Neglected aspects of hormone mediated maternal effects

Kumar, Neeraj

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Chapter 1

General introduction and thesis overview

Neeraj Kumar

1 DEVELOPMENTAL PLASTICITY AND PARENTAL EFFECTS

To understand the influence of developmental plasticity in evolutionary processes is an outstanding challenge in evolutionary biology (West-Eberhard 2003). Developmental plasticity is the ability of a genotype to give rise to multiple phenotypes in different environmental conditions during development of an individual. In vertebrates, developmental plasticity frequently involves parental effects, which are the effects on the phenotype of offspring that are not determined by the offspring's own genotype but by the phenotype of its parents, that can be influenced by their environment (Mousseau & Fox 1998; Uller 2008; Youngson & Whitelaw 2008).

Parental effects can influence the offspring fitness. For example, if exposed to natural enemies, degrading local conditions, or increased competition, mothers can produce offspring with more resistant, dispersive, and/or competitive phenotypes (Marshall & Uller 2007). Parental effects can be particularly effective during prenatal development when the embryo is particularly sensitive for environmental influences, and during which time the fate of important phenotypic traits, such as formation of sex-specific gonads and neural circuitries, can be irreversibly affected. Mothers, in particular, can induce such (maternal) effects during prenatal development, leading to long-lasting stable modifications in offspring phenotype (Mousseau & Fox 1998).

Although generally thought to be adaptive, parental effects can give rise to parent-offspring conflict (Godfray 1995; Trivers 1974) when the fitness outcome of these effects is suboptimal for the parent or the offspring or both (Marshall & Uller 2007). For example, as insulin hormone reduces blood glucose levels, in order to obtain more of maternal glucose the fetus can induce insulin resistance in the mother, which can have severe health consequences for the mother (Haig 1993). Likewise, to have increased flow of maternal resources to the fetus it can induce constriction of maternal blood vessels, which the mother can counteract by vasodilation that can put unnecessary strain on the mother's heart (see review by (Del Giudice 2012)).

In almost all vertebrate taxa (e.g. fish (Brown et al. 2014; Guiguen et al. 2010; Pri-Tal et al. 2011), reptiles (Clairardin et al. 2013; Paitz & Bowden 2011), birds (Gil 2008; Schwabl 1993; von Engelhardt & Groothuis 2011), mammals (Del Giudice 2012; Drea 2011; Harris & Seckl 2011)), developing embryos are exposed to varying amounts of maternal hormones. The effects of this variation in prenatal hormone exposure on development and behaviour is a hot topic of research. Especially steroid hormones, and in particular the androgen testosterone (T) have received much attention over the last decades.

2 HORMONE MEDIATED MATERNAL EFFECTS: AVIAN MODEL

2.1 Role of steroid hormones in maternal effects

Steroid hormones in vertebrates can have a profound influence on phenotypic development. Early exposure to these hormones can result in long-lasting organizing effects on sexual differentiation of gonads, brain and behaviour, leading to individual variation both within same sex and between opposite sex individuals (Cooke et al. 1998; Rhen & Crews 2002). Steroids can have multiple effects on phenotypic development by their action on multiple targets (developmental pleiotropy), and therefore they can provide a proximate basis for trade-offs, i.e. beneficial change in one trait that is causally linked to detrimental change in another trait (Ketterson et al. 1992). For example, steroid hormones when injected in freshly laid bird eggs were found to be correlated with a number of both early and long-term organizational effects on the offspring's phenotype (reviewed by (von Engelhardt & Groothuis 2011)) such as hatching time, hatching success, metabolic rate, immune function, endocrine function, growth, competitiveness, reproduction, mate choice, and survival rate.

The net outcome of increased hormone exposure is thus likely to depend on a complex trade-off between effects on several traits. Hence, steroid hormone actions may provide a bridge between proximate and ultimate approaches to study effects of prenatal exposure to maternal hormones, as physiological and behavioural targets of hormone actions can be studied together with their ecological functions. Moreover, as hormone levels in mothers can be systematically adjusted to the current environment (described below), they offer an excellent pathway to communicate the environment of the mother to the offspring. Hence, steroid actions may serve as a potential mechanism for maternal effects.

