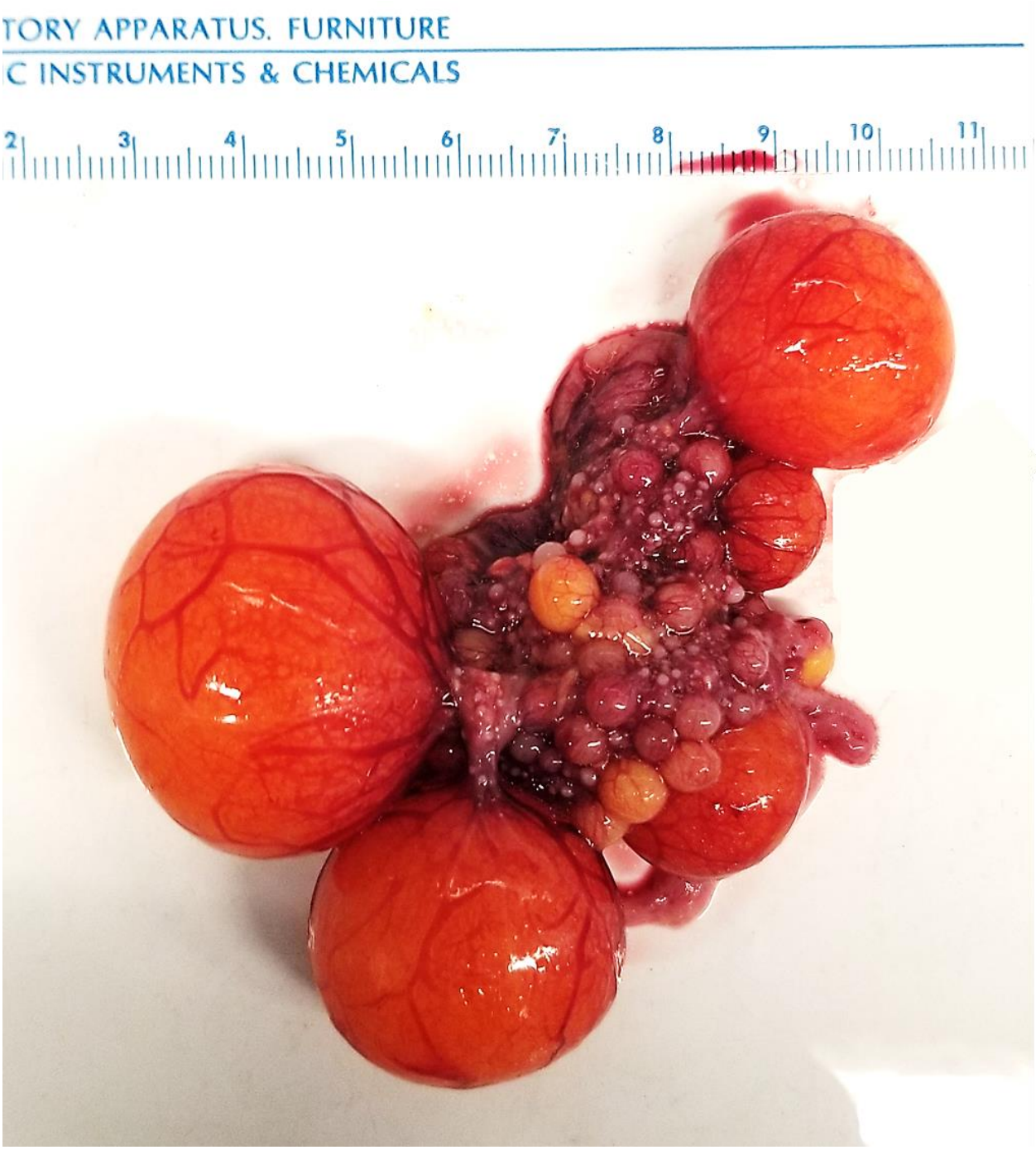

Figure 2.6. Example of a normal ovary. Three-year-old hen that reached the end point of the experiment.

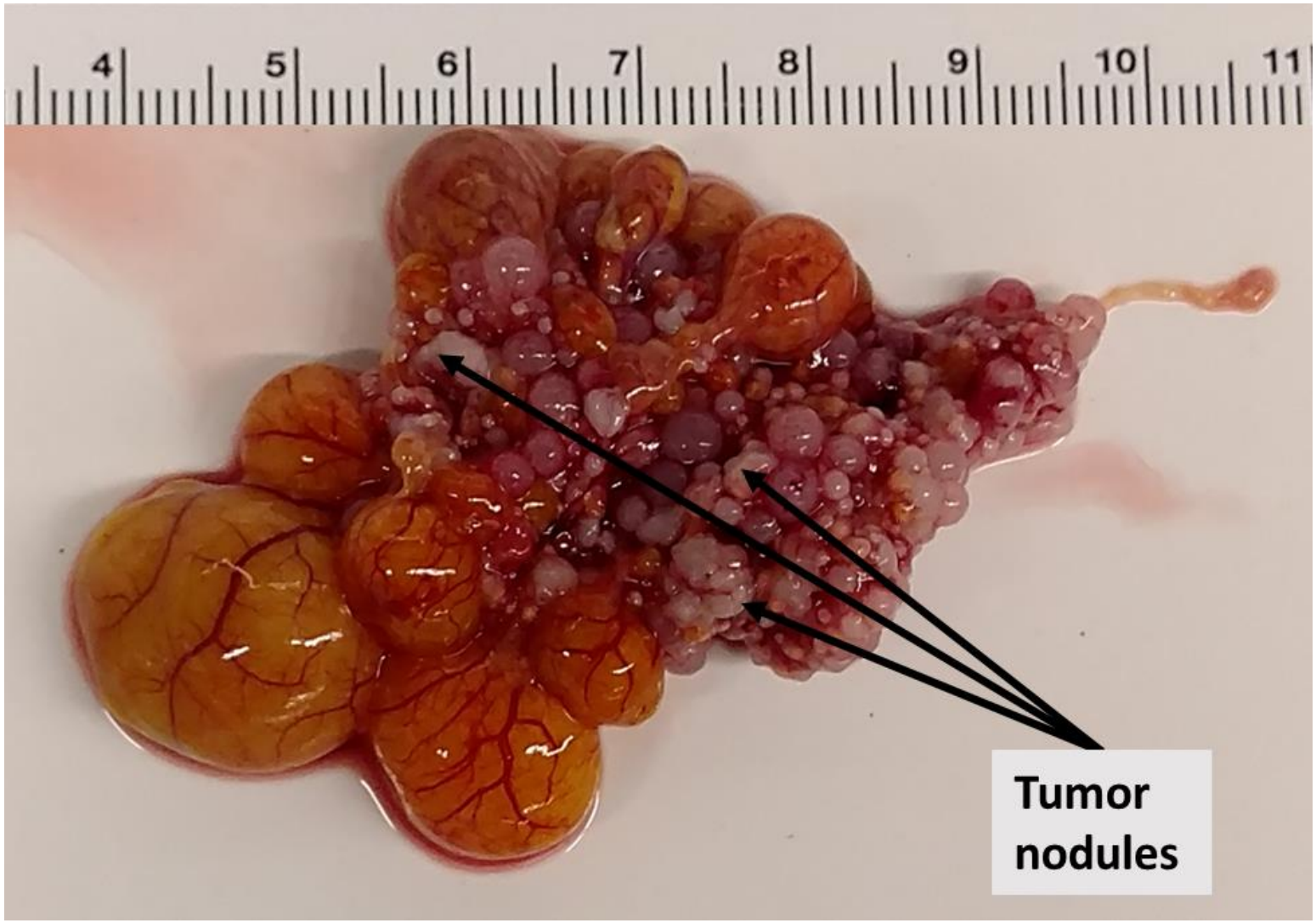

Figure 2.7. Stage IV Serous Ovarian Cancer. Two and a half-year-old laying hen in the melatonin-treated group. This animal presented illness and was euthanized 3 months after enrollment. Metastasis and ascites were present.

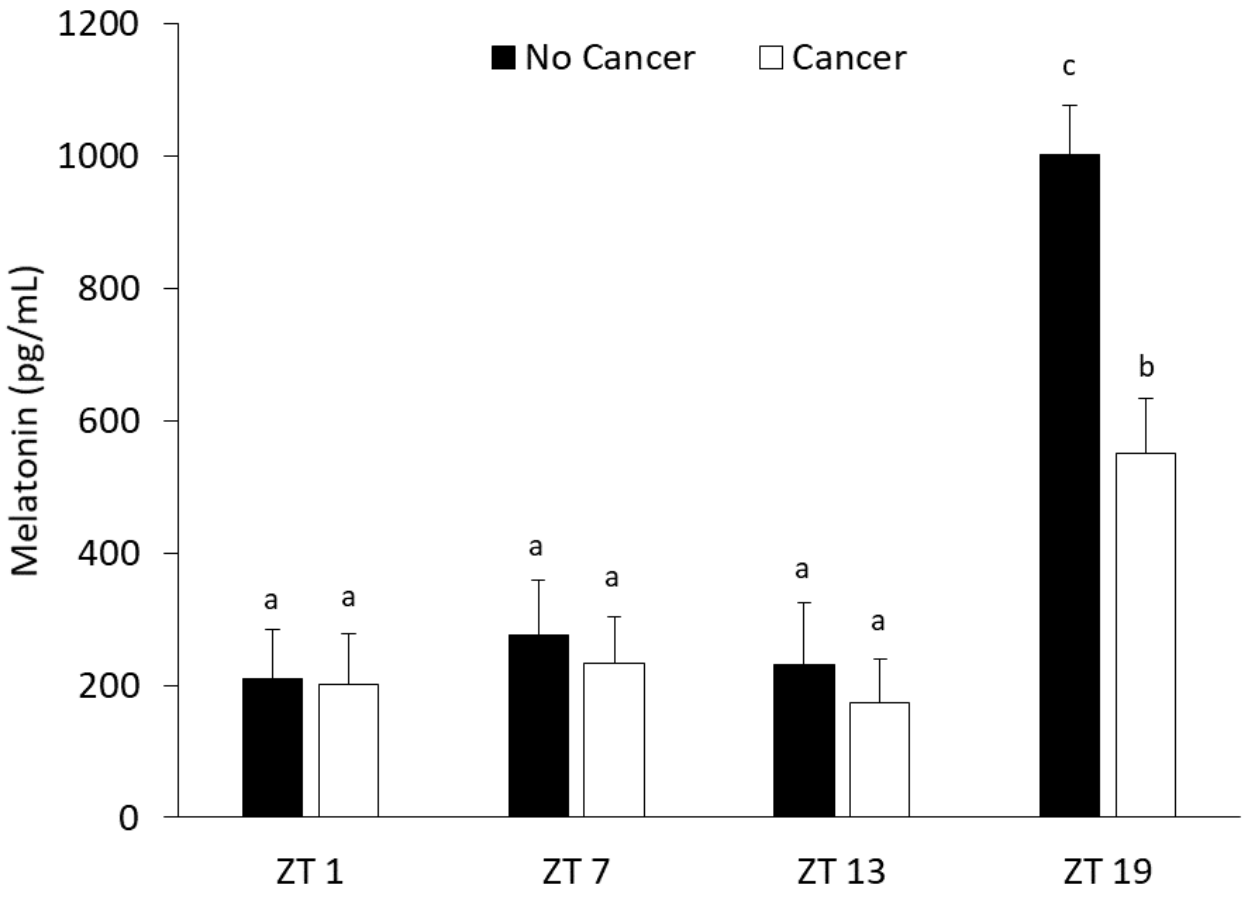

Figure 2.8. Melatonin levels in hens developing ovarian cancer or not throughout the study. Bars represent plasma melatonin levels according to cancer status. A subset of treated and control animals (n = 4 – 7 per group) was assayed. Different letters indicate significant differences ($P < 0.05$) between groups and times. An interaction was found between treatment and time ($P = 0.02$).

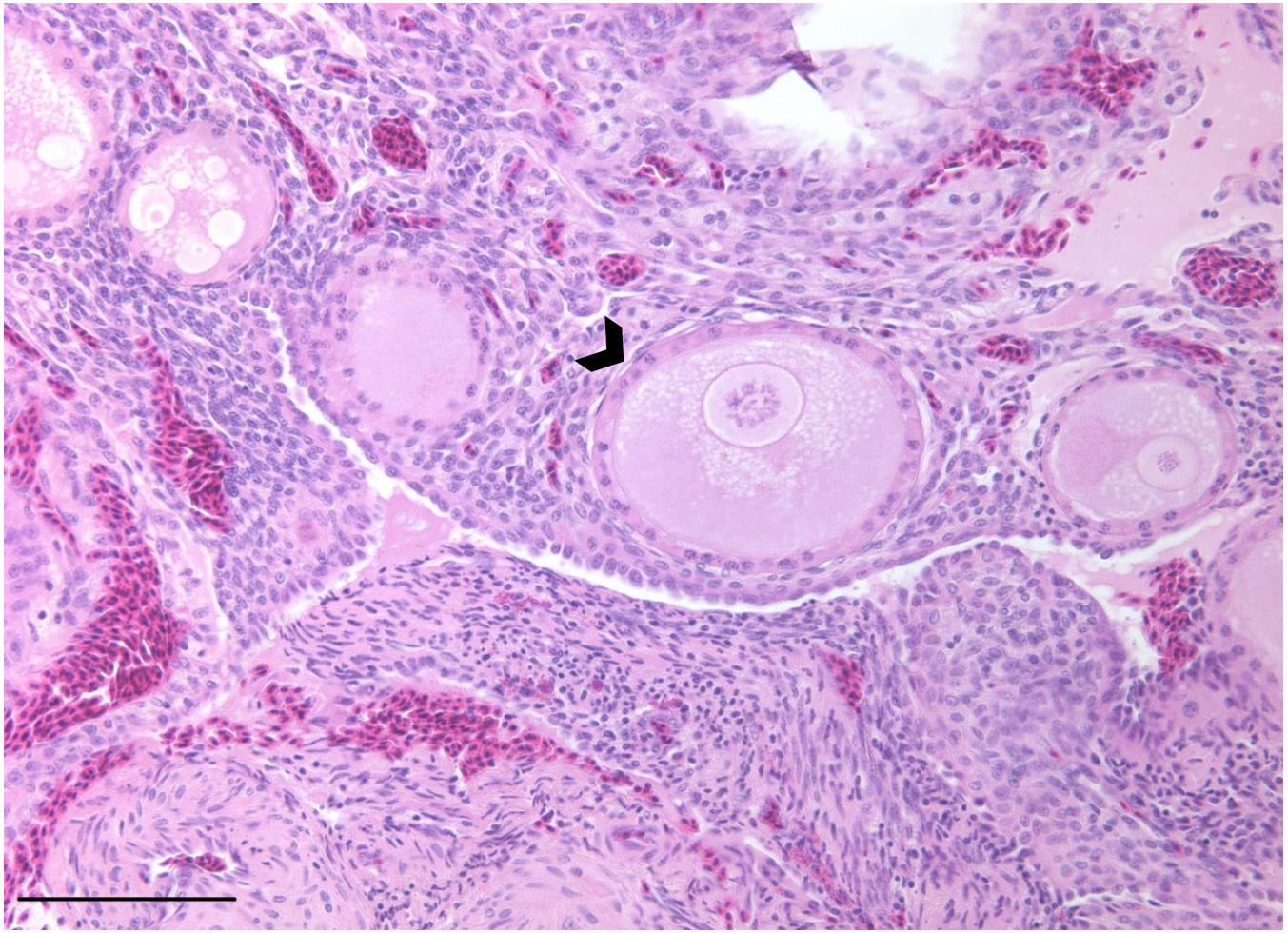

Figure 2.9. Normal Ovary from a 3-year-old laying hen belonging to the melatonin group. Different size follicles. Note the follicle (arrowhead) lined with a single layer of granulosa and nucleus of the oocyte with chromatin material. A smaller follicle is also present. Ovarian surface epithelium line the crevice below the larger follicle. Normal ovarian stromal cells with basophilic nuclei and eosinophilic cytoplasm are present. Scale bar represents 50 µm.

