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Physiological factors influencing female fertility in birds

Katherine Assersohn, Patricia Brekke and Nicola Hemmings

Article citation details
R. Soc. open sci. **8**: 202274.
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Review timeline

Original submission:	15 December 2020
1st revised submission:	17 May 2021
2nd revised submission:	8 July 2021
Final acceptance:	12 July 2021

Note: Reports are unedited and appear as submitted by the referee. The review history appears in chronological order.

Review History

RSOS-202274.R0 (Original submission)

Review form: Reviewer 1

Is the manuscript scientifically sound in its present form?
Yes

Are the interpretations and conclusions justified by the results?
Yes

Is the language acceptable?
Yes

Do you have any ethical concerns with this paper?
No

Have you any concerns about statistical analyses in this paper?
No

Recommendation?
Accept with minor revision (please list in comments)

Comments to the Author(s)
I would not consider this to be a comprehensive review. But based on the objectives listed at the beginning of the paper, I am not sure it is meant to be. There are several other papers on the topic in poultry in particular that are not included in this review.

In the sperm:egg interaction section of the review, I know of several papers that are not cited. There is at least one theory of thought that i did not think was included and that is the interaction of the yolk with the IPVL in the non-germinal disc areas of the egg. I provided the authors some of those references but it has been a while since I have reviewed the literature on that topic and i would not consider this to be a comprehensive list.

The rest of my comments and suggestions are in the attached file (see Appendix A).

Review form: Reviewer 2

Is the manuscript scientifically sound in its present form?
Yes

Are the interpretations and conclusions justified by the results?
Yes

Is the language acceptable?
Yes

Do you have any ethical concerns with this paper?
No

Have you any concerns about statistical analyses in this paper?
No

Recommendation?
Major revision is needed (please make suggestions in comments)

Comments to the Author(s)
I have not followed the avian ovarian physiology literature in several years, but I am knowledgeable in the area of reproductive ecology and behavior. So, I feel I should be able to provide some insight from that perspective. My overall impression of this manuscript is very favorable. It is an important topic, and the authors are correct that fertility problems have been poorly defined historically and that female fertility is a vastly understudied area. The authors seem to cover the topic thoroughly, so it is my opinion that this manuscript would make an important and much-needed contribution to the field. With all that said, however, I am not convinced that Royal Society Open Science is the best venue for its publication, given the journal's own account of favoring papers that are of very broad, general interest. Indeed, I am not sure how broad and general the interest in physiological mechanisms of female bird fertility is. If the paper were on mechanisms of vertebrate or perhaps mammalian fertility, it might be of broader interest. But its current focus on female birds would seem to lend itself better to a bird journal, perhaps, or a journal more specifically oriented toward reproductive physiology, such as Biology of Reproduction.

As I indicated, however, I do feel that it is a worthwhile contribution, but some changes, as follows, would make it better. First, it is difficult to understand its overall organization. I wonder if a table of contents would be appropriate. I found it difficult to understand how each section tied in with the one before it and the one after it and then how they all fit together to address the central question: what causes infertility in female birds? One possibility would be to organize the paper around the anatomy of figure 2, walking the reader through each part of egg formation and how fertility can be affected in that part. Additionally, somewhere around line 41, it would be extremely helpful to list as many mechanisms for failed syngamy as possible in order to set up the rest of the paper. The paper could then be better organized around these. Currently, it seems vaguely organized around aspects of Figure 2, but a more deliberate breakdown of the specific ways syngamy can fail, perhaps in order of timing, would greatly facilitate the organization of the paper and therefore understanding it. Currently the paper reads like a long list of what we know about reproduction with little direct tie-in to failed syngamy, especially in any sort of chronological or other logical order.

I think about fertility from an adaptive perspective. Given the presence of a multitude of life-history trade-offs (particularly the trade-offs between number of offspring and quality of offspring within a single brood and also the trade-off between number of current offspring and number of future offspring), perhaps the term "failure" is inappropriate when describing when fertilization does not occur. There may be times when it benefits the female from a fitness perspective to avoid fertilization, even if it means the wasted time and energy that went into production of an infertile ovum or egg. Given that, at least in wild, altricial birds, far more investment occurs in feeding young than in forming the egg for the young, a female suddenly faced with an unexpected challenge (such as unexpected energy demands, predatory risks, or poor quality mate) might benefit by selectively blocking fertilization to avoid costly stages later during nestling rearing when conditions might not merit the investment. For example, the female may be constrained to laying 4 eggs, but challenging food conditions might mean that, this particular bout, 3 eggs might be more adaptive, so she can at least raise 3 good offspring rather than 4 poor ones. In this case, one way to save energy would be to adaptively prevent fertilization of one of the ova, giving rise to 4 eggs but only 3 mouths to feed. Ultimately, I think use of the terms "fail" and "succeed" when it comes to syngamy or fertilization is loaded and assumes fertilization is always adaptive, when there might be situations in which it is not adaptive.