2.2 The avian model

Maternal effects are already well established in vertebrates (Mousseau & Fox 1998). Egg-laying (oviparous) vertebrates have the following advantages to study hormone mediated maternal effects: (i) the development of the embryo occurs outside the body of the mother, i.e. inside the egg. Therefore, it enables relatively easy experimental manipulations without interfering with the mother, as well as adequate measurements; (ii) after egg-laying the mother cannot further influence the offspring prenatal development through hormonal provisioning, which occurs during a relatively small time-window of typically less than a week. This makes it possible to map environmental factors to maternal condition and hormone deposition, which facilitates establishing a direct link between maternal hormone provisioning and offspring phenotype development.

Among the oviparous vertebrates, birds are in particular an attractive model for studying hormone mediated maternal effects because (i) birds produce relatively large eggs, making it possible to study maternal effects for individual embryos, and (ii) bird ecology is often well-known and can be studied in the field, facilitating studies of the adaptive significance of maternal effects.

2.3 Maternal steroids in bird eggs

Hubert Schwabl discovered the presence of a substantial amount of steroid hormones – T, androstenedione (A4), dihydrotestosterone (DHT), estradiol (E2), and corticosterone (CORT) in avian eggs (Schwabl 1993). As unfertilized eggs also contain steroids, these hormones are certainly of maternal origin and are hence referred as maternal steroids. Since then, maternal steroids have been measured in eggs of a variety of bird species, and found to show systematic within-clutch, between-clutch, and between-species variations. For example, a systematic increase over egg laying order in yolk T concentrations in canary (Schwabl 1993), and black-headed gull (Eising et al. 2001) was found, but a systematic decrease over egg laying order in yolk DHT in zebra finch (Schwabl 1997a) (Fig. 1).

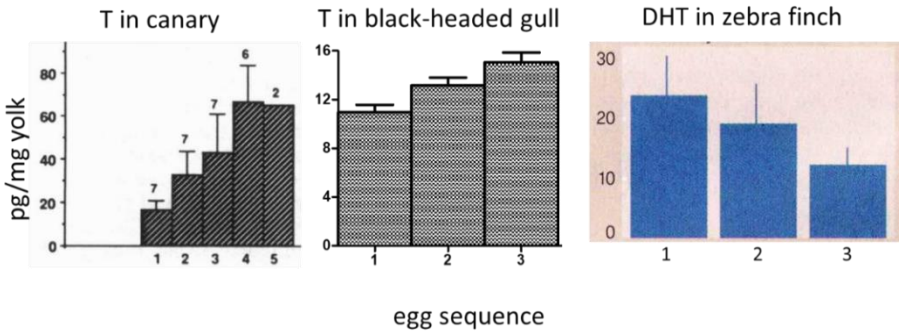

Figure 1. Examples of systematic variation in yolk hormone deposition in relation to the laying order in bird eggs (graphs are adapted from the references cited in the text).

The maternal steroid levels were also found to vary with the mother's environment (Gil 2008; Hahn 2011; von Engelhardt & Groothuis 2011; Welty et al. 2012). For example nest density, intrusions at nest sites and colony size (Gil et al. 2006b; Groothuis & Schwabl 2002; Mazuc et al. 2003; Pilz & Smith 2004; Reed & Vleck 2001; Schwabl 1997b; Whittingham & Schwabl 2002); mother's social rank (Müller et al. 2002); male quality (Dentressangle et al. 2008; Gil et al. 1999, 2004, 2006b; Gwinner & Schwabl 2005; Kingma et al. 2009; Loyau et

al. 2007; Michl et al. 2005; Tanvez et al. 2004); food (Gasparini et al. 2007; Verboven et al. 2003); and parasite prevalence (Gil et al. 2006a; Tschirren et al. 2004). Together with the above mentioned results of the hormone injection experiments in ovo, this indicates the correlational functional consequences of increased exposure of the embryo to maternal steroids, and provides a basis for the hypothesis of maternal hormone actions as a potential mechanism for maternal effects (reviewed by (Groothuis et al. 2005b)) (see Fig. 2).