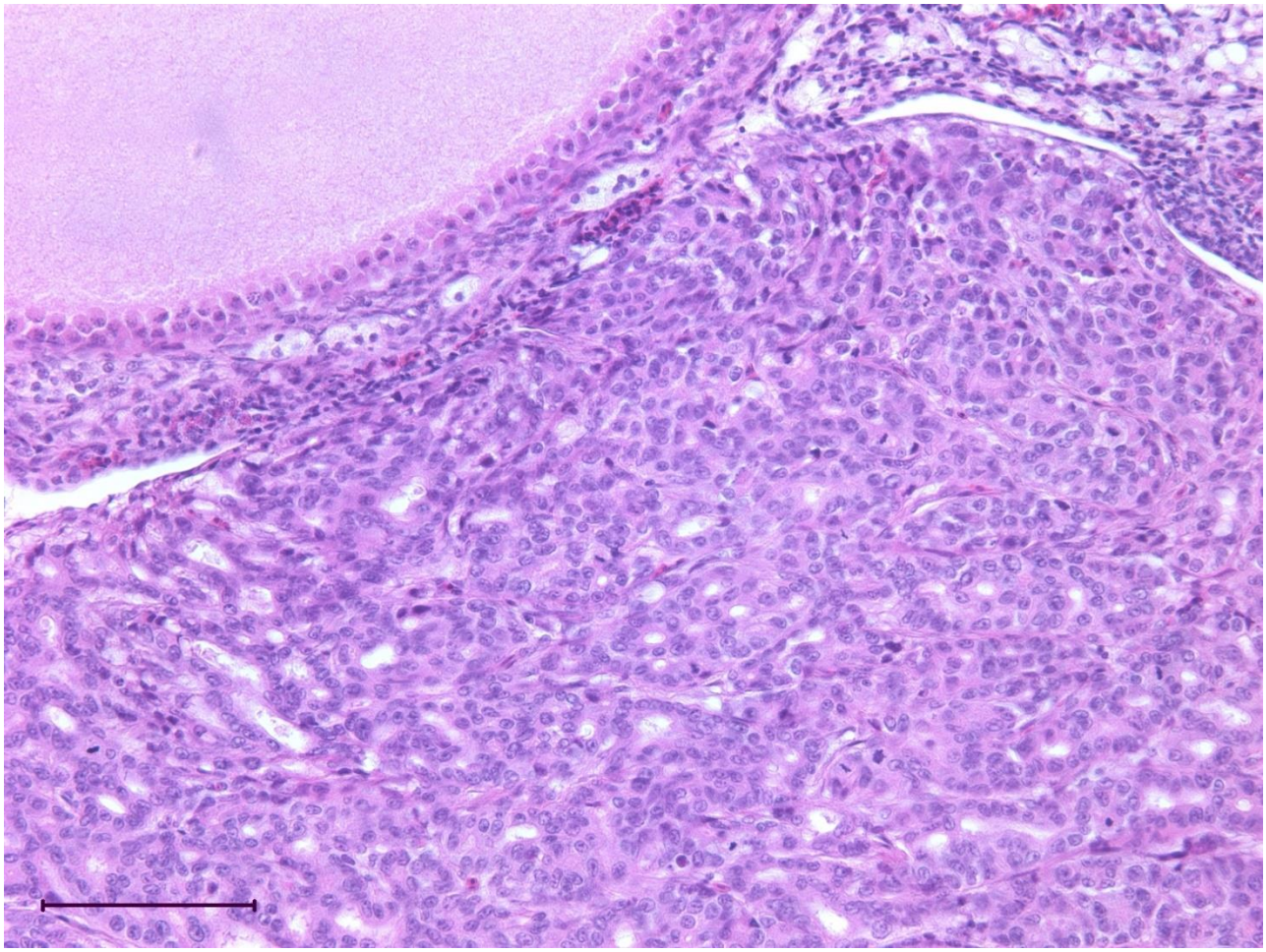

Figure 2.10. Serous Epithelial Ovarian Cancer from a 2.5-year-old laying hen belonging to the melatonin group. Note follicle in the top left corner lined with granulosa cells and surrounded by thecal cell layer. The small cells with clear cytoplasm and small centrally located dense nucleus are normal and commonly found within the theca cell layers and throughout the ovarian stroma. Tumor cells are arranged in a glandular pattern with little stroma between the glands. Scale bar represents 100 µm.

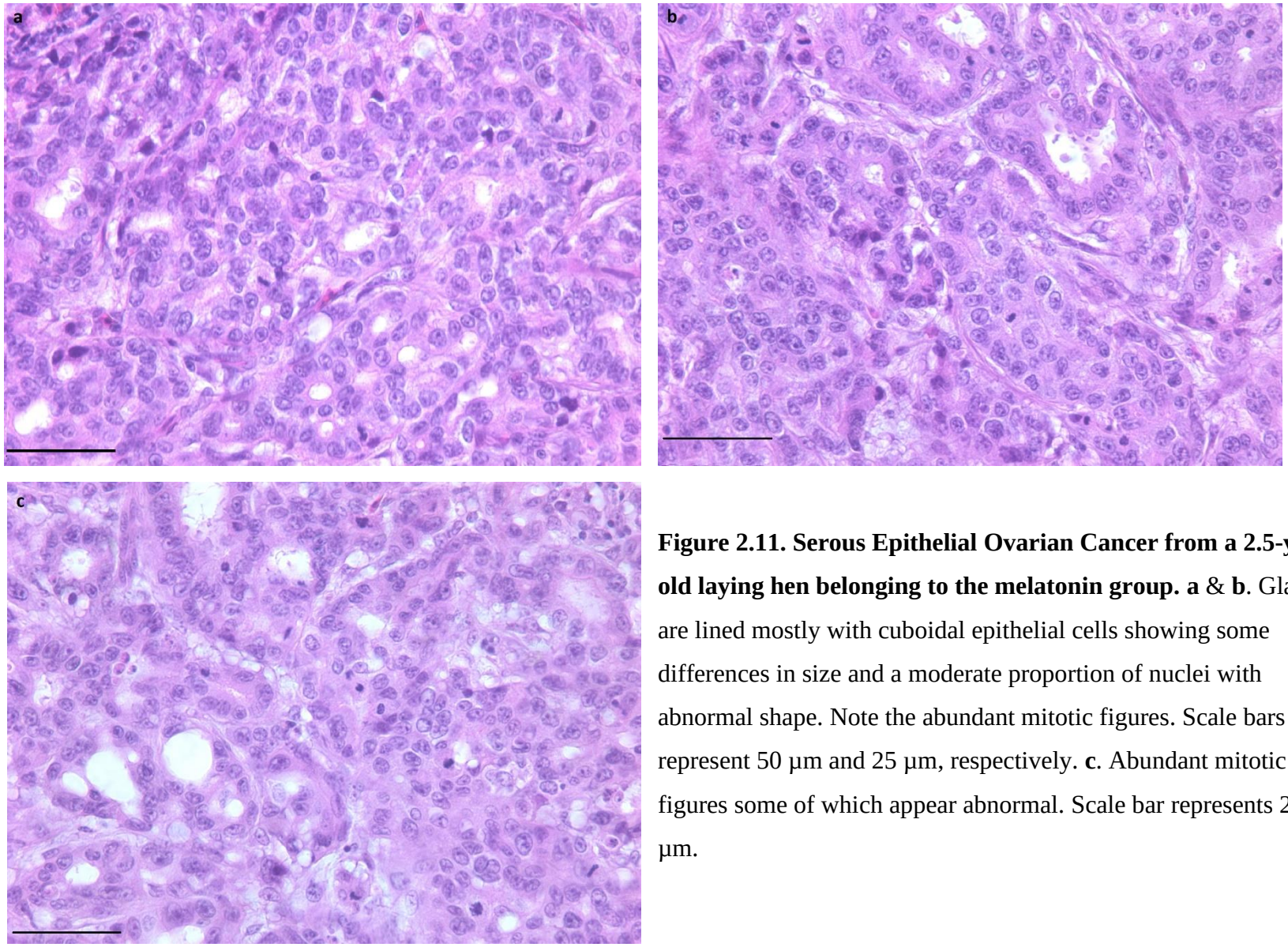

Figure 2.11. Serous Epithelial Ovarian Cancer from a 2.5-year-old laying hen belonging to the melatonin group. a & **b**. Glands are lined mostly with cuboidal epithelial cells showing some differences in size and a moderate proportion of nuclei with abnormal shape. Note the abundant mitotic figures. Scale bars represent 50 µm and 25 µm, respectively. **c**. Abundant mitotic figures some of which appear abnormal. Scale bar represents 25 µm.

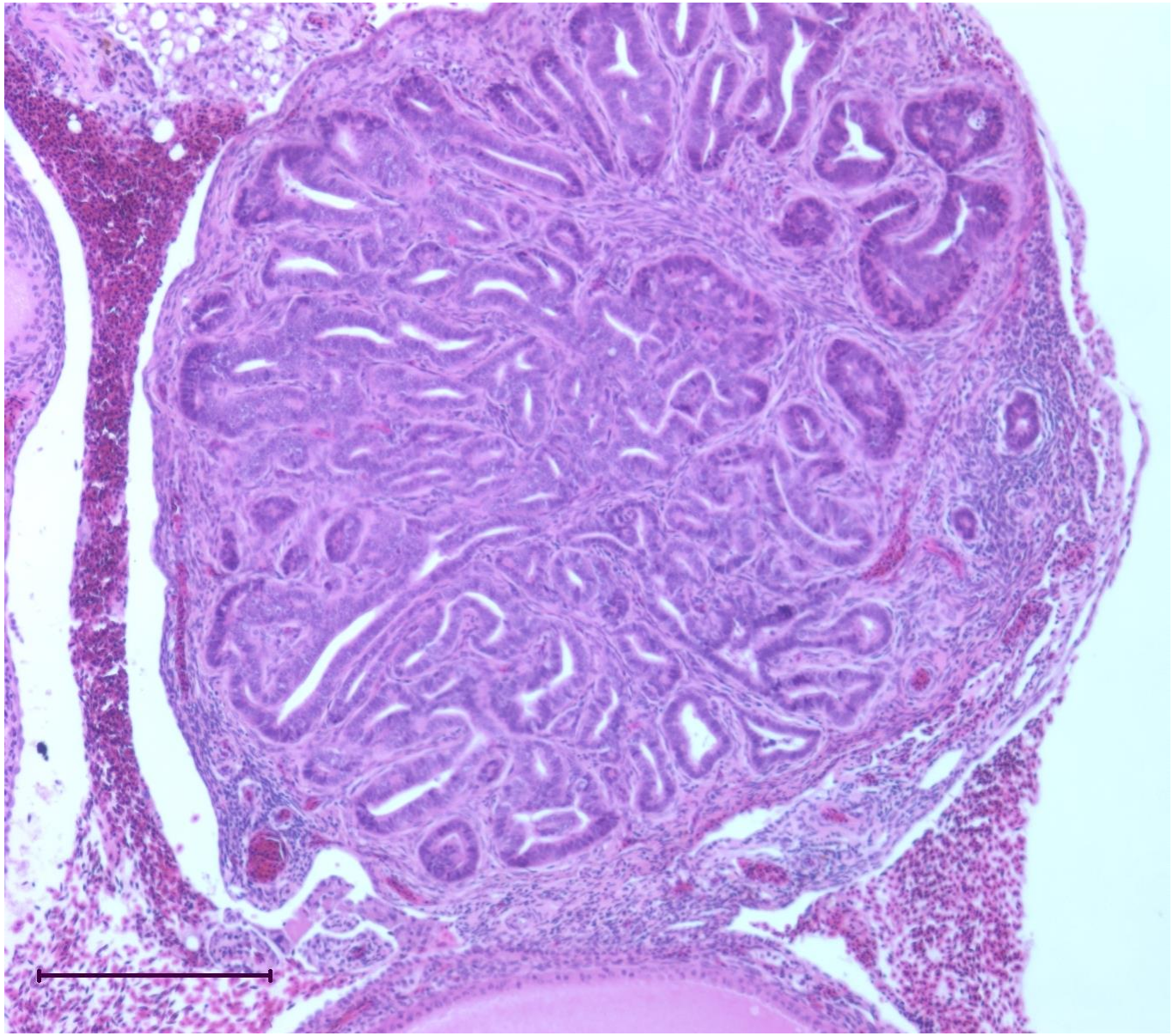

Figure 2.12. Endometrioid Epithelial Ovarian Cancer from a 3-year-old laying hen belonging to the control group. Note Follicle at the bottom of the photo. Back to back glands with little stroma between. Scale bar represents 100 µm.

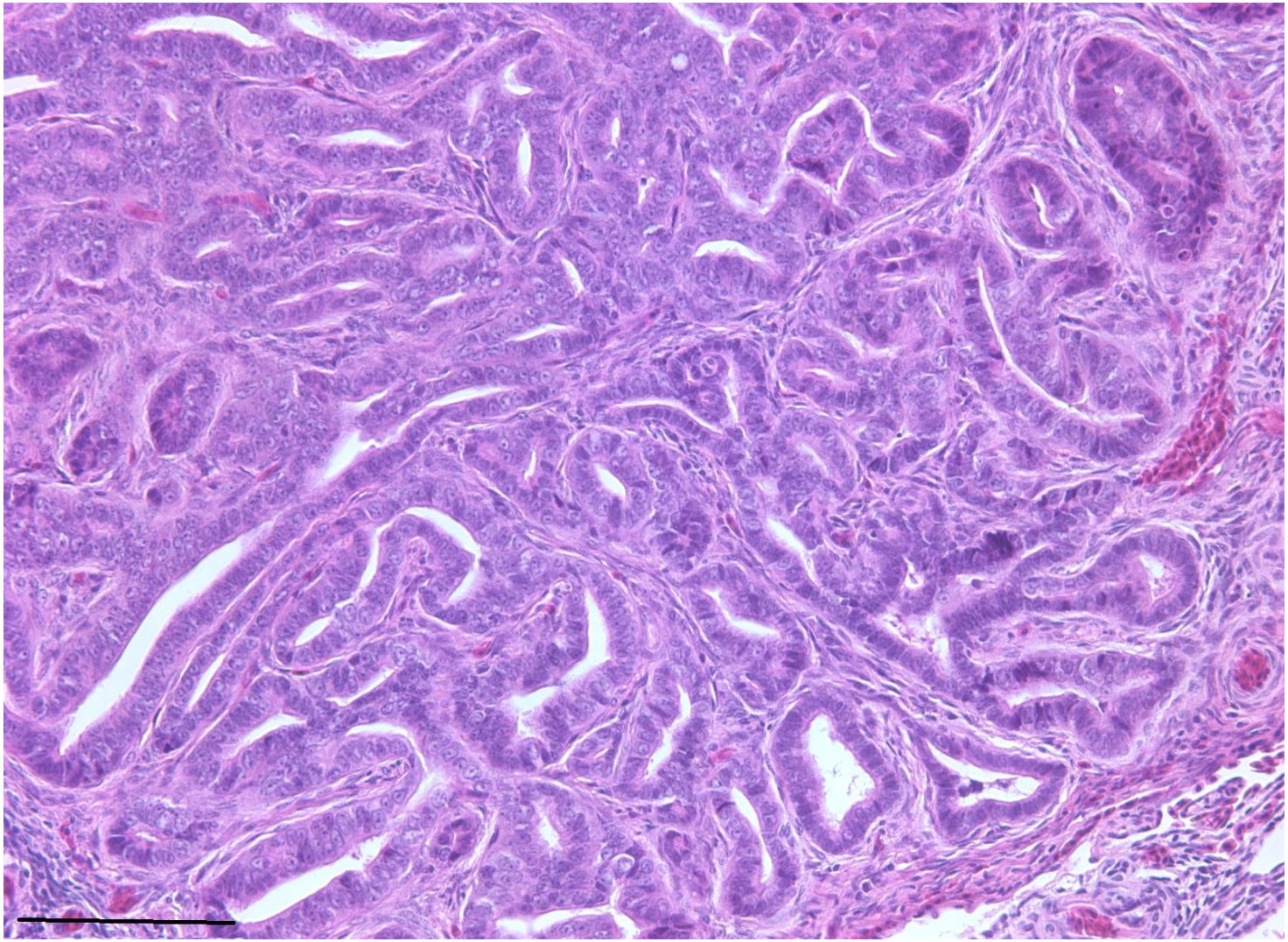

Figure 2.13. Endometrioid Epithelial Ovarian Cancer from a 3-year-old laying hen belonging to the control group. Note rare mitotic figures and single cell layer glands. Scale bar represents 50 µm.

