Investigating Maternal Hormones in Avian Eggs: Measurement, Manipulation, and Interpretation

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ABSTRACT: The last decade has witnessed a surge in studies on steroid hormones of maternal origin present in avian eggs and affecting offspring development. The value of such studies for the understanding of maternal effects and individual differentiation is endorsed and a series of methodological and conceptual issues in the current approaches is discussed. First to be addressed is the topic of correct sampling of eggs or yolk for hormone analyses. Changes in yolk hormone levels during the incubation period and the uneven distribution of hormones within the egg are discussed. Different ways of calculating hormone levels and the importance of collecting data for specific a priori hypotheses are explained. Next to be discussed are the pros and cons of different techniques for manipulating yolk hormone levels and their proper scaling to naturally occurring levels. Third, several issues hampering the interpretation of results from descriptive and experimental studies are addressed. These concern biased embryonic mortality, clutch size, and egg quality that may confound the interpretation of the effect of egg position in the laying order, and the possibility of sex-specific effects and long-term effects. Also discussed are the probability of context-dependent results (due to, e.g., other egg components affecting egg quality, parental quality, and environmental factors), the difficulty in demonstrating adaptive effects due to individual optimization, and the lack of insight in the underlying physiological processes. Finally, it is concluded that this field has shown much progress but that it would profit from a more careful consideration of methodology and from a better integration of behavioral ecology and endocrinology.

KEYWORDS: maternal effects; maternal hormones; steroids; androgens; development; birds; egg quality, nongenomic inheritance

INTRODUCTION

The past decade has witnessed a surge in studies on patterns of maternal steroid hormone deposition and especially their effects on the offspring in avian species. Since the milestone paper by Hubert Schwabl,\(^1\) which showed that substantial amounts of androgens are present in eggs of the canary and the zebra finch, androgens, estrogen, and corticosterone have been found in all avian eggs that have been analyzed for these hormones, from a number of both precocial and altricial species.\(^2\)

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These hormones are of maternal origin, since they are present in freshly laid eggs, long before the embryo would be able to produce hormones itself, and even in unfertilized eggs. Several studies have shown that the concentrations of these hormones, especially androgens, vary systematically with the position of the egg in the clutch and with several environmental factors such as social competition, food availability, mate attractiveness, laying date or photoperiod, and maternal parasite load (reviewed in Ref. 2). Moreover, a rapidly increasing number of studies demonstrated the wide array of the effects of these maternal hormones on the offspring, such as on hatching time, early muscular growth, early postnatal growth of body mass and structural size, early begging and competitive behavior, and prefledging survival. In addition, evidence is beginning to emerge that prenatal exposure to maternal androgens exerts long-lasting effects on morphology and behavior. 2

These findings are of general importance. They demonstrate that parents can influence the development of their offspring not only by genetic inheritance but also by nongenetic inheritance. The advantage of the latter is that it can provide the parent with a relatively flexible tool to adjust offspring development to prevailing environmental conditions. Hormones are powerful tools for such a parental—or maternal—effect. Early exposure to steroid hormones can have a wide array of important organizing 3 and activating effects, 4 influencing not only sexual differentiation 3 but also the differentiation within the same sex, 5 perhaps leading to different personalities. 6 Furthermore, the production of steroid hormones such as androgens and glucocorticoids is strongly influenced by environmental factors, 7,8 and therefore offers an excellent pathway for the translation of these environmental factors, as perceived by the mother, to her offspring. Prenatal exposure to maternal hormones, such as androgens and glucocorticoids, has now been documented in several taxa of vertebrates other than birds (e.g., fish, 9 lizards, 10 turtles, 11 and mammals, 8,13 including humans 13). Thus, the study of maternal hormones and their effects provides an excellent possibility to study general principles of mechanism, function, and evolution of nongenomic maternal effects.

Birds are especially adequate for such studies. The avian embryo develops in a sealed environment, the egg, outside the mother’s body. Furthermore, avian eggs are relatively large, facilitating the sampling and manipulation of egg hormones without the interference of the mother. In addition, most avian species are studied under field conditions, facilitating the analysis of the function of maternal hormone deposition under natural circumstances. This has most likely stimulated the recent surge in studies of avian egg hormones. The time has now come to critically evaluate these studies, and this is the aim of two complementary contributions to this issue: one focusing on the methodology of the measurement of egg hormones, 14 and this paper, which focuses on conceptual issues and problems with the biological interpretation of the data.