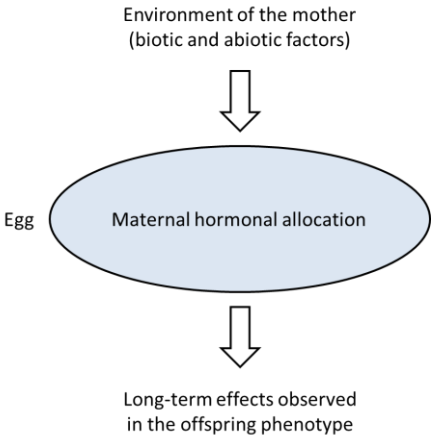

Figure 2. The most commonly used framework for studying hormone mediated maternal effects in birds that only shows correlations between maternal hormonal allocation (often mimicked by experimentally injecting hormone into the egg) and offspring phenotype. However, it does not explain what the underlying mechanisms are and neglects the role of the embryo in this process (as described in section 3), which are the key challenges in this field of research.

3 KEY CHALLENGES IN THE RESEARCH FIELD (THESIS OVERVIEW)

3.1 Maternal allocation

A large number of studies have focussed on the factors affecting maternal hormonal allocation, but the following questions still need to be addressed, as their answers may affect the interpretation of hormone mediated maternal effects.

3.1.1 Are radioimmunoassay-based hormone measurements in eggs reliable? (chapter 2)

In studies injecting hormones in the egg, the dose of hormone manipulation is calculated based on the levels in the egg that are mostly measured using radioimmunoassays (RIA's). The kits and antibodies used in RIA's are not standardized for measuring steroids in eggs. This can lead to unreliable hormone measurements with much exaggerated estimates due to antibody cross-reactivity with uncharacterized substances in the egg (i.e. matrix effect, where 'matrix' represents a particular tissue of a particular species). Even calibrating a RIA kit using steroid stripped egg matrix, such as by charcoal treatment, does not really solve the issue. This is because use of charcoal or any other such substance could potentially not only remove the hormone but also part of the matrix itself, so that the validation of the kit is then performed in a changed matrix that cannot be translated to the original matrix. Furthermore, it should also be noted that although RIA kits for measuring a target hormone may contain in the kit brochure the cross-reactivity values with non-target compounds, those cross-reactivity values would only be applicable for the same matrix for which the kit was optimized and not for the egg matrix. Additionally, even if the cross-reactivity were to be the same in the egg matrix, it would still be virtually impossible to correct for cross-reactivity in order to accurately calculate the levels of the target compound due to unknown amounts of the cross-reacting substances in the egg matrix (Quillfeldt et al. 2011; Rettenbacher et al. 2009).

Tandem mass spectrometry in combination with liquid chromatography (LC-MS/MS) on the other hand is a much more reliable method as discussed extensively in the literature (Shackleton 2010; Taylor et al. 2015; Wudy et al. 2018). Instead of using antibodies, mass spectrometry is based on detecting mass to charge ratio of fragmented ions of a target compound. This method interpolates the concentrations of a target steroid from a calibration curve prepared using the ratio of a known amount of an added stable isotope labelled analog as an internal standard to the unlabelled target compound. As the labelled internal standard is structurally and chemically nearly identical to the unlabelled target steroid, and is added before starting the procedures for hormone extractions, it corrects for matrix effects, and accounts for recovery losses during the sample preparation procedures and for possible ion suppression in the mass spectrometer.

In **chapter 2** we tested whether RIA's measured higher T concentration compared to LC-MS/MS when the same egg yolk samples were measured by both methods. We further tested whether the difference between the two methods depends on the species (between-species effect), the type of matrix (between-matrix effect such as egg yolk and blood plasma), and the egg laying order (within-species effect on eggs from the same mother which differ in their laying order in the clutch, as the egg composition, and hence the matrix effect, might change per laying order).

The reason that we compared the two methods was because if RIA's would give exaggerated values, this would question the reliability of previous hormone injection studies, as they would be using doses far outside the physiological range and thereby not necessarily reflecting biologically relevant effects. Further, if the extent to which RIA's would give exaggerated measures would depend on the species and egg laying order, that would further indicate a possible misinterpretation of maternal allocation, embryonic modification of maternal hormones, as well as between-species comparisons (meta-analyses) when using RIA's.

3.1.2 Do steroid levels in freshly laid eggs truly reflect maternal allocation? (chapter 3)

The systematic variation in yolk hormone levels measured in freshly laid eggs (i.e. at oviposition) is assumed to be due to differential maternal hormone deposition depending on a certain context, for instance the laying order of eggs, mate quality, social hierarchy of the female in groups, seasonal variation in predation risk, food availability, parasite prevalent, etc. However, the yolk formation and hormone deposition in the yolk is finished by the time of ovulation as no more yolk can be added to the egg after ovulation, which typically occurs one or more days prior to egg laying. It has been shown that yolk hormone levels decline strongly already early during incubation of laid eggs (Eising et al. 2003; Elf & Fivizzani 2002; Paitz et al. 2011; Wilson & McNabb 1997). If the hormone levels also change already between the times of completion of yolk deposition and egg laying (while the egg is still inside the mother's reproductive tract), the levels measured at oviposition might not reflect the actual maternal allocation.