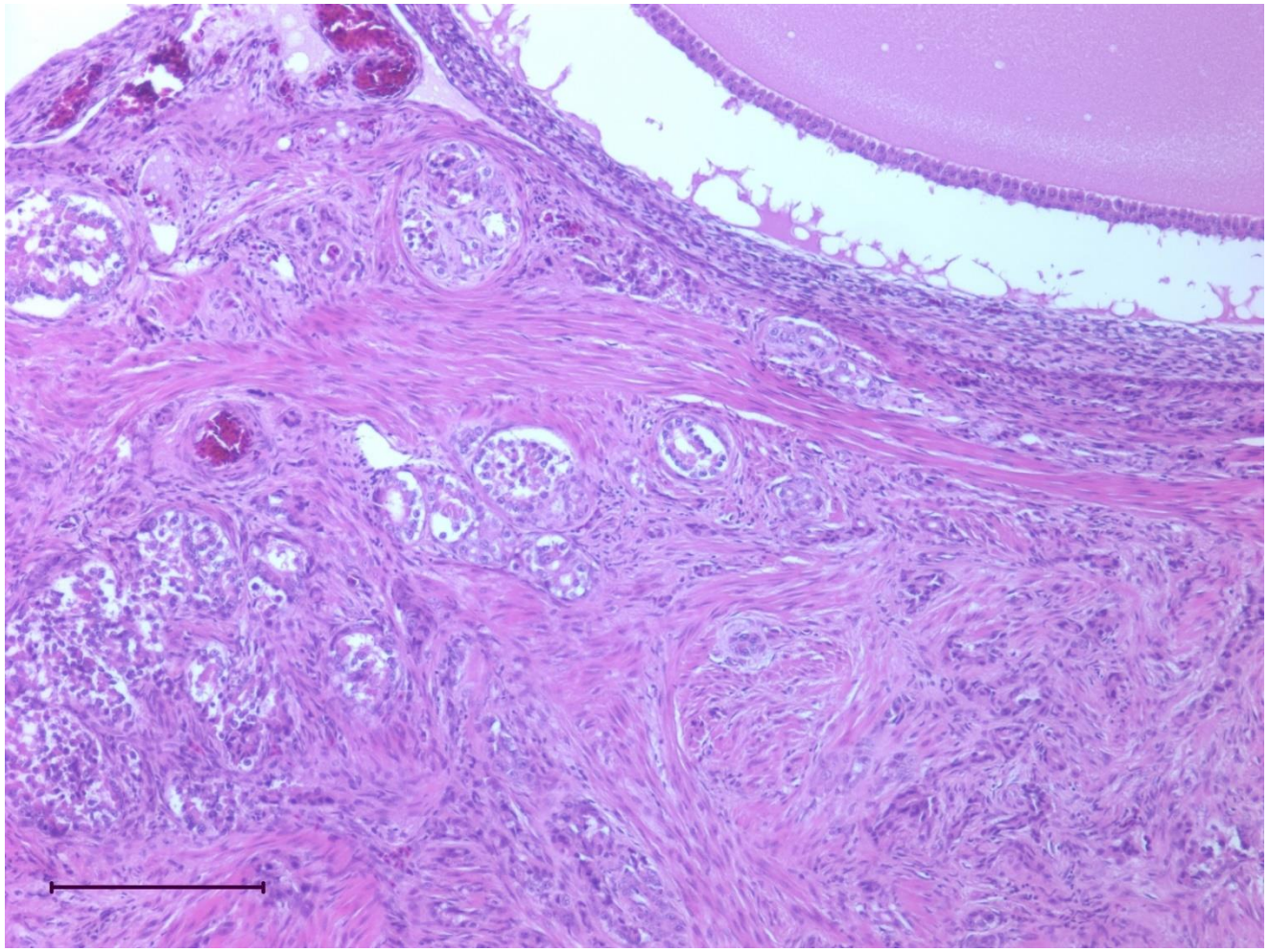

Figure 2.14. Clear-Cell Epithelial Ovarian Cancer. Three-year-old laying hen with no signs of illness and was diagnosed only after the experiment was terminated. This hen belonged to the control group. Note the follicle on the top right corner and tumor cells below, some with clear cytoplasm. Mitotic figures are rare. Scale bar represents 100 µm.

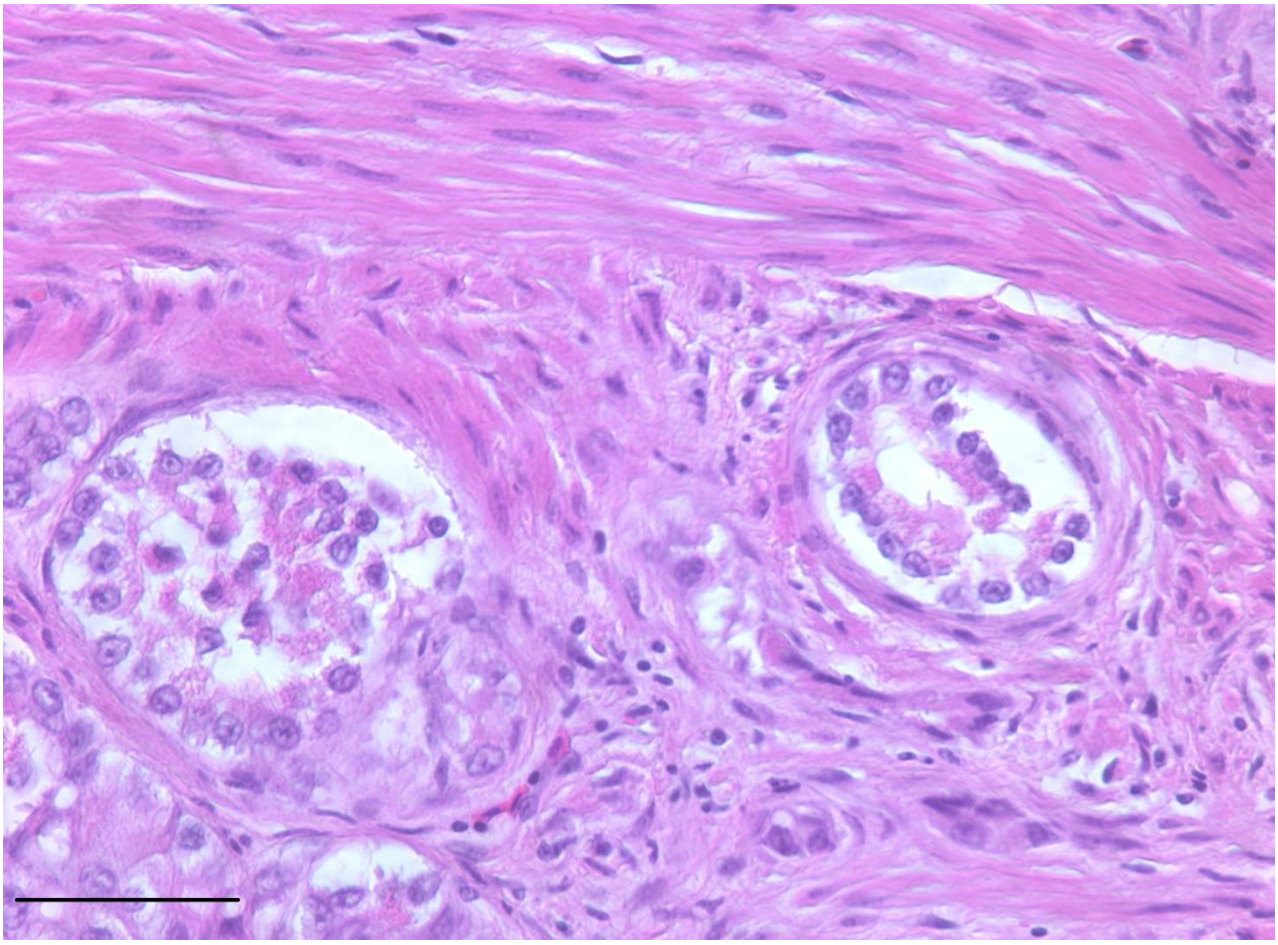

Figure 2.15. Clear-Cell Epithelial Ovarian Cancer in a three-year-old laying hen with no signs of illness. Note cells with clear cytoplasm on an enlargement of figure 2.14.

Egg production can predict ovarian cancer

Egg production was assessed retrospectively three months prior to ovarian cancer diagnosis in both melatonin (Mel) and control (Ctrl) groups. There was a significant difference between hens that developed ovarian cancer and those that did not (Fig 2.16; $P < 0.05$). No differences were observed between the control and melatonin group. Importantly, when egg production was divided by treatment and by cancer presence throughout the experimental period, there was a significant difference between hens that developed OC and those that did not (Fig 2.17). Animals that would later develop OC, had significantly lowered egg production rates than animals that never presented with OC ($P< 0.0001$).

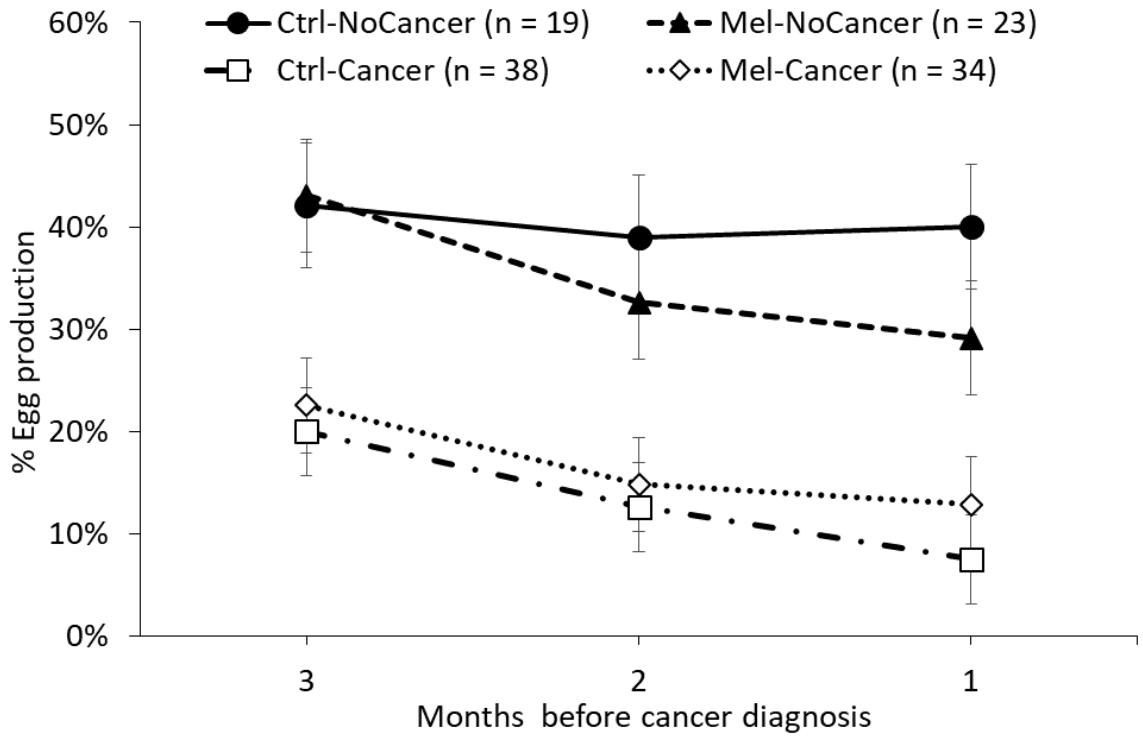

Figure 2.16. Percent egg production by treatment and by ovarian cancer status. For hens with ovarian cancer, "month 0" was the month of cancer diagnosis. For no cancer hens, 'month 0" was the average month of cancer diagnosis for those hens that developed ovarian cancer (Month 0 = 11 month after enrollment). There was statistical difference at all time points between cancer and no cancer hens ($P < 0.0001$) and no difference between treatments.

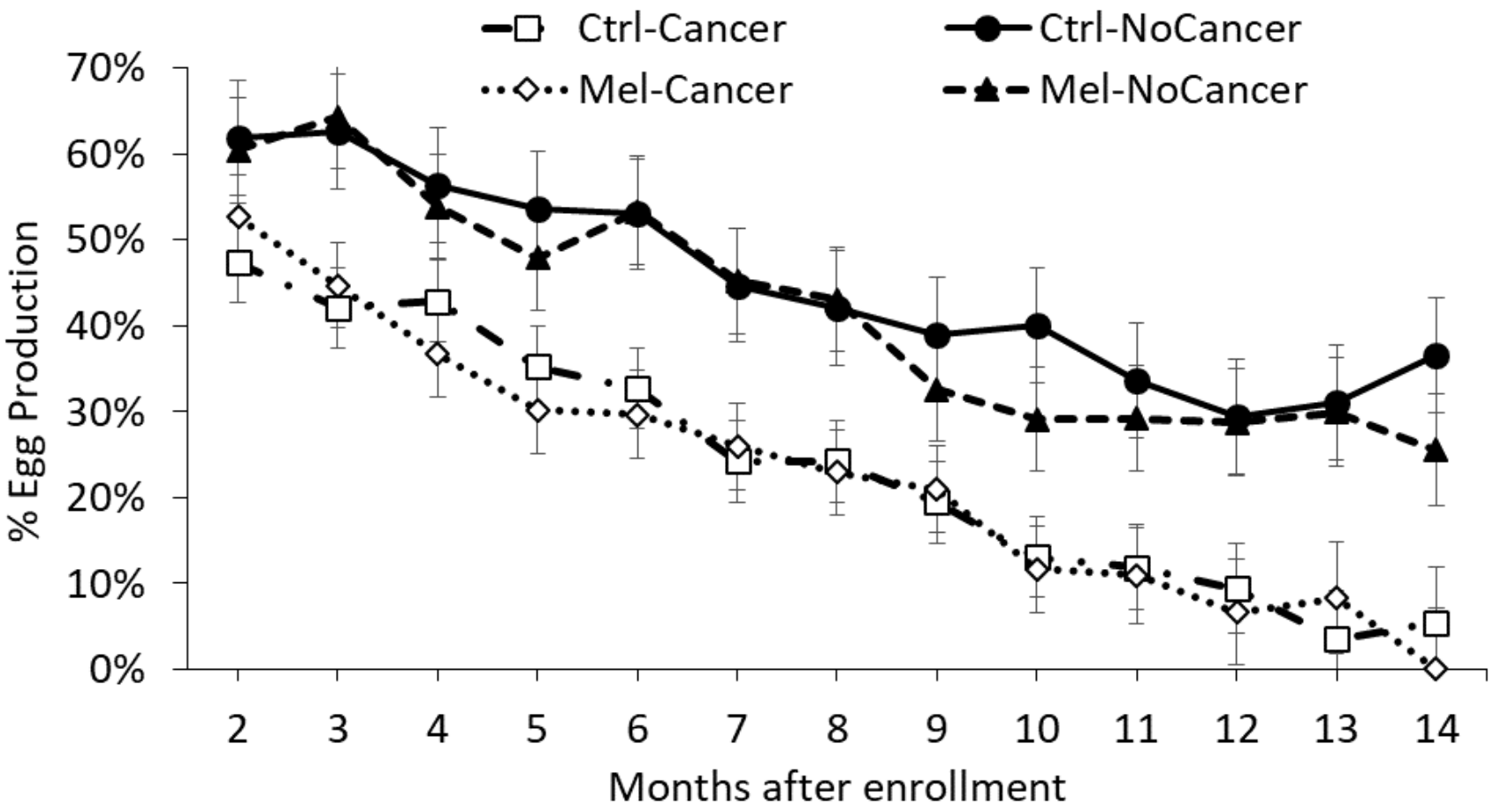

Figure 2.17. Egg production by cancer throughout the duration of the study. A significant difference was observed between animals that developed cancer and those who did not ($P < 0.0001$).