**COLLECTING DATA: WHERE, WHEN, AND WHAT?**

*Where and When*

By far, most studies have measured egg steroid hormones in the yolk of the egg. These hormones are not evenly distributed within the yolk of freshly laid eggs but differ in their concentration among the concentric layers of the yolk that reflect the
The circadian pattern of yolk deposition. This may have consequences for the interpretation of hormone levels in yolk samples. However, most studies use samples of homogenized whole yolks. Because it seems that after a few days of incubation the structure of yolk layers disappears (own observation), and injection of a lipophilic substance revealed substantial diffusion of this substance over the whole yolk after a few days, the functional significance of the difference in hormone levels among yolk layers may be absent. However, this distribution should be investigated in more detail by injecting labeled hormones into the egg and determining after a few days the recovery from several locations.

Such experiments should also check the possibility that hormones, deposited in the yolk during egg formation, diffuse to other parts of the egg in the course of incubation. If so, the analysis of hormone concentrations of the whole egg may be a more accurate measurement of embryonic exposure to these hormones than the analysis of the yolk only. Such diffusion may explain why yolk concentrations of steroid hormones substantially decrease after 1 day of incubation. This decrease may lead to substantial bias in reported yolk hormone levels when timing of incubation is not carefully controlled for.

In addition, in the course of development embryos start to produce steroid hormones themselves in a sex-dependent manner. Substantial amounts are produced in, or after, the second week of incubation, and this may explain the increase in egg steroid hormone levels at the end of the incubation period. In addition, lower levels may be present already in the first week because enzymes for the production of androgens and estrogens have been detected 2 days and 5 days, respectively, after the onset of incubation. This may lead to an overestimation of levels of maternal hormones. However, data of embryonic steroid production in birds come from only a limited number of species, and to what extent and by what time in development the endogenously produced hormones reach the yolk is as yet unclear. Another possibility to consider is that the enzymes for hormone synthesis, being present early in development, may also convert the maternal hormones to other active or inactive metabolites. This may perhaps explain the function of the presence of large amounts of androstenedione in the egg, a precursor for other androgens that are more biologically active. Therefore, as long as we lack sufficient knowledge of the diffusion, production, and conversion of yolk steroid, eggs should be analyzed for the presence of maternal hormones as early in the incubation period as possible, ideally freshly laid. This would also avoid the potential problem that the mother deposit enzymes in the yolk that may metabolize the hormones in the period after the onset of incubation, a possibility so far not studied but that requires more attention.

What

Bird eggs contain several steroid hormones, such as testosterone, androstenedione, dihydrotosterone, estradiol, and corticosterone. They originate either from the mother or from the embryonic gonads. Although the effects of the hormones produced by the embryo itself on sexual differentiation and on the adult phenotype are well known, information on the different effects of these hormones when of maternal origin is still very incomplete. There is a general tendency to focus on maternal testosterone. This is understandable since androstenedione is by itself biologically not very active, and testosterone can be converted to both 5-α-
dihydrotestosterone and estradiol, all three being potent hormones. However, androstenedione is an important prohormone that can be converted to both testosterone and estradiol. Levels of maternal androstenedione are usually high compared with those of other hormones, so androstenedione may constitute an important source for the embryo to produce testosterone and estradiol (T and E). Experiments manipulating specifically A4 levels and assessing yolk and embryonic plasma levels of T and E are urgently needed to test this hypothesis. Corticosterone and estradiol are present in the egg in much lower levels than androgens and have received much less attention. The low levels of corticosterone and estradiol may be a protection against detrimental effects of these hormones on the vulnerable embryo, since persistent exposure to elevated levels of corticosterone is well known to be detrimental, and estrogens affect sexual differentiation in birds. Nevertheless, the small amounts of these hormones may still be biologically relevant for chick development. Recently, yolk corticosterone has received more attention, and the results suggest that it may have important effects on the offspring.