In **chapter 3** we addressed the question: do hormone levels in bird eggs measured at oviposition truly reflect maternal allocation in the yolk deposited in mature follicles, both in terms of absolute levels as well as relative differences between eggs of different laying order. To this end, we used eggs of the rock pigeon (*Columba livia*). Rock pigeons lay two eggs per clutch, and at oviposition the second eggs contain systematically higher levels of yolk androgens compared to the first eggs (Hsu et al. 2016). This allowed us to test whether the relative differences in eggs based on the laying order remain the same at ovulation as at oviposition, the latter being the most commonly used time point for estimating maternal hormone allocation.

If the hormone levels at oviposition do not reflect maternal allocation, including the relative difference between eggs that differ in laying order, this would indicate that the estimates of egg injection dose from oviposition are inadequate. This would have important consequences for the interpretation of both the results of such experiments and their ecological and evolutionary interpretations. This could also potentially explain the discrepancy in the outcomes of hormone injection experiments (that some studies find a

positive effect on a certain trait while others find no effect or even a negative effect) as the dose of hormone treatment might not reflect maternal allocation. Further, if laying order based relative differences in hormone metabolism between ovulation and oviposition were due to embryonic activity, this would open up new perspectives on hormone mediated family conflict already in this early phase of embryonic development, highlighting the importance to address questions such as whether this early hormone metabolism is maternal or embryonic. We partly addressed the latter question by experimentally testing whether maternal enzymes in the yolk were responsible for the very early hormone conversion. We did this by comparing hormone levels in follicular yolks incubated with or without proteinase-k treatment, an enzyme that degrades maternal enzymes in the yolk. The two treatment groups would differ in hormone levels after incubation only if maternal enzymes in the yolk were responsible for the very early hormone conversion.

3.2 Role of the embryo

Most studies on hormone mediated maternal effects use the framework that assumes a passive role of the embryo (Fig. 2). But recent developments in the field, mostly through some pioneering studies by Bowden and colleagues (Paitz & Bowden 2008, 2013; Paitz & Casto 2012; Paitz et al. 2011; Vassallo et al. 2014; von Engelhardt et al. 2009), point towards the importance to also focus on the embryo's perspective. That is, the role of the embryo in translating maternal hormonal allocation to their phenotypic effects, or in circumventing such effects when they are unfavourable for the embryo, including underlying mechanisms, such as embryonic uptake and/or metabolism, as well as its adaptation to maternal hormone levels in the yolk. This alternative framework is depicted in Fig. 3, and is discussed in detail in the following sections, where we deal with three important questions.

3.2.1 Are maternal androgens taken up by the embryo from the yolk in their original form? (chapter 4)

In order to be functional, maternal egg hormones must reach the embryonic tissues. However, the embryo can produce steroids itself and therefore a target steroid found in embryonic tissue might not be of maternal origin. In order to distinguish between steroids of maternal and embryonic origin, radioactive steroids have been injected into bird egg yolks, and the radioactivity was found in the embryonic tissues (Benowitz-Fredericks & Hodge 2013; von Engelhardt et al. 2009). However, these studies did not verify the molecular identity of the radiolabelled compound in the embryonic tissues. The original hormones could have been metabolized by embryonic enzymes before being taken up by the embryo, as suggested by some pioneering studies (Paitz & Casto 2012, Paitz et al. 2011, Vassallo et al. 2014) and in **chapter 5** of this thesis.