Effect of oral melatonin supplementation on the probability of developing ovarian cancer

The addition of melatonin to the diet of laying hens had no significant effect on time to cancer (Fig. 2.18). The hazard ratio (HR) of developing ovarian cancer was 1.044 for the Mel group (95% confidence interval 0.653 – 1.668). The mean time to cancer was 342 days for the Ctrl group and 318 days for Mel group (Fig. 2.18). Location did not exert an effect on time to cancer either (Fig. 2.19). The HR for cancer was 0.683 (95% CI 0.425 – 1.098) for the top cages. Mean time to cancer was 343 days for hens located on top and 319 days for bottom cages (Fig. 2.19).

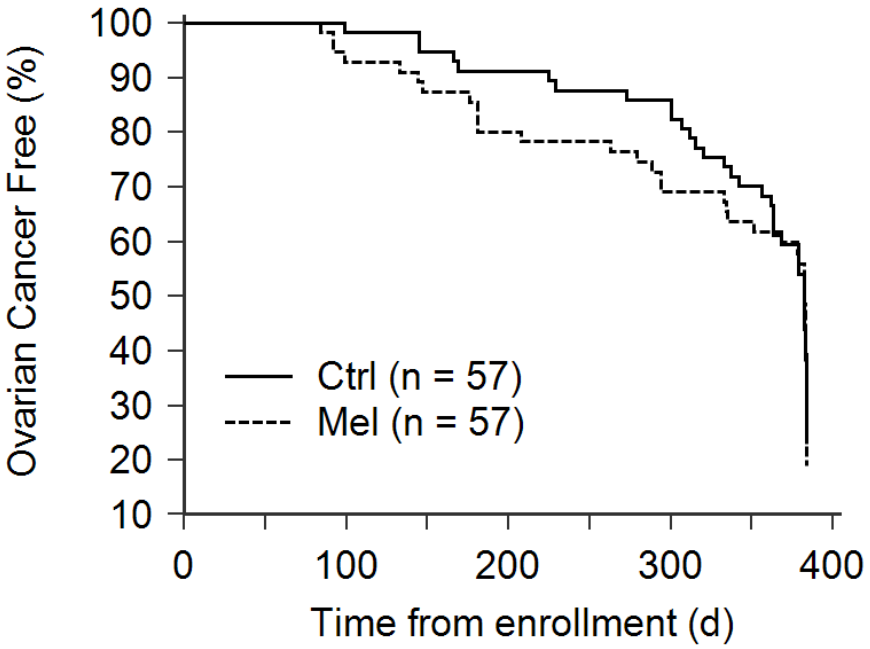

Figure 2.18. Time to cancer by treatment group. A Kaplan-Meier survival curve shows time to cancer in hens from the control (Ctrl) and the melatonin (Mel) groups. No difference is observed between groups (P = 0.85).

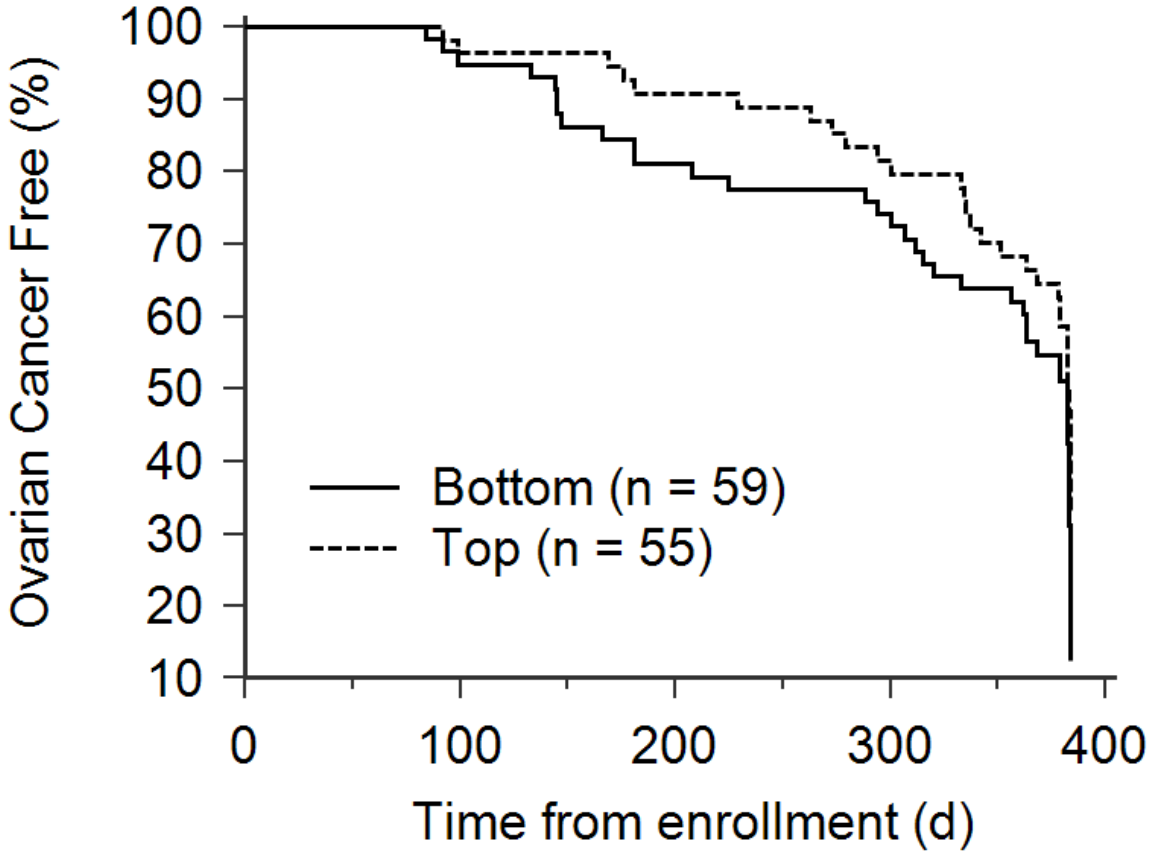

Figure 2.19. Time to cancer by location. A Kaplan-Meier survival curve shows time to cancer in hens allocated in bottom and top cages. No difference is observed between location groups (P = 0.11).

Effect of oral melatonin on survival probability

No significant difference was observed between control (Ctrl) and melatonin (Mel) hens regarding survival probability. The death hazard ratio (HR) was 1.169 for Mel versus Ctrl animal, with a 95% confidence interval of 0.689 -1.984. Mean time to death was 333 days in Ctrl animals and 297 in Mel hens (Fig. 2.20). Location did not have statistical significance in survival either, showing a HR of 0.761 (95% CI 0.447 – 1.297) for hens located in top cages. Mean time to death was 314 and 316 days for bottom and top cages, respectively (Fig. 2.21). The time to death by OC was not significantly different by treatment (Fig. 2.22) nor by location (Fig. 2.23) (HR 0.959, 95% CI 0.502 – 1.831 for Mel hens; HR 0.745, 95% CI 0.389 – 1.429 for hens on top cages). Mean time to death by cancer was 336 days for the Ctrl group and 324 for the Mel group. Mean time to death by cancer was 321 and 340 days for bottom and top cages, respectively.

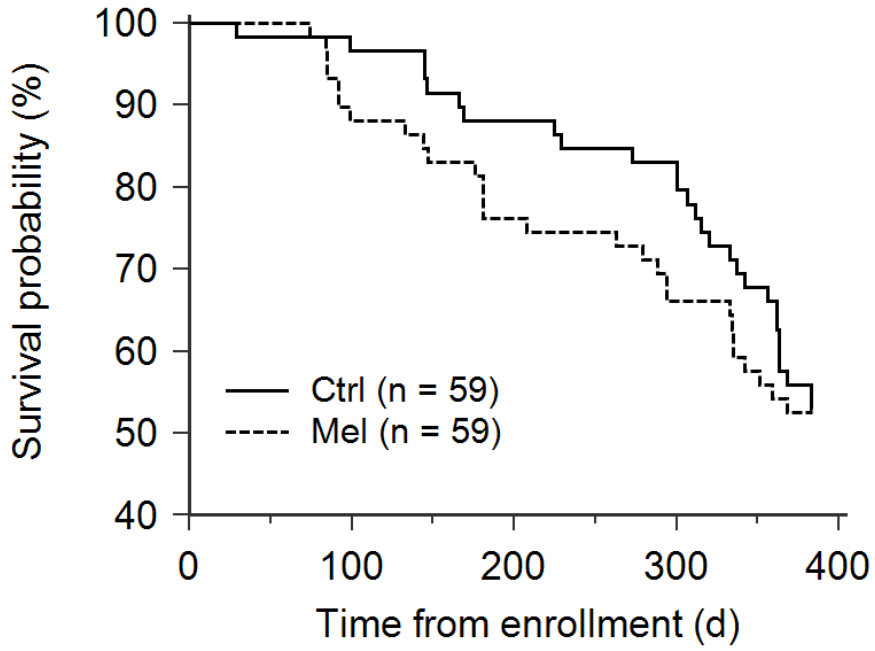

Figure 2.20. Time to death by treatment group. A Kaplan-Meier survival curve shows time to cancer in hens allocated in the control (Ctrl) and melatonin (Mel). No difference was observed between groups (P = 0.56).

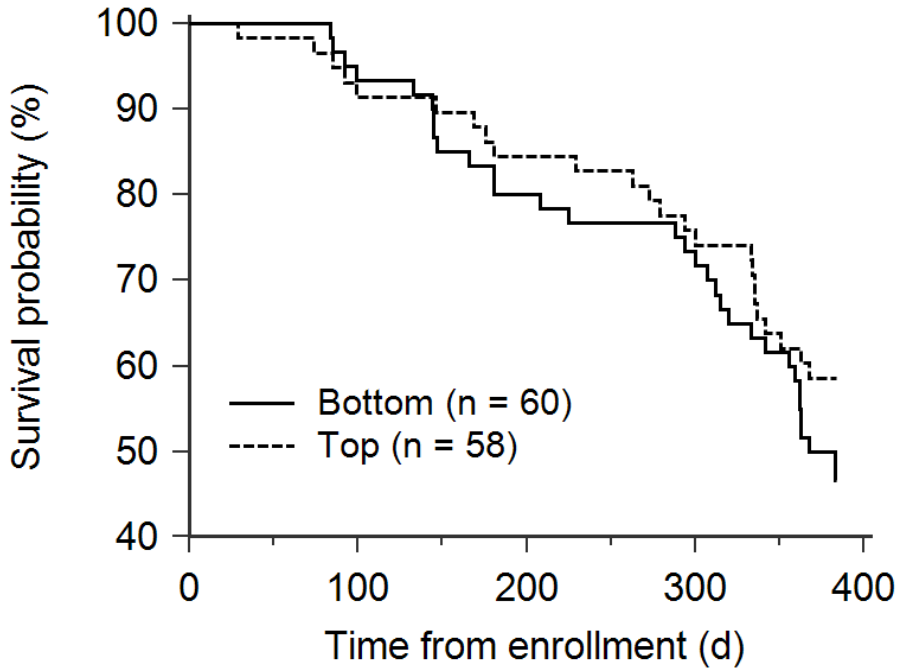

Figure 2.21. Time to death by location. A Kaplan-Meier survival curve shows time to cancer in hens allocated in bottom and top cages. No difference was observed between location groups (P = 0.32).

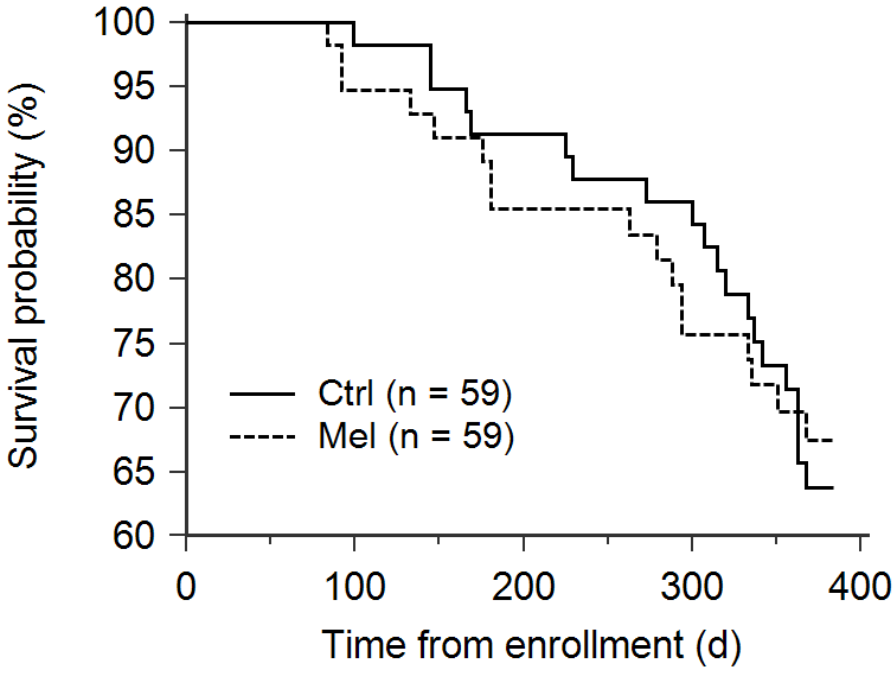

Figure 2.22. Time to cancer death by treatment. A Kaplan-Meier survival curve shows time to cancer in control (Ctrl) and melatonin (Mel). No difference was observed between groups (P = 0.9).

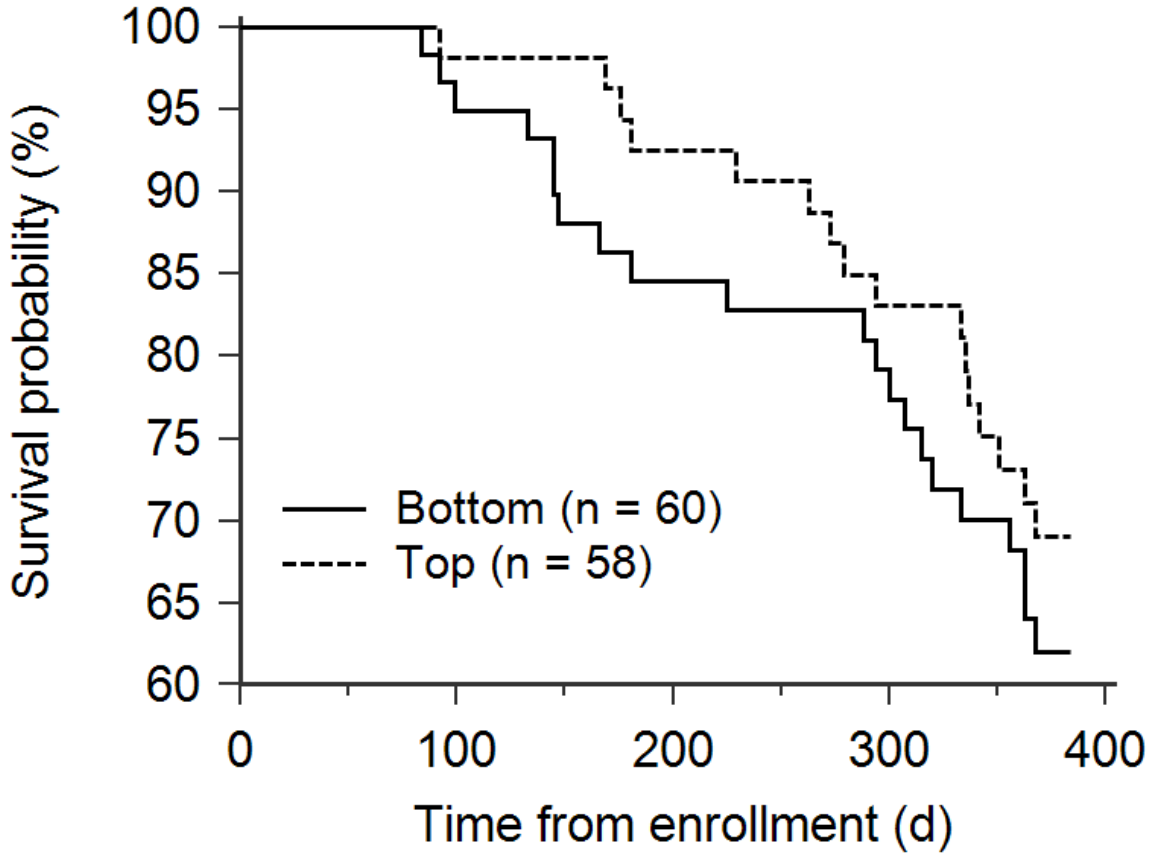

Figure 2.23. Time to cancer death by location. A Kaplan-Meier survival curve shows time to cancer in hens allocated in bottom and top cages. No difference was observed between groups (P = 0.38).