In view of the presence of several steroid hormones in avian eggs, some studies have measured more than one hormone, especially all three androgens. However, without specific a priori hypotheses about the expected patterns of deposition or their effects on the chick, this has the serious risk of a positive bias in results, since multiple analyses increase the chance of yielding statistically significant findings, resulting in type II errors. Clearly, it is important to gain more knowledge about the specific effects of the different hormones by conducting a series of experiments to compare the effects of injections of the different hormones, separately and in combination, in eggs of the same species. On the basis of the question, these hormones should be injected in the similar concentrations (to compare the effectiveness of the hormones) or in the levels that occur in unmanipulated eggs (to study the effect of different maternal hormones as deposited by the mother).

Most studies present the hormone levels as the amount of hormone per milligram or milliliter of yolk. Other studies use the total amount of hormone in the whole yolk. This method requires the determination of total yolk weight for each egg. Because of the influx of water soon after incubation, scaling to the start of incubation is important here. In blood plasma, the concentration of the hormone is generally the most appropriate parameter, but this is not necessarily the case for the study of yolk hormones. Yolk mass varies between and within clutches and may not always be associated with the body mass of the newly hatched chick. In such a case, the amount of hormone per unit of yolk may not be an adequate estimate of how much hormone reaches the embryo per unit body mass. Scaling the amount of hormone to total egg weight may not be a solution either, since weight differences between eggs may be due to the shell or to water content that do not contribute to the body mass of the chick.

In addition to yolk weight, other factors may influence hormone deposition, and their measurement and inclusion in the analysis can greatly enhance our understanding of the patterns in yolk hormone levels. First, the pattern of hormone deposition over the laying sequence as well as total levels of a certain hormone in a clutch can be substantially influenced by environmental or parental parameters (reviewed in Ref. 2). As a consequence, one study of a single population in a particular situation will provide a limited basis for the characterization of yolk hormone levels in that species if no additional contextual information is given. Furthermore, if the pattern
across the laying sequence is indeed flexible, then one egg of a particular position in a clutch would not sufficiently characterize hormone deposition in that clutch. Second, such environmental or maternal parameters may mask interesting patterns. For example, we found in great tits (Parus major) that there was on average no pattern in hormone concentration over the laying sequence. However, when we analyzed the data in relation to the results of artificial selection on exploratory behavior, we found that one selection line showed a consistent increase, and the other a consistent decrease in androgen deposition over the laying sequence. 24

Finally, evidence is beginning to emerge that levels of yolk hormone differ between eggs of male and female embryos, 25–28 which can become a confounder if avian mothers would be able to manipulate the primary sex ratio of their eggs. This problem can be solved by molecular sexing but would require a few days of incubation to allow sufficient embryonic growth for sampling DNA. Because incubation may affect yolk hormone levels (see earlier), it would be necessary to determine also the change of hormone levels across incubation time. Recent data suggest that after an initial decrease in hormone levels (24 h of incubation), 15, 19 but see Ref. 20, these levels remain relatively stable for at least a few days. The importance of sex as a confounding variable is as yet unclear, since at the moment the evidence for substantial maternal control of the primary sex ratio in birds is ambiguous. 29

How

The measurement of hormones in the egg; their extraction and separation; and the accuracy, specificity, precision, and sensitivity of the assay sometimes need more validation than provided, and we discuss this in a separate article in this issue. 14

MANIPULATION OF EGG HORMONE LEVELS

To study the effects of yolk hormones, it is essential that the levels of these hormones be manipulated independently of other aspects of the egg and maternal quality. This is because yolk hormone levels may be associated with other aspects of egg quality, such as egg mass, 30 other yolk substances, such as carotenoids, 31 and aspects of maternal quality. 32 Thus, associations between unmanipulated levels of egg hormones with offspring performance can reveal only inconclusive results with respect to the effects of yolk hormones. Manipulating yolk hormone levels by manipulation of the mother (by modification of environmental conditions or by pharmacological tools) is inadequate, since it is likely to affect other aspects of egg quality and/or maternal quality as well, again confounding the results.