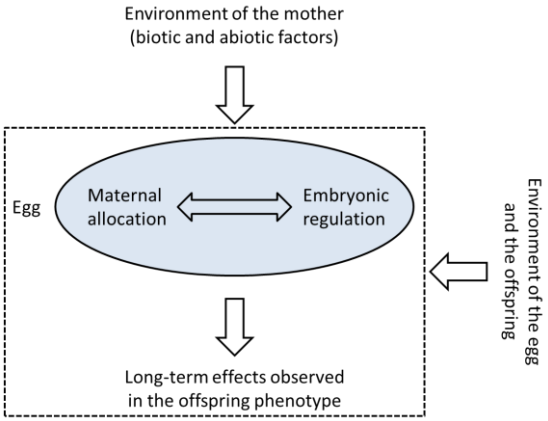

Figure 3. A comprehensive framework to study hormone mediated maternal effects, which not only takes into account the mother's but also the embryo's perspective (see Fig. 2 for a comparison), as well as the environment of the egg and the offspring. This includes an active role of the embryo in dealing with the maternal hormonal signals in (1) proximate manner: uptake and utilization, or degradation, of the maternal hormones at time, location, and amount as needed for the embryo itself; (2) ultimate manner: context-dependency of the role of the embryo, such as other environmental cues in the egg composition, trade-offs between the advantageous and detrimental effects of prenatal exposure to maternal hormones, family conflicts, etc.

In **chapter 4** we tested whether the injected stable isotope labelled T and A4, two prominent maternal androgens in the yolk, were taken up by the embryo. In contrast to the studies using radiolabelled steroids, we verified the molecular identity of the injected stable isotope labelled compounds and differentiated them from their metabolites using mass spectrometry, a method that can distinguish between naturally occurring and heavy isotope labelled compounds that differ only with respect to their mass.

If maternal androgens do not reach embryonic tissues but only their metabolites, this would suggest that the effects of increased exposure to maternal hormones are either mediated by the metabolites (but most of them are generally thought to be much less potent), or the effects take place very early (before the maternal steroids are metabolized by the embryo), or perhaps both take place in a temporally regulated manner (early effects by the hormones and late effects by their metabolites).

3.2.2 Are maternal gonadal steroids metabolized by embryos differentially depending on egg laying order? (chapter 5)

A few pioneering studies indicate metabolism of maternal yolk steroids by the embryo by conjugation, using conjugating enzymes such as sulphotransferase and glucuronosyltransferase (Paitz & Casto 2012; Paitz et al. 2011; Vassallo et al. 2014; von Engelhardt et al. 2009). By conjugating, the embryo can convert biologically active maternal hormones into their inactive forms, as conjugated steroids might not bind to steroid receptors; whereas embryonic de-conjugation, using enzymes such as sulphatase and glucuronidase, can convert them back to active forms. This way, the embryo can have an active control over when, and to what extent, to use maternal hormones. As suggested earlier by Paitz and Bowden (2008, 2013) and von Engelhardt (2009), this opens the possibility that embryos of oviparous species have in fact active control over their endocrine environment as in mammalian species (Cottrell & Seckl 2009) (reviewed by (Braun et al. 2013; Del Giudice 2012)), which would be favoured by natural selection (Del Giudice 2012; Mock & Forbes 1994; Müller et al. 2007; Wilson et al. 2005; Winkler 1993).

However, the detailed scope for such role of the embryo in translating maternal gonadal hormones is not well understood, especially in bird species, the most widely used model in the field. This includes the overall metabolic outcomes of the detailed steroid metabolic pathways (Fig. 4), such as conversion of less potent metabolites to more potent ones or vice-versa, the quantitative dynamics of embryonic metabolism (e.g. where, when, and to what extent conjugation/de-conjugation takes place), metabolic differences based on embryo's laying order in the clutch, and hormone uptake and utilization by the embryo.

The main aims of **chapter 5** were to compare the metabolic profile of incubated fertilized and unfertilized eggs to discern the maternal and embryonic contribution to the steroid metabolism; and to compare the metabolic outcomes of maternal steroid hormones between fertilized eggs of first and last laying order, which would indicate scope for differential embryonic activity based on egg laying order. Here also we took advantage of the rock pigeon, in which the first and second embryos of a clutch are exposed to different levels of maternal androgens (Hsu et al. 2016). This provides an appropriate natural context to test whether the embryos of first and second eggs can utilize or metabolize maternal hormonal signals differently, as the initial hormone differences between different eggs of a clutch were found to be correlated with the chick development and behaviour (Eising et al. 2001). We analysed a wide spectrum of hormone profiles and their metabolites (Fig. 4) in order to map the androgen and estrogen pathways of metabolism, including conjugated compounds.