Discussion

To the best of our knowledge, ours is the first study to investigate the value of melatonin to prevent and potentially treat ovarian cancer in a prospective study. The laying hen is the only spontaneous ovarian cancer (OC) model with a high enough incidence to investigate cancer biology and test treatments in long-term and large-scale experiments. We employed this model to test the prophylactic and/or therapeutic use of oral melatonin in peak-risk-aged hens. Because OC incidence increase begins at approximately 2.5 years, we provided melatonin-supplemented diet for a year, from 2 to 3 years of age. This period could be considered equivalent to supplementing women around the time of menopause, when risk increases. Supplementing the commercial feed with a low dose of melatonin, provoked a significant plasma increase during the night.

The dose for the current experiment was selected based on a previous study where plasma melatonin was increased during both night and day by their "low" dose (Taylor et al., 2013). In the present experiment, we obtained a 2-fold melatonin increase at night in Mel versus Ctrl hens with minimal difference in daytime using a low dose (17.36 ug/kg of body weight) of melatonin in the diet. We were able to increase nocturnal melatonin without disturbing its physiological rhythms. Melatonin has been postulated as the likely link between disruption of circadian rhythms and the increased risk for diseases (Cohen et al., 1978, Schernhammer et al., 2001, Bhatti et al., 2013, Carter et al., 2014, Zhao et al., 2016). Chronic disruption of circadian rhythms leads to an increased risk of various diseases, including cancer (Masri and Sassone-Corsi, 2018, Sulli et al., 2018, Ye et al., 2018). In the present study, melatonin was only elevated at nighttime. The mechanism by which only nocturnal melatonin is elevated, despite diurnal consumption, could involve the anatomical and digestive particularities of the chicken. Feed transit in the avian gastrointestinal tract is slowed to ensure physical break down and nutrient absorption (Shires et

al., 1987, Denbow, 2015). This process requires the passage through the crop and through the gizzard for muscular grinding of the food, before the nutrients are assimilated in the small intestine. The mean passage time in the gastrointestinal tract has been reported to range from 5 to 9 hours in Leghorns and broilers (Shires et al., 1987, Denbow, 2015). The delayed assimilation of nutrients may explain why melatonin is increased only during the night in treatment animals.

There is a well-established association between ovulation and ovarian tumorigenesis, as lifetime number of ovulations impact the risk of developing OC (Bassuk and Manson, 2015, Yang et al., 2016, Peres et al., 2017). The association between ovulation and ovarian tumorigenesis was initially proposed in 1971 by Fathalla (Fathalla, 1971), when he proposed that "incessant ovulation" leads to OC. Indeed, strategies that reduce the number of ovulations in a lifetime also reduce the risk of developing OC, such as oral contraceptives, parity, and lactation (Whittmore et al., 1992). The use of oral contraceptives decreases the risk of OC by 30 to 50% (National Cancer Institute, 2018). In agreement with human clinical data, oral contraceptive compounds (estrogen- or progestin-based) reduced ovulation (as for egg numbers) and ovarian cancer incidence in laying hens (Treviño et al., 2012). In addition, hens that often fail to ovulate, the so called "mutant restricted ovulator" have greatly reduced OC incidence (Giles et al., 2010). It is reasonable to assume that ovulation and, therefore, egg production plays a role in ovarian carcinogenesis. The laying hen is of particular use in this regard since ovulations can be objectively measured by egg production. In the present study, laying rates were not altered by dietary supplementation of melatonin. Over the last decades, laying hens have been consistently selected based on egg production, which could explain the increase of spontaneous OC that we observed (~50%) compared to approximately 35% found in previous studies (Fredrickson, 1987, Urick et al., 2008, Barua et al., 2009, Treviño et al., 2012).

We had anticipated that oral melatonin supplementation would be protective against ovulatory oxidative stress. Although reactive oxygen species (ROS) are involved in ovarian physiology, such as follicle rupture during ovulation, excessive ROS can contribute to the development of malignancies, as reviewed by Tamura et al. (Tamura et al., 2012). Antioxidant properties of melatonin are thought to play a key role maintaining redox homeostasis in the ovary, as it highly concentrates in preovulatory follicle follicular fluid (Nakamura et al., 2003). Furthermore, melatonin protects the oocyte from oxidative damage (Tamura et al., 2008). Oral supplementation is an appropriate route to elevate ovarian melatonin levels, as other investigators have reported that melatonin reaches the ovary from the circulation because no ANAT (a crucial enzyme for melatonin production) was found in the ovary (Tamura et al., 2012). It is possible that the dosage selected in our study was not sufficient to counteract the cumulative damage of ovulation. Alternatively, as hens were enrolled in their second cycle of laying, it is possible that ovaries had already begun malignant transformation.

Whether melatonin has a role in ovulation is not clear. The administered dosage did not alter egg production; therefore, it can be assumed that ovulation was unaltered by the dose used. Previous studies reported an increased in egg production using doubling doses of oral melatonin (Liu et al., 2018). Based on the unaltered egg production found in this study, it is likely that higher doses of oral melatonin are needed to influence laying rates.

Interestingly, our data show that among these proficient producers, chickens that develop OC decrease their laying rates in the months preceding cancer diagnosis. These results are in agreement with previous studies that showed a significant decrease in egg production preceding clinical ovarian cancer (Urick et al., 2008, Barua et al., 2009, Giles et al., 2010, Treviño et al., 2012). Laying rates dropped significantly 3 months prior to clinical disease. Likely, before

clinical disease manifestation, ovarian cycling slows and egg production declines. A direct translation to human medicine is difficult to make due to the usual occurrence of OC after menopause. However, having an early indicator strengthens the animal model and offers the possibility of performing prophylactic trials. In this regard, knowing that decreased egg production may be an early indicator of ovarian cancer, a pitfall of our experiment is that animals were not randomized by historical egg production, and data regarding egg production prior to enrollment is not available. However, at enrollment, all animals were laying without apparent abnormalities.

Location had an unexpected impact in several outcomes of this study. Hens located in top cages (~1.82 m from the floor, ~51 lux) produced more eggs than those in bottom cages (~1.34 m from the floor, ~40 lux). This finding is in agreement with studies in laying hens that showed greater hen-day production with higher light intensity (Renema et al., 2001). In addition, the effect of light on both melatonin and reproduction in birds may indirectly influence ovarian cancer development. A study in turkeys found remission of ovarian adenocarcinoma with exposure to short-day (8L:16D) photoperiod (Moore and Siopes, 2004). It is possible that light contributes to tumorigenesis either directly through an effect to inhibit melatonin or indirectly by an effect on egg production. We found no difference in plasma melatonin between positions, although we may have sampled extensively enough. Alternatively, the decreased egg production in the bottom cages may reflect the increase in OC in these cages (stage II and III, Table 2). As we did not have historical egg production prior to the study, we cannot address overall egg production. In this study, we have observed that hens exposed to higher light intensity during the day (top cages) present a lower incidence of stage II and III ovarian cancer, but no difference in individual stages.

Several of the reported beneficial effects of melatonin are associated with a reduction in tumor growth. Anti-proliferative properties were shown to be mediated by CDK inhibition in ovarian cancer. Shen et al. (Shen et al., 2016) detected an accumulation of OC cells in G1 phase and a reduction of CDK 2 and 4 expression upon melatonin treatment in a dose-dependent fashion. Other investigators demonstrated that melatonin blocks cell proliferation and growth through inhibition of ovarian cancer stem cell invasiveness, migration, and epithelial to mesenchymal transition (EMT) (Akbarzadeh et al., 2017). Petranka et al. showed that melatonin reduces cell numbers and its nuclear receptor agonist induces apoptosis in OC lines (Petranka et al., 1999). Melatonin was also shown to upregulate p53 and to induce apoptosis in in vivo models (Chuffa et al., 2016). Despite all the attributes of melatonin, we did not detect a statistical reduction in ovarian cancer. The reason why we did not observe an effect of melatonin in our model of ovarian cancer is unclear. The selected dosage, although elevated melatonin while maintaining the physiological pattern, may not have been sufficient to decrease OC incidence. Also, it is possible that ovarian carcinogenesis had begun before enrollment to our study, and that mutagenic changes had already occurred making prophylactic measures futile.

Future investigations are warranted based on these results to elucidate the role of melatonin in the prevention and/or treatment of ovarian cancer, and to evaluate the molecular mechanisms involved in a possible melatonin effect.

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APPENDIX I

HIGH DOSE OF ORAL MELATONIN SUPPLEMENTATION

Introduction

There is a growing body of evidence regarding the potential of melatonin for the prophylaxis and treatment of ovarian cancer. Our previous experiment tested a low dose (17.36 mg/kg) of melatonin in our efforts to conserve the normal daily cycle of melatonin. Although that treatment raised plasma melatonin during the night, it failed to significantly affect ovarian cancer incidence. In this experiment, we selected a higher dose of melatonin to be administered to the hens. These two experiments should have been contemporary, but due to the lack of sufficient number of animals available, the present experiment started a year later. Here, we present preliminary results of this study.

Materials and Methods

All procedures and methodology were similar to those used in the previous experiment. Briefly, sample size was calculated based on a reduction in cancer incidence from 35% to 20%, with a power of 80% and a type I error of 5%. Animals were randomly assigned to the melatonin group (300 ug/kg of body weight; Mel n = 55) or the control group (Ctrl n = 55). Two animals were lost early in the experiment and are not considered for analysis. Feed was prepared weekly to conserve the freshness of melatonin. All animals are housed under standard temperature and humidity conditions in Cornell University Poultry Farms facilities and exposed to 16:8 L:D lighting schedule. All procedures are performed in accordance to IACUC regulations. Individual daily egg production was recorded. Monthly body weights are assessed as a parameter of overall

health. All hens were monitored daily and those presenting signs of illness compatible with ovarian cancer were euthanized in a CO_2 chamber. Blood samples were collected immediately before euthanasia. Ovary, oviduct, liver and intestine samples were collected immediately after death, fixed overnight with 10% formalin, and transferred to 70% ethanol.

Blood samples were collected at ZT1 (7 am), ZT7 (1 pm), ZT13 (7 pm), and ZT19 (1 am) from different hens (n = 9 - 12 per treatment group and time). Unfortunately, some samples were lost on transportation and resulted in 5 samples for Mel group at ZT 19.

Plasma melatonin measurement was performed as in our previous experiment, using a commercial RIA kit (Direct Melatonin RIA, 79-MELHU-R100, Alpco. Salem, New Hampshire, USA). Based on earlier testing of chicken samples using this kit, night samples were diluted 1:5 and daytime samples were diluted 1:2 using diluent provided with the kit.

Preliminary Results

Plasma Melatonin Levels with Oral Supplementation

Circulating plasma melatonin was significantly elevated at all time points (ZTR1, ZT7, ZT13, ZT 19) in the melatonin treated group compared to the control group (Fig. I.1, Table I.1). There are no daily cycles of melatonin in the treated group. A significant effect of treatment, time, and treatment by time interaction was observed in plasma melatonin ($P < 0.0001$, $P = 0.0079$, $P = 0.0374$, respectively).

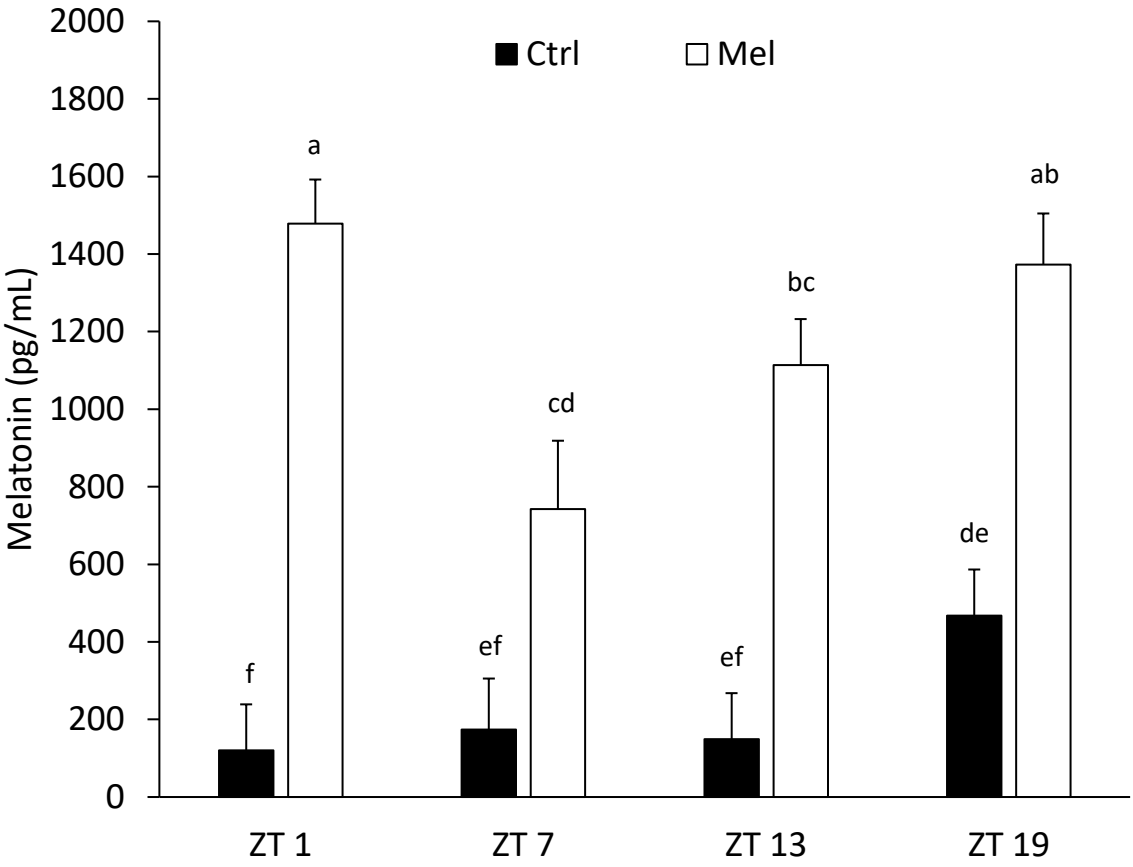

Figure I.1. Plasma melatonin levels in selected hens at ZT1 (7 am), ZT7 (1 pm), ZT13 (7 pm), and ZT19 (1 am). Animals were in the trial for four months prior to sampling. Different letters above the bars indicate statistic al difference between groups and/or time points.

	Ctrl	**n**	**Mel**	**n**
ZT1	115.01	11	1575.87	10
ZT7	169.27	9	765.44	5
ZT13	144.63	11	1168.82	11
ZT19	455.51	11	1375.55	11

Table I.1. Melatonin values (pg/mL) and number of animals sampled at each time point.

Body Weight

Melatonin treated hens were consistently heavier ($P = 0.01$) than control hens throughout the elapsed experimental time. Every monthly measurement of body weight showed significance (Fig. I.2). A significant effect of time was observed ($P < 0.0001$), but there was no interaction between time and treatment ($P = 0.73$).