There are several techniques for treating avian eggs with hormones. An easy technique is dipping eggs in a solution containing the hormone, which then diffuses through the eggshell into the egg. 33, 34 This has, however, the disadvantage that the effective dose that reaches the embryo, or at least the yolk and albumen, is unknown unless the effectiveness of the treatment is determined by assaying eggs before and after treatment or by studying the fate of a labeled hormone dissolved in the solution. Therefore, injection of known amounts into the egg appears to be more appropriate. This can be done in the air chamber, in the albumen, or in the yolk itself. In all cases, a known amount of hormone is dissolved in a solvent such as sesame oil. The first
option may have the advantage that there is a relatively small chance that infections will affect the embryo in an early and vulnerable stage, before the bill penetrates the membrane of the air chamber. However, we (T.G.) found a relatively low hatching success with this technique, which may have been due to the oil that attaches to the membrane separating the air chamber from the rest of the egg, hampering air exchange. Furthermore, also in this case the effective dose is unknown. Therefore, the best option is injection of known amounts of hormone into the yolk, which is actually the location where the mother deposits the hormones. The distribution of the hormone after injection needs to be established and may depend on the solvent used. If the hormone does not dissolve in the yolk and is taken up in a high dose in a brief period of time, the injection of a natural amount of hormone may result in a pharmacological dosage.

Three issues need to be considered for all these techniques. First, it is essential that the dose used be scaled to the endogenous levels normally occurring in the species studied. In doing so, one must realize that the injected dose is added to the endogenous level already present. Unfortunately, such scaling is not always carried out. To complicate matters, it may be that levels of yolk hormones sampled in the year or population to which the injection is scaled are different from those in the year or population in which the experiment is actually carried out. A combination of biopsies and injection of the same egg would be ideal in this case.

Second, because injected levels are added to those already present, the resulting yolk level of the hormone will almost inevitably be at the upper level of the natural range. However, the hormone may have dose-dependent effects that are as of now hardly studied. The only study we are aware of that applied different doses of androgens in the same population of eggs \(^{18}\) found dose-dependent effects on growth and immune function of the chick, suggesting that this issue deserves much more attention.

Third, the injection technique lowers hatchability of the egg, although this can in our experience improve considerably with practice. Although no difference in hatching success has yet been reported between eggs treated with the hormone in solvent and eggs injected with solvent only, a low hatching success may still be cause for some concern, not only for ethical reasons and statistical power but also for another reason: Using only well-hatched chicks may select for chicks of high quality. If the effect of the hormone depends on the quality of the chicks, or when there is a ceiling effect in measuring aspects of chick quality, such selection may bias the results to smaller or larger differences between experimental and control chicks.

In behavioral endocrinology, a standard approach to test the effect of a hormone is not only to enhance but also to lower exposure to the hormone. This result is usually achieved by using a receptor agonist or antagonist or by blocking the action of a specific enzyme needed for the production of the hormone. Unfortunately, we have as yet no tool to lower embryonic exposure to maternal hormones specifically. Lowering hormone deposition by affecting the egg-laying female can easily lead to confounding variables (see earlier). Injection of flutamide, a blocker of androgen receptors, in the egg has been applied \(^ {35,36}\) but the half-life of this blocker in the egg and the timing of its uptake by the embryo is unknown. Therefore, it may block not only the effect of maternal androgens but also the effect of androgens produced by the embryo itself. To complicate this matter even further, it is conceivable that maternal androgens exert their effects very early in development, before the classic an-
drogen receptors are present, by nongenomic mechanisms. In that case a classic receptor blocker would be of no use. Experiments in which the development of receptors is determined and in which androgen injections are conducted at different stages of development should provide the necessary information on this matter.

**PROBLEMS OF INTERPRETATION**

Apart from the problems of biased survival and uncontrolled covariates that have been mentioned previously, there are several other problems and pitfalls with the interpretation of results from descriptive and experimental studies. First, recent evidence indicates that yolk hormones may exert sex-specific effects on the offspring,\(^2,37\) which requires sex-specific analysis of the data. For example, in a study on American kestrels it was found that experimentally elevated androgen levels in the first egg exerted detrimental effects on the chicks, whereas normally all eggs in clutches of this species have such elevated levels except for the first egg.\(^38\)

The European kestrel is a classic example of a species showing primary sex ratio adjustment,\(^39\) and it may be that the sex that is most vulnerable for the effect of elevated androgen levels was overproduced in the first egg.