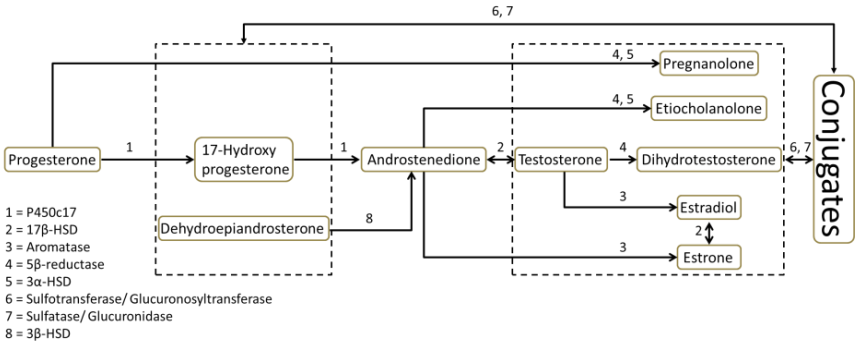

Figure 4. The gonadal steroid metabolic pathways of the analysed compounds including 10 free and 8 conjugated forms. Only the compounds within the dashed boxes can be conjugated. Numbers represent the enlisted enzymes involved in the pathways with the following abbreviations – P450c17: steroid 17 alpha-hydroxylase/17,20 lyase; HSD: hydroxysteroid dehydrogenase.

We analysed the metabolic outcomes over the first 4.5 days of incubation, as this is the timeframe before the embryo's gonadal differentiation and start of its endogenous hormone production (Andrews et al. 1997; Woods et al. 1975; Yoshida et al. 1996).

If embryos metabolize maternal hormones differently based on their laying order, this would indicate that the embryo is able to adjust its metabolic capacity according to the maternal signal, as maternal hormone deposition differs over the laying order. That would also suggest that embryos may play their own role in the hormone mediated family conflict.

3.2.3 Does the embryo expresses steroid receptors in its extra-embryonic membranes and regulates their levels in response to yolk steroid levels? (chapter 6)

The mechanisms underlying hormone mediated maternal effects in birds are largely ignored, hampering further progress in this active field of research (Groothuis & Schwabl 2008). A large number of studies have injected androgens into the egg yolk. In order to be functional, injected androgens must reach the embryonic tissues and those tissues must have androgen receptors (AR). In **chapters 4 and 5** we showed that very early in incubation yolk androgens were substantially and rapidly metabolized to supposedly biologically inactive forms instead of being taken up by the embryo. Moreover, steroids are lipophilic, and how the embryo is able to take up these hormones in its circulation to exert their effects on body tissues remains an enigma. So how do maternal hormones in the yolk exert any effect on the offspring?

In **chapter 6** we tested the hypothesis that the embryo expresses androgen (AR) and/or estrogen (ER) receptors in its extra-embryonic membranes (EMs). The EMs are at the

immediate interface of the maternal egg yolk containing the maternal hormones and the circulation of developing embryo, making them a potential structure for mediating effects of maternal hormones. We also tested whether the egg androgen treatment induced changes in AR and/or ER expression levels in embryonic tissues, including the EMs, which would reflect embryonic adaptation to maternal yolk hormone levels.

If the embryo expresses steroid receptors in the EMs very early, and if maternal steroids in the yolk can bind to those receptors, this could potentially explain how maternal steroids exert their effects even if they do not reach embryonic body tissues (see **chapter 4**) but are instead substantially metabolized very early (see **chapter 5**). This would also solve another long standing puzzle of how maternal gonadal steroids can exert effects without affecting embryonic sexual differentiation (Carere & Balthazart 2007). Additionally, if the embryo can adjust its receptor expression depending on yolk steroid levels, that would further indicate an active role of the embryo in translating maternal steroids to their effects.

In summary, we highlight and address a number of challenges in studying hormone mediated maternal effects, with a specific emphasis on the role of the embryo in these processes. These concern – reliable measurements of egg steroids (**chapter 2**), correct determination of maternal hormonal allocation (**chapter 3**), a potential lack of embryonic uptake of maternal yolk androgens in their original form (**chapter 4**), differential embryonic metabolism of maternal hormones (**chapter 5**), and a potential mechanism of action of maternal steroids via steroid receptors and their regulation in the EMs (**chapter 6**). In **chapter 7** we present a synthesis by summarising the key findings, drawing conclusions, and outlining future perspectives.