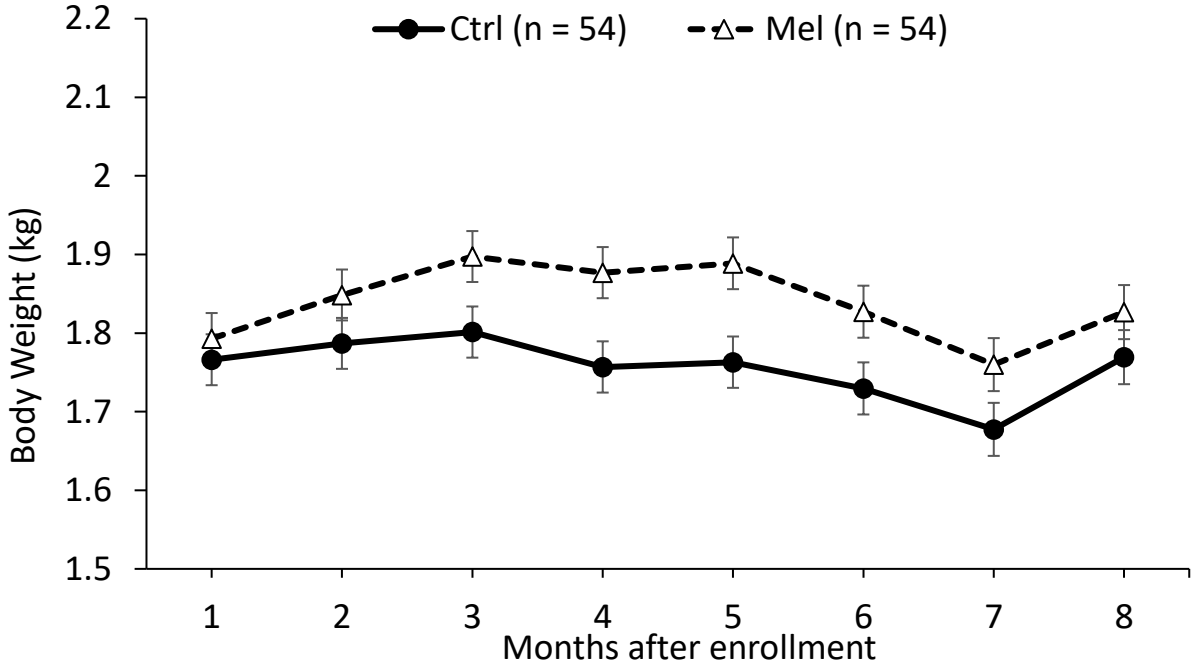

Figure I.2. Monthly body weights in control (Ctrl) and melatonin group (Mel). After month 1, there was statistical difference between groups ($P = 0.01$). Every point in the graph depicts the average of all animals within the group.

Egg production

Animals in the melatonin group showed significantly lower egg production than control hens in subsequent months. This difference has been maintained throughout the experiment (Fig. I.3). No statistical difference was detected between groups in egg weight (data not shown).

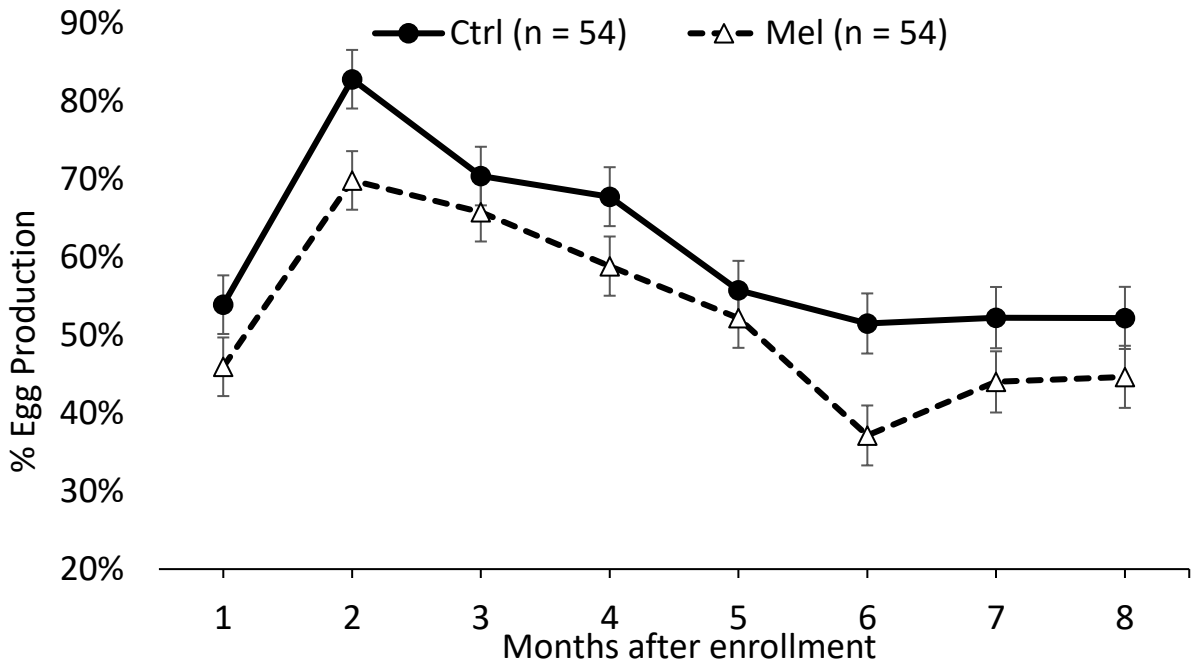

Figure I.3. Percentage egg production in control (Ctrl) and melatonin (Mel) treated hens. Dots indicate average of all hens belonging to that group. There was a significant effect of treatment ($P = 0.019$).

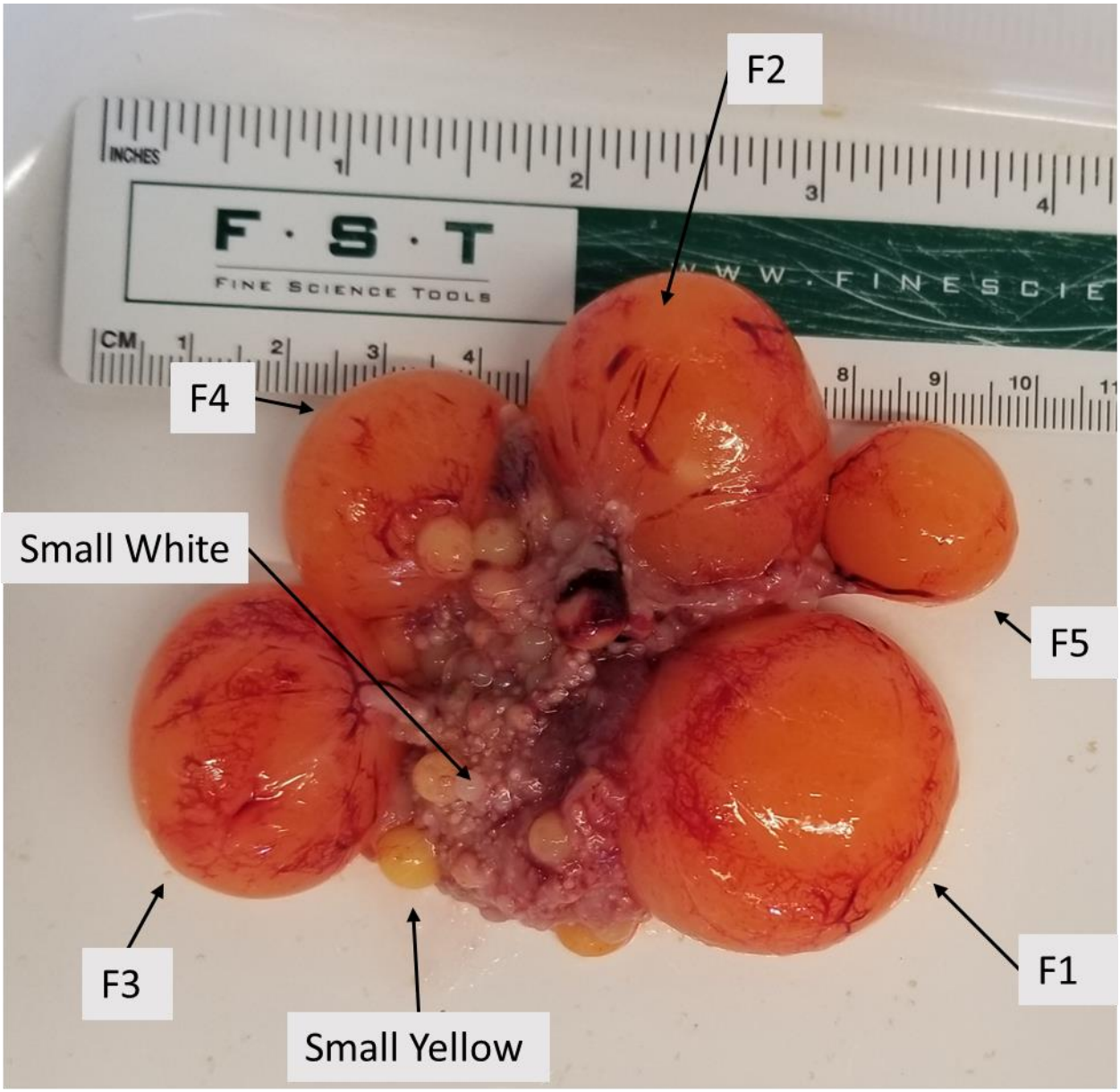

Figure I.4. Normal ovary. Two-year-old hen found dead almost three months after enrollment. Note the normal follicular hierarchy with small white and yellow follicles, as well as F1, F2, F3, F4, and F5.

Mortality

To date 18 hens (16.7% of the total sample size) died, 9 in the melatonin group and 9 in the control group. In the melatonin group, two of them presented ovarian cancer and 7 died of unrelated illness. In the control group 3 hens showed signs of ovarian cancer and 6 died of unrelated illness (Table I.2). Examples of a healthy ovary and an advanced ovarian cancer are shown in Figures I.4 and I.5, respectively.

Table I.2. Mortality

	Ovarian Cancer % (n/n)	No Ovarian Cancer % (n/n)	Total % (n/n)
Melatonin	3.7% (2/54)	13% (7/54)	16.7% (9/54)
Control	5.6% (3/54)	11.1% (6/54)	16.7% (9/54)

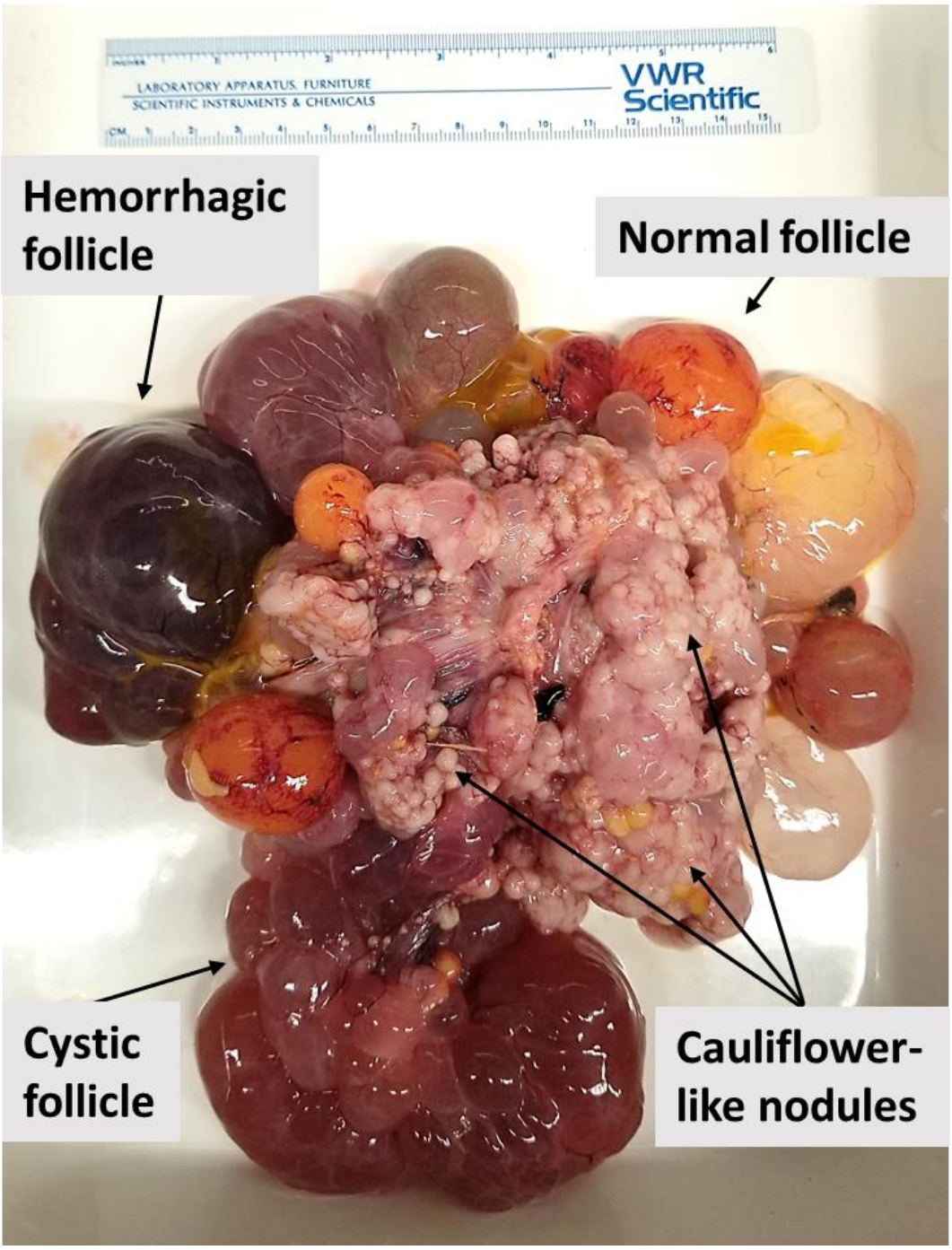

Figure I.5. Ovarian cancer. Two and a half-year-old hen with some nodules in intestine, pancreas, and oviduct, euthanized 7 months after enrollment for presenting signs of illness. Note the presence of hemorrhagic, cystic, nodular (cauliflower-like) tumorous follicles.

Discussion

Oral melatonin supplementation with 300 ug/kg has been used in this experiment to evaluate ovarian cancer incidence. At 8 months into the experiment, there is no obvious difference in mortality related to treatment. We anticipate that the experimental period will be 14 months, and we will be then able to assess ovarian cancer mortality rates and incidence.

A statistical increase of plasma melatonin was found at all time points (ZT) with the present supplementation (300 ug/kg) with minimal daily oscillations in contrast to the elevation of nocturnal melatonin only during the night when a low dose (17.36 ug/kg) was administered. Chronic circadian disruption has been linked to multiple diseases, as reviewed by Stevens (Stevens et al., 2007). Under normal conditions, the body's "master clock", the suprachiasmatic nucleus (SCN) orchestrates physiological functions of target tissues via the upregulation of clock genes, as reviewed by Bell-Pedersen (Bell-Pedersen et al., 2005). The SCN depends on melatonin to receive information regarding time of day and season, and melatonin receptors are highly abundant in the SCN (Weaver et al., 1989, Dubocovich et al., 2003). Overall homeostasis relies on the delicate balance between central and peripheral components of the timekeeping axis. Recent reports have extensively reviewed the link between circadian biology and metabolism and described deregulation of daily rhythms in metabolic disorders (Reinke and Asher, 2019). It is possible that the unexpected increased body weight observed with melatonin-supplemented diet reflects such a metabolic disruption, as chronic circadian disruption caused overweight and leptin resistance in mice (Kettner et al., 2015). Overweight and obesity have been long associated with circadian rhythm disruption, and melatonin is one of the key factors, as reviewed by Cipolla-Neto (Cipolla-Neto et al., 2014). It has been suggested that the poultry industry could use circadian disruption to enhance weight gain in broilers. Indeed, dietary

supplementation with melatonin increases feed conversion and weight early in broilers' life (Clark and Classen, 1995). In agreement with this report, we observed increased body weight on the animals fed with melatonin-supplemented diets. The specific cellular signaling in the central and peripheral oscillators remain to be studied in laying hens and could be evaluated at the endpoint of this experiment. The potential impact of melatonin to maintain metabolic homeostasis warrants further evaluation of the endocrine effects and possible mechanisms of action.