Second, the effects of a specific hormone may depend on synergistic effects with other hormones or on other substances present in the egg. For example, maternal androgens may lower immune function of the chick,\(^18,28,40\) but this effect may be counteracted by elevated levels of carotenoids enhancing immune function.\(^41\) Therefore, the effect of yolk hormone manipulation may differ between species, populations, clutches, or even within clutches of the same female in case eggs differ in composition of other substances. For example, to study the effect of elevated androgen levels in last-laid eggs of black-headed gulls (*Larus ridibundus*), we used first-laid eggs of a clutch, which contain relatively low levels of androgens, and elevated their levels to those of last-laid eggs.\(^42\) For our protocol, last-laid eggs could not be used because they contain high levels of androgens, and an additional elevation might have resulted in a supraphysiological level. We found results in the expected direction, but the effect of the induced androgen level in first-laid eggs may be different from similar levels in last-laid eggs due to the influence of other egg components that differ in concentration between these eggs.\(^28\) This possibility may also explain the paradoxical effect found in the study on American kestrels, discussed previously.

This point relates to the important possibility of individual optimization in maternal hormone deposition that may severely hamper the interpretation of experimental results. Yolk hormones such as androgens can have not only beneficial but also detrimental effects on the chick (such as immune suppression\(^18,28,40\)), and the production of the hormone may lead to elevated and detrimental levels in the mother, too (lowering, e.g., clutch size, reviewed in Ref. 2). Therefore, androgen deposition may be adjusted to the deposition of other egg substances and to maternal condition. Furthermore, in trading off costs and benefits of hormone deposition, avian mothers may optimize the latter in relation to environmental conditions, too. For example, avian mothers may adjust the transfer of androgens in relation to food availability (affecting the need for sibling competition, which is enhanced by maternal androgens,\(^42\) reviewed in Ref. 2) and the risk of infectious diseases (androgens decrease
immune function; see earlier). In theory, when all eggs contain the optimum level of androgens, experimental elevation of these hormones in the egg will always lead to detrimental effects on the chick, which would easily lead to the wrong conclusion that transfer of maternal androgens is not adaptive (FIG. 1, last row of the table). In the case where mothers adjust hormone deposition to their own (rearing) condition, one could avoid such paradoxical effects by cross-fostering the eggs to a random selection of other mothers. To investigate the adjustment of hormone deposition to environmental parameters, one could first test the effect of a certain environmental factor on the level of hormones in the yolk and then inject the hormone in those eggs that are produced under the condition that induced low hormone deposition. The effect of this treatment can then be analyzed in a design in which the eggs are cross-fostered to both environmental conditions (FIG. 1). Results of such experiments have not yet been published, but one such study is currently being conducted in our laboratory. It is also conceivable that the effect of yolk hormones depends on the genetic quality of the chick.43 This could be tested by a combination of egg treatment and artificial insemination with semen of high- and low-quality males within the same design, as depicted in FIGURE 1.

Another issue that complicates the interpretation of the function of maternal hormones in the egg is the recent finding that they can have long-term consequences for the offspring, well after fledging and into adulthood.15,37,44 If enough offspring sur-

**FIGURE 1.** Experimental design to test context-dependent effects of elevated levels of maternal hormones assuming, that adjustment of hormone levels to the environment are beneficial for the chick. ABBREVIATIONS: Env: environment; Hhigh: high level of the yolk hormone; Hlow: low level of the yolk hormone; Hinj: hormone injection; Oinj: oil injection; cf: cross-fostering; + indicates beneficial effects on the offspring, − detrimental effects; bold signs indicate the most important part of the design.
vive to the reproductive stage, it would mean that monitoring short-term effects of
the manipulation of yolk hormones is not sufficient for a conclusion about the func-
tional significance of these hormones. For a reliable estimate of the fitness effects of
exposure to maternal hormones in the egg, monitoring long-term survival and repro-
duction in the field is required, which is much more difficult than estimating survival
until fledging.

A related issue is that maternal hormone deposition might be shaped by evolution
to maximize fitness of the mother, not of her offspring. For example, in the case of
deteriorating circumstances, mothers may benefit from reducing brood size at the
cost of some individual chicks. In that case the number of chicks surviving until
fledging in that particular reproductive bout may be the wrong estimate for maternal
fitness, and her lifetime reproductive success should be estimated—again a much
more challenging task than the estimation of current reproductive success.