Beneficial effects of melatonin on egg production depend on the dosage. Evidence indicates that there is a limit for melatonin enhancement of egg production (Jia et al., 2016). Dietary melatonin at a concentration of 0.625 mg/kg significantly enhanced egg production in laying hens. However, 2.5 and 10 mg/kg did not impact egg production (Liu et al., 2018) Our dose is 300 ug/kg of body weight (equivalent to 30 mg/kg diet), and therefore much higher. The laying reduction could alternatively be explained by unknown factors related to weight gain and disruption of daily patterns of secretion of melatonin.

In the future, we will determine the effect that 300 ug/kg of oral melatonin supplementation has on ovarian cancer incidence. Endocrine and metabolic parameters should be evaluated to understand the pathways involved and mechanisms of action. Furthermore, in vitro experiments should be pursued to test potential melatonin signaling.

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APPENDIX II

ROLE OF THE PINEAL GLAND IN OVARIAN CANCER

Introduction

Since the discovery of melatonin as an extract of the bovine pineal gland in the late 50s (Lerner et al., 1958), much research has been conducted to understand the biology and importance of this hormone. Most studies implicate melatonin as a timekeeping hormone with roles in biological and seasonal rhythms (Reiter et al., 2010). Several pathologies emerge when melatonin synthesis is disrupted, as reviewed by Zawilska and by Reiter (Zawilska et al., 2009, Reiter et al., 2016). Cancer is one example (Blask et al., 2014). Early research focused on breast cancer (Cohen et al., 1978, Schernhammer et al., 2001, Poole et al., 2015), and was then followed by observational (Bhatti et al., 2013, Carter et al., 2014, Zhao et al., 2016), and experimental (Lissoni et al., 1999, Moore and Siopes, 2004, Akbarzadeh et al., 2017) studies in other hormone-dependent cancers, like ovarian cancer (OC). We aimed to evaluate how chronically diminished melatonin from early life affects OC incidence in the laying hen animal model, via the surgical removal of the pineal gland. Pinealectomy (PNX) was shown to be a viable strategy to reduce melatonin in the chicken (Fagan et al., 2009). We evaluate if the surgical removal of the pineal gland impacts ovarian cancer incidence in the domestic laying hen. Although others have performed PNX in chicken, no study has conducted long-term research of its impact on OC.

Materials and Methods

Housing

All animals were exposed to the same lighting schedule (16 hours of light and 8 hours of darkness), and housing conditions throughout the experiment. All hens received *ad libitum* access to commercial feed (Agway) and water. During the experimental period, no major procedures other than occasional blood collection, monthly weighing are performed. Weights are recorded every month to monitor growth patterns across groups, as an indirect measure of health.

Pinealectomy

Two hundred female chicks were assigned randomly to either the pinealectomy (PNX) or the control (CTRL) group. Animals in the PNX group had their pineal removed between 7 and 11 days of age following a previously published protocol (Foss et al., 1972, Fagan et al., 2009). No surgery was performed on the Ctrl birds. Nine chicks were lost in the PNX group between the first and the fifth months, before data regarding body weights and egg production were recorded. Four additional chicks underwent sham operation that consisted of the same surgical procedure as the pinealectomy. Sham-operated animals were considered part of the Ctrl group.

Briefly, chicks were anaesthetized in an induction box with 5% isoflurane and then kept on a mask at 2% for the surgical procedure (Fig. II.1). Toe pinch was used to assess anesthetic depth. A CO_2 scavenger container was employed to control anesthetic waste. The surgical area was disinfected extensively with chlorhexidine. A small skin incision in the mid sagittal head region (where the comb grows in the adult) was performed to have visual access to the skull. Then, two J-shaped cuts through the skull were carefully performed from the axial plane to the sides of the head to form a flap of approximately 1 cm. The delicate skull was gently flipped to expose the cerebrum and cerebellum. Using optical-grade forceps, the pineal was removed. Skin

was sutured using Vicryl-6-0. Recovery was allowed in the room under close monitoring with hand warmers. Butophanol (analgesic, 0.23-0.3 ml of 1 mg/ml solution, subcutaneously (SQ)), robenacoxib (anti-inflammatory, 2-10 mg/kg, intramuscularly (IM)), doxycycline (antibiotic, 0.3 ml of 10 mg/ml solution, IM), and warm subcutaneous saline solution (electrolytes, 0.5 ml, SQ) were administered in the immediate post-operation period to control pain and potential infections.

Melatonin levels at night

All hens were bled between 20 – 22 months of age. Because melatonin rises approximately an hour after darkness, peaks around midnight and declines one hour before lights are on (Pelham, 1975, Wang et al., 1998), blood samples were collected during the dark phase, between ZT17 and ZT22 (11 pm and 4 am). An attempt was made to collect a blood samples of 2 ml from all birds under dim green lights. Heparinized syringes were used for blood collection and sodium citrate was added prior to plasma storage at -80˚ Celsius.

Melatonin RIA

Plasma samples were assayed for melatonin level using a commercial RIA kit, with validation as described in Chapter 2. Melatonin measurements from one assay are included in this appendix (CTRL n = 38, and PNX n = 32). All samples were diluted 1 part of plasma sample in 5 parts of kit diluent, as directed by the manufacturer's manual of use. The low pool intra-assay CV was 21% and the high pool intra-assay CV was 18%. Samples were excluded from statistical analysis using two criteria: CV higher than 15%, and samples falling below the lowest percentage binding of the standard curve (10.57%). Also, outliers were identified using an outlier test and were excluded from analysis (CTRL 1 outlier, PNX 3 outliers).

Necropsy and tumor classification

The endpoint of this study is defined as age of 36 months or development of ovarian cancer signs, whichever occurs first. At that time, birds will be euthanized and necropsied to evaluate the presence of ovarian gross disease and to collect ovary, oviduct, intestine and liver samples. If present, ovarian cancer will be staged as previously described (Treviño et al., 2012). Histological slides will be stained and assessed for pathological characteristics to detect stage I cases and to identify histotype. The brains of deceased animals will be evaluated for gross presence of the pineal gland to validate the surgical procedure (Fig. II.2). Cerebral tissues will be submitted for histological analysis to the Diagnostic Histology Laboratory for routine slide preparation and will be assessed for microscopic remaining of pineal tissues.

Figure II.1. Seven-day-old chick anesthetized and prepared for pinealectomy.

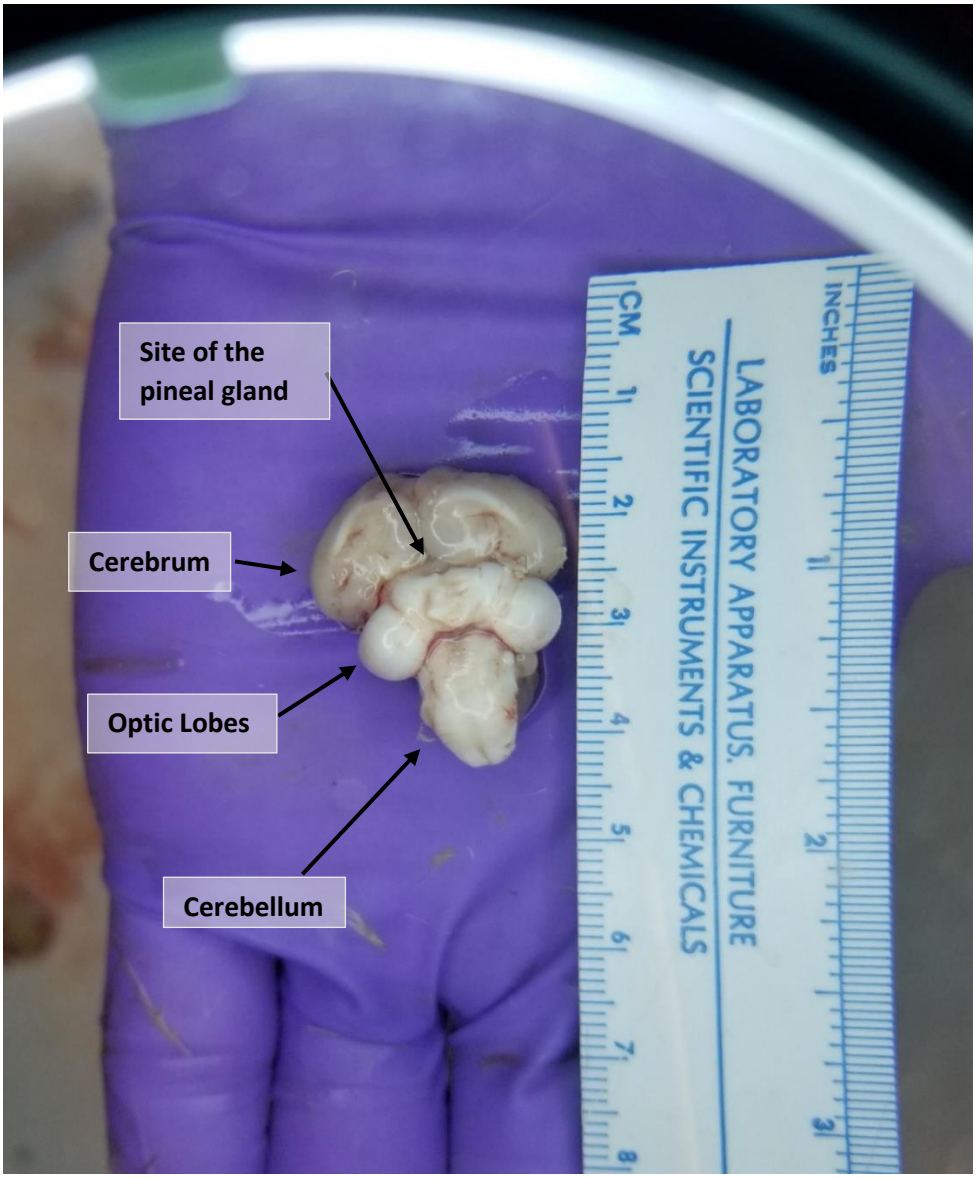

Figure II.2. Chicken brain gross morphology. Two and a half-year-old hen brain at necropsy. This hen belonged to the PNX group and was euthanized for ovarian cancer-related signs of illness. The pineal gland is normally located beneath the cerebrum. Note that the cerebrum is artificially displaced cranially unintentionally. An additional piece of tissue was found and conserved for histological examination to determine pineal origin.

Preliminary Results

Plasma melatonin

Plasma melatonin showed statistical difference between groups (P = 0.04; Fig. II.3). There is high variation among samples of all groups as demonstrated by the inter- and intra-assay CV values (see the Materials and Methods section). For the samples collected during the dark phase, a rhythmic secretion pattern is observed with peaks around the middle of the night followed by a decline as night advances (data not shown).

Body weights

Both groups showed a steady increase in body weight which plateaued at approximately 18 months (Fig. II.4). Body weights were significantly different between groups from the age of seven months throughout the experimental period. At month 13, there was a mite infestation which affected both groups. As part of the mite treatment, animals had to be moved to a different building for 6 weeks. The acute body weight drop reflects the stress caused by this situation.

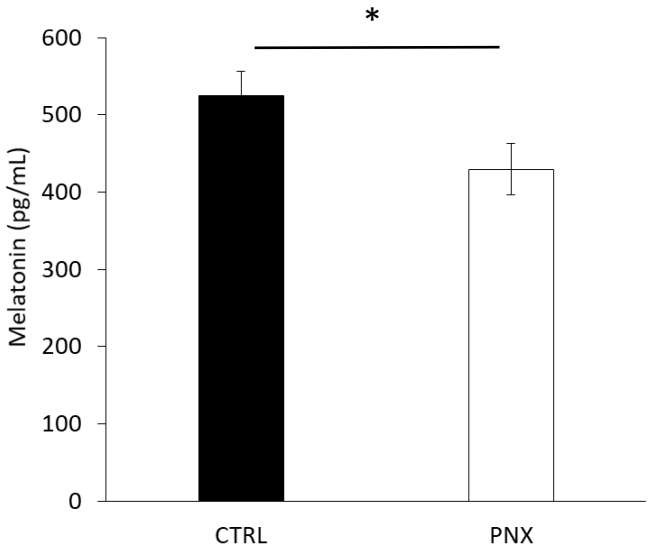

Figure II.3. Plasma melatonin during dark phase (11 pm to 4 am) of a subset of control (CTRL n = 39) and pinealectomy (PNX n = 32) hens. (P = 0.04). Samples were collected during the night, on three consecutive nights, over 2.5 years after the pinealectomy procedures.

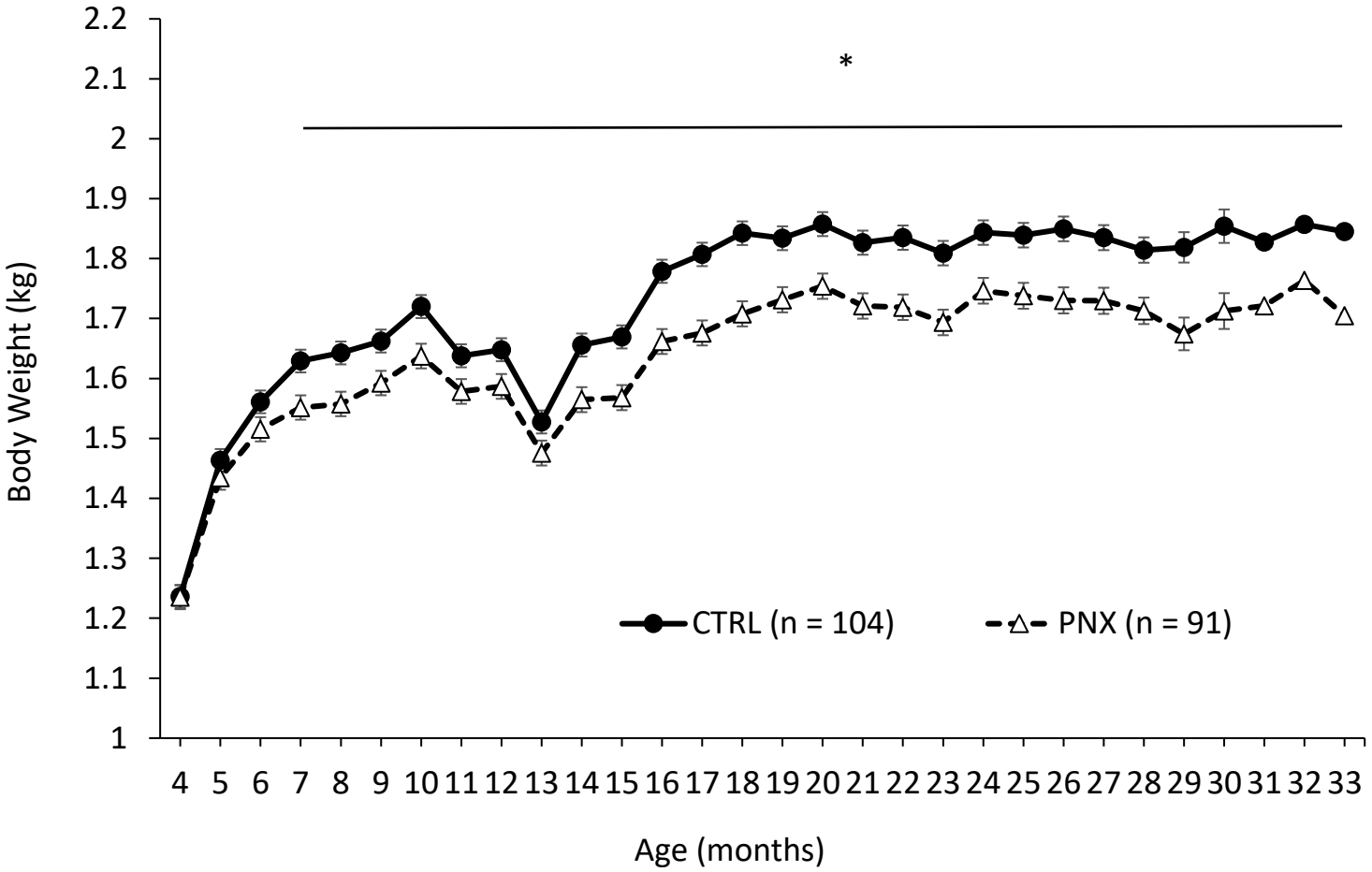

Figure II.4. Monthly body weights (kg) of PNX (n = 91) and CTRL (n = 104) hens. Seven to eleven-day-old female chicks had their pineal gland removed. Control and pinealectomized hens were monitored monthly for body weight throughout the experimental period to this point. Asterisk denotes a *P* value = 0.0001. Average from all body weights are depicted in this graph

Egg production

Individual egg production was recorded starting at the seventh month of age (Fig. II.5). Earlier egg production is not available for individual hens. One hundred percent egg production reflects the production of one egg per day. There was no difference in egg production between groups. All animals reached 100% egg production, equivalent to almost one egg per 24 hour period. The acute drop in production at 13 months reflects the stress caused by the moving out of the building for 6 weeks and then back, as explained previously. No statistical differences were observed in egg weights between groups (Fig. II.6).

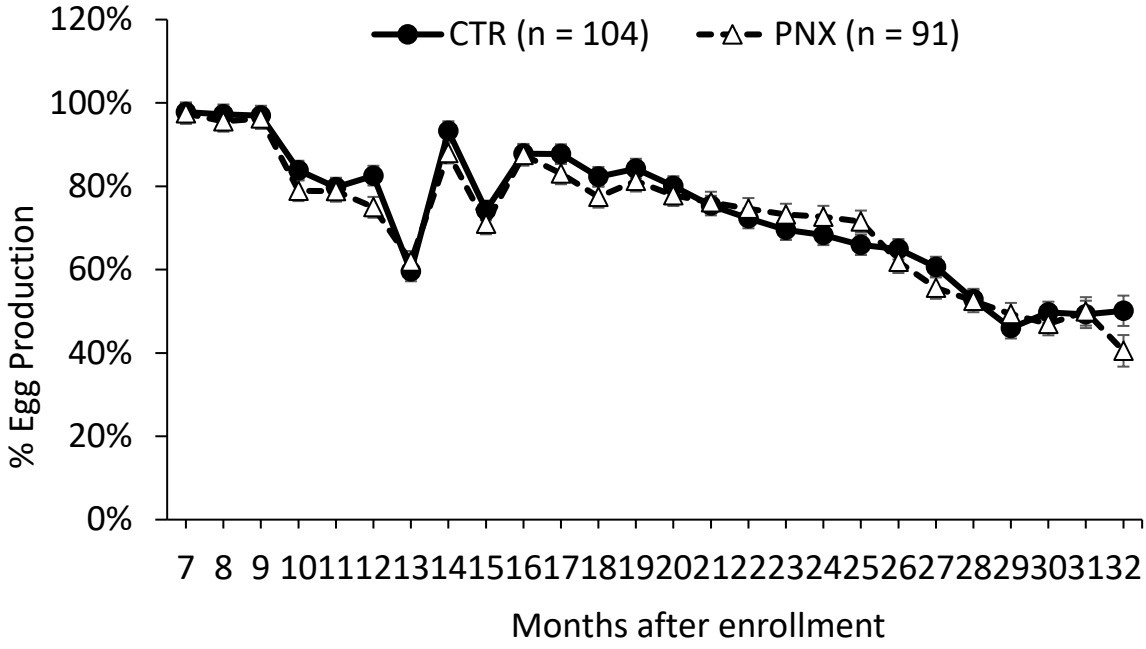

Figure II.5. Percentage egg production in control (CTR) and pinealectomy (PNX) hens. Each dot in the graphs show the average produced by each group. There was no statistical difference between groups (*P* value = 0.34).

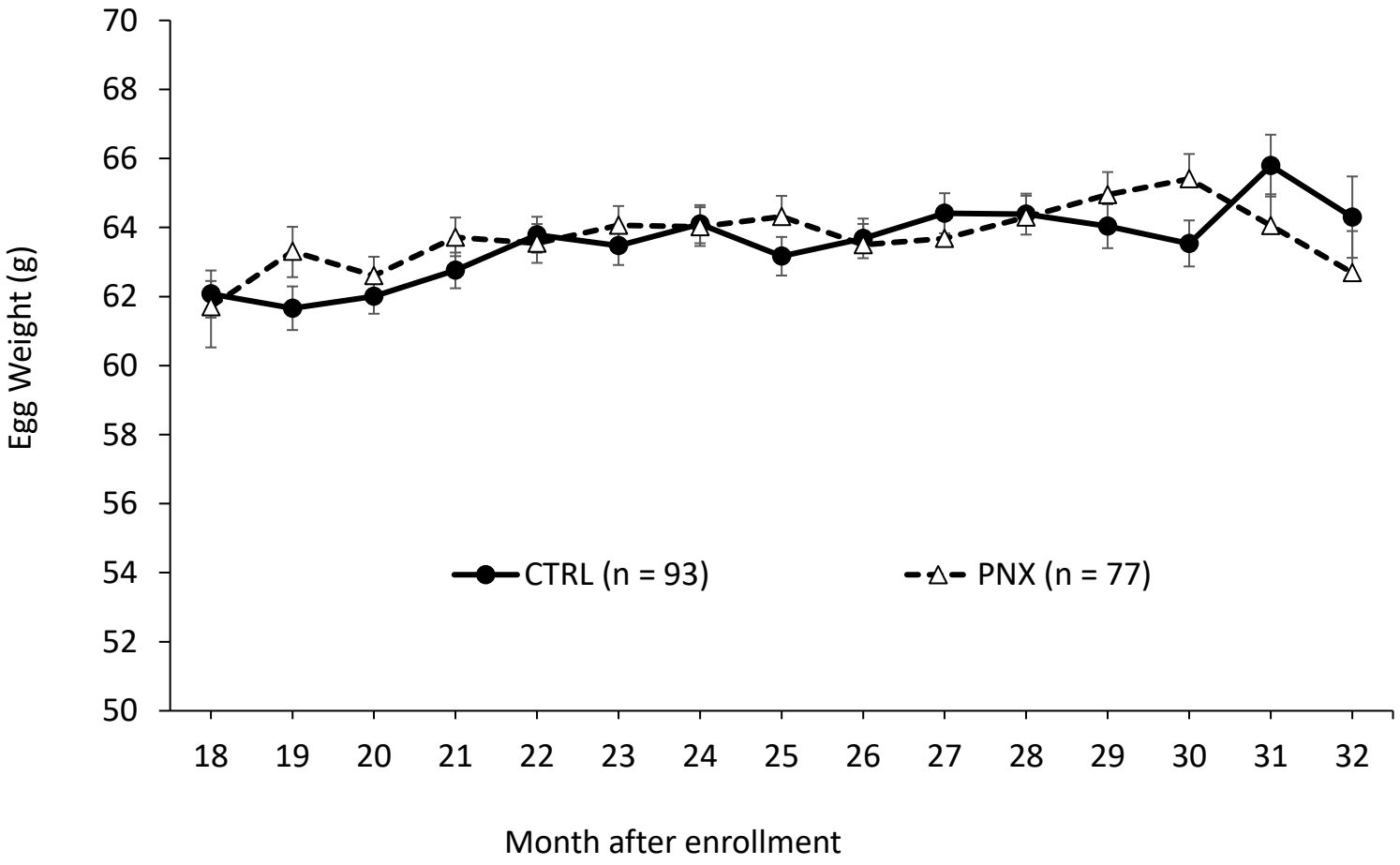

Figure II.6. Egg weight from 18 months of age to the present in control and pinealectomy groups. Eggs were weighed once a month on one day. The eggs present at the weighing time were included in the analysis. This figure shows the average per group. No statistical difference was observed between groups (*P*-value = 0.66).

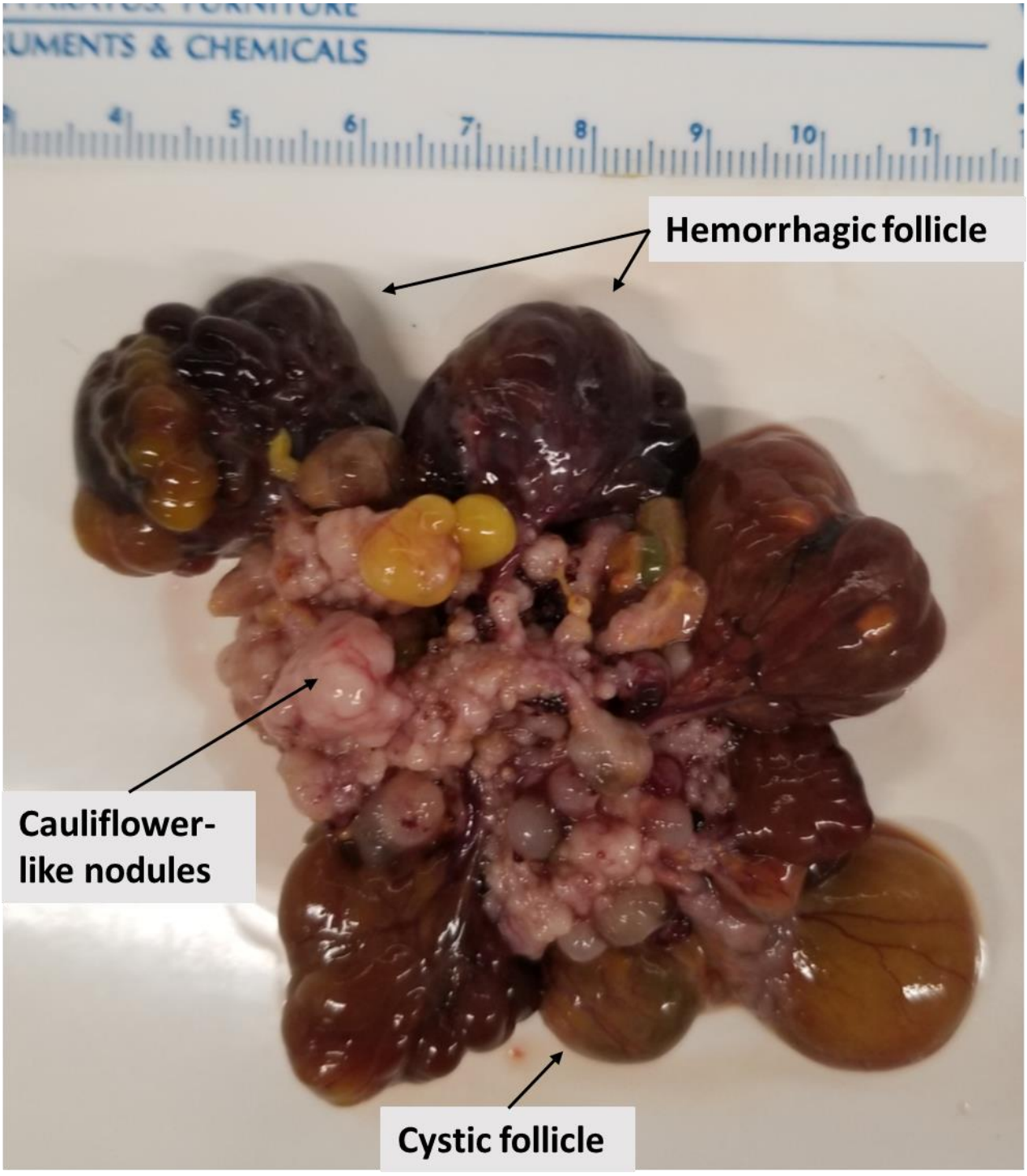

Figure II.7. Stage IV Ovarian cancer. Two-and-a-half-year-old hen the belonged to the control group. Note the cauliflower-like, cystic, and hemorrhagic follicles.

Cancer incidence

An example of the gross morphology of ovarian cancer is shown in Figure II.7. We observed no significant difference between the pinealectomy (PNX) and control (CTRL) groups regarding mortality nor ovarian cancer incidence, as shown in Table II.1.

Table II.1. Preliminary cancer incidence and mortality

	Treatment group			Location			*P*-value	
	CTRL % (n/n)	PNX % (n/n)	Adj. OR (95%CI)	BOTTOM % (n/n)	TOP % (n/n)	Adj. OR (95%CI)	Treatment	Location
Ovarian Cancer Incidence	10.6 (11/104)	7.7 (7/91)	1.35 (0.47-3.89)	10.7 (12/112)	7.2 (6/83)	0.67 (0.23-2.00)	0.56	0.47
Mortality	26.0 (27/104)	17.6 (16/91)	1.61 (0.75-3.46)	22.3 (25/112)	21.7 (18/83)	1.00 (0.46-2.15)	0.22	0.99

Discussion

Based on the growing evidence linking melatonin deficiency with cancer risk, we designed an experiment to assess the effect of chronic suppression of melatonin in the development of ovarian cancer using the laying hen animal model. We hypothesized that melatonin suppression from early life increases the incidence of ovarian cancer. To confirm that the surgical procedures caused a decline in circulating melatonin, we tested plasma samples of all animals using a commercial radioimmunoassay kit. A small but significant difference was observed between groups on plasma melatonin. The high variation between individuals may be influencing the outcome of the assays, as has been shown in human medicine (Arendt et al., 1979). It is important to note that the eyes are a significant source of melatonin in birds. We do not know if chronic absence of melatonin causes compensatory mechanisms implicating other tissues such as eyes (Bernard et al., 1997, Thomas et al., 1998). Alternatively, it is also possible that the pineal gland has regenerative properties and that any microscopic piece remaining can re-grow the gland and re-instate pineal functions. To determine the presence of remaining pineal tissue, post-mortem histological and gross examination will be conducted (Fig. II.5). While some reports suggest that extra-pineal melatonin behaves as a paracrine or autocrine factor in those cases (Cipolla-Neto and Amaral, 2018), others postulate that gastrointestinal melatonin can contribute to plasma levels after pinealectomy (Bubenik, 2002).

We observed a significant impact of pinealectomies on body weight from seven months of age (Fig. II.3), which coincides with the peak of egg production (Jacob et al., 1998). It was recently suggested that melatonin abolition by pinealectomy causes deregulation of the cyclic patterns of leptin, and cortisol in hamsters (Chakir et al., 2015). In chicken, pinealectomy caused a reduction in body weight, a feed intake decline before 4 months, followed by an increase in

feed consumption afterwards (Yamauchi et al., 1990). In agreement, our results showed that pinealectomy induces body weight reduction and suggest that melatonin may impact metabolism and that its deficiency contributes to weight loss. Whether it is caused by early impaired growth rather than by metabolic regulation remains to be uncovered. The mechanisms involved in such effect remain to be uncovered. Metabolic hormones could be measured on these animals to understand their participation on body weight differences.

Whereas egg production was reduced in melatonin-supplemented hens, neither egg production nor egg weights were altered by pinealectomy in the present study. Data have suggested that there is a dual effect of exogenous melatonin on egg production, it exerts a positive effect when the dose is less than 0.625 mg/kg of feed, whereas it has no impact at higher concentrations (Jia et al., 2016, Liu et al., 2018). It is possible that a chronic reduction in circulating melatonin through PNX plays no role in egg production. Previous publications found no differences in plasma luteinizing hormone (LH) in laying hens after pinealectomy, and the surgeries did not alter ovulation or egg production (Johnson and Van Tienhoven, 1984). Supplemented melatonin may interact with endocrine factors that govern egg production, which are unaltered since there wasn't an elevation in melatonin. It is also possible that the lack of a difference reflects either unsuccessful surgeries and/or compensatory mechanisms.

Melatonin biological relevance as a chronobiotic and as an anticancer has been actively investigated in the recent years. However, evidence regarding melatonin mechanisms of action in ovarian cancer in the laying hen is still scarce. We anticipate final results from this trial within a year. Future research should direct efforts to identify pathways affected by melatonin and to strengthen the only spontaneous animal model for ovarian cancer, the laying hen.

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