Perhaps the most dominant functional hypothesis in the field is that maternal an-
drogens adjust sibling competition, resulting from hatching asynchrony within the
brood1 (reviewed in Ref. 2). Female birds lay each egg with an interval of about 1
day or more, but incubation—and therefore embryonic development—generally
starts before the last egg is laid, so that first-laid eggs hatch before late-laid eggs.
This hatching asynchrony leads to age and size hierarchies among the nest members
that compete for parental food provisioning. Therefore, many studies are interested
in the patterns of maternal yolk hormones with respect to the position of the egg in
the laying sequence within the clutch. Indeed, in most cases yolk hormone levels in-
crease or decrease over the laying sequence.2 A potential puzzle here is that clutches
may substantially differ in number of eggs, and egg hormone levels may be related
to clutch size. For example, if small clutches contain higher hormone levels than
large clutches—even though laying order does not affect hormone levels at all—eggs
of an early position appear to have on average higher levels than those of a later po-

tion when clutch size is not accounted for. On the other hand, if hormone levels in-
crease with laying order, larger clutches appear to have on average higher levels than
smaller clutches. Therefore, data should be plotted and analyzed, taking into account
both laying position and clutch size. This is also important since in addition to the
position of the egg in the laying sequence, clutch size itself may strongly affect
hatching asynchrony (smaller in smaller clutches) and sibling competition (lower in
smaller clutches).

In general, if one would like to test the hypothesis that the pattern of hormone
deposition over the laying order functions as a tool to adjust sibling competition or
the effect of hatching asynchrony (for a discussion of this possibility, see Ref. 2),
then we need to know not only hormone levels in relation to the position of the egg
in the laying sequence but also the actual degree of hatching asynchrony (that can be
manipulated by the mother by varying her onset of incubation in the course of egg
laying), as well as other factors that may affect sibling competition such as food
availability. Such data are almost entirely lacking (but see Refs. 45 and 46). More-
over, the position of the egg in the laying sequence is often confounded with egg
quality, since egg weight and composition usually change systematically with laying
position.28,31

Finally, we invite researchers in this field to make a more cautious use of some
terminology. Maternal hormone transfer into the egg is often referred to as an invest-
ment by the mother that reflects a certain strategy of allocating resources to different
eggs. Our current knowledge does not justify this interpretation. There is currently no convincing evidence that hormones are costly resources or that their deposition into the egg is costly for the mother. The steroid hormones themselves are produced in very small amounts starting from cholesterol, which is abundantly present. It has also not been demonstrated that the transfer of hormones into the egg necessarily requires that the mother herself be exposed to these hormones too, which may inflict some cost on her. The mechanism of hormone deposition is as yet unclear, in particular whether hormone deposition into the egg can be regulated independently from the regulation of hormone levels in the circulation of the female. An independent regulation would suggest that avian hormone deposition is designed by evolution, indicating that it is an adaptation rather than an exaptation.47 Although such an independent regulation is conceivable, evidence is ambiguous at the moment (for an extensive discussion of this issue, see Ref. 2). Some studies have found a positive relationship between maternal levels of androgens and the concentration of these hormones in the egg of these females,15,48 but others have not.49,50 More insight into the physiological mechanism of hormone deposition is urgently needed, which can be obtained both by sampling the plasma of females around the time of yolking the egg and sampling that egg directly after it is laid, as well as by experimental studies. The largest problem for the descriptive approach is the substantial temporal dynamics of plasma levels of hormones during egg laying48 and of hormone deposition in the yolk,17,51 although it may be of great help to use the different yolk layers to estimate the actual time of deposition.

CONCLUSION

Over the past decade it has been shown that avian species offer excellent opportunities to study hormone-mediated maternal effects, and the field has made great progress. Both descriptive and experimental studies suggest that the hormone deposition in avian eggs reflects a maternal tool to maximize her fitness. So far, androgens have received most attention, perhaps because they are present in the egg in large amounts. However, the time is ripe to study other hormones in more detail as well.

Also, there are as yet almost no studies regarding the physiological mechanisms and regulation of hormone deposition into the egg, and of the uptake and action on the developing embryo—such studies are now urgently needed. Optimally, the study of egg hormones should take place at the interface of the ultimate and proximate approach, integrating such diverse fields as behavioral ecology, animal physiology, and embryology. Finally, some aspects of the methods used, and some conceptual problems, especially with regard to the adaptive value of hormone deposition, need more careful consideration, and the field can benefit from new experimental approaches.

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