Lines 408-410: Up to this point, the manuscript has mainly focused on what we know in domestic species, each time referring to how little is known in wild species. The reader is left wondering what IS known for wild species. So far, the manuscript mostly reads as a review in domestic species despite what seemed to be a focus early on on the importance of this topic to wild species.

Lines 686-687: It is this possibility specifically that I think of above all else when it comes to possible ways for fertilization to fail, and yet apparently nothing is known. Nonetheless, if the authors could go into greater detail, even if just speculation, I think it would resolve a lot of wondering by their readers.

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Other comments:

Lines 28-29: Delete.

Line 40: Is this the most broadly used and accepted definition of infertility? If not, reword to say something like "We define infertility as . . . "

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Lines 457-458: Increased relative to what? To a time more distant from ovulation?

Decision letter (RSOS-202274.R0)

We hope you are keeping well at this difficult and unusual time. We continue to value your support of the journal in these challenging circumstances. If Royal Society Open Science can assist you at all, please don't hesitate to let us know at the email address below.

Dear Miss Assersohn

The Editors assigned to your paper RSOS-202274 "Physiological factors influencing female fertility in birds" have now received comments from reviewers and would like you to revise the paper in accordance with the reviewer comments and any comments from the Editors. Please note this decision does not guarantee eventual acceptance.

We invite you to respond to the comments supplied below and revise your manuscript. Below the referees' and Editors' comments (where applicable) we provide additional requirements. Final acceptance of your manuscript is dependent on these requirements being met. We provide guidance below to help you prepare your revision.

We do not generally allow multiple rounds of revision so we urge you to make every effort to fully address all of the comments at this stage. If deemed necessary by the Editors, your manuscript will be sent back to one or more of the original reviewers for assessment. If the original reviewers are not available, we may invite new reviewers.

Please submit your revised manuscript and required files (see below) no later than 21 days from today's (ie 19-Apr-2021) date. Note: the ScholarOne system will 'lock' if submission of the revision is attempted 21 or more days after the deadline. If you do not think you will be able to meet this deadline please contact the editorial office immediately.

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Thank you for submitting your manuscript to Royal Society Open Science and we look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Best regards,
Lianne Parkhouse
Editorial Coordinator
Royal Society Open Science
openscience@royalsociety.org

on behalf of Professor Kevin Padian (Subject Editor)
openscience@royalsociety.org

Editor Comments to Author:

Thank you for your submission. As you will see the reviewers are generally favorable but have some rather different concerns. We ask you to address these carefully in your revision. Best wishes.

Reviewer comments to Author:
Reviewer: 1
Comments to the Author(s)
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one version identifying all the changes that have been made (for instance, in coloured highlight, in bold text, or tracked changes);
a 'clean' version of the new manuscript that incorporates the changes made, but does not highlight them. This version will be used for typesetting if your manuscript is accepted.

Please ensure that any equations included in the paper are editable text and not embedded images.

Please ensure that you include an acknowledgements' section before your reference list/bibliography. This should acknowledge anyone who assisted with your work, but does not

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Attach your point-by-point response to referees and Editors at Step 1 'View and respond to decision letter'. This document should be uploaded in an editable file type (.doc or .docx are preferred). This is essential.

Please ensure that you include a summary of your paper at Step 2 'Type, Title, & Abstract'. This should be no more than 100 words to explain to a non-scientific audience the key findings of your research. This will be included in a weekly highlights email circulated by the Royal Society press office to national UK, international, and scientific news outlets to promote your work.

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1) One version identifying all the changes that have been made (for instance, in coloured highlight, in bold text, or tracked changes);
2) A 'clean' version of the new manuscript that incorporates the changes made, but does not highlight them.
-- An individual file of each figure (EPS or print-quality PDF preferred [either format should be produced directly from original creation package], or original software format).
-- An editable file of each table (.doc, .docx, .xls, .xlsx, or .csv).
-- An editable file of all figure and table captions.
Note: you may upload the figure, table, and caption files in a single Zip folder.
-- Any electronic supplementary material (ESM).
-- If you are requesting a discretionary waiver for the article processing charge, the waiver form must be included at this step.
-- If you are providing image files for potential cover images, please upload these at this step, and inform the editorial office you have done so. You must hold the copyright to any image provided.
-- A copy of your point-by-point response to referees and Editors. This will expedite the preparation of your proof.

At Step 6 'Details & comments', you should review and respond to the queries on the electronic submission form. In particular, we would ask that you do the following:
-- Ensure that your data access statement meets the requirements at https://royalsociety.org/journals/authors/author-guidelines/#data. You should ensure that you cite the dataset in your reference list. If you have deposited data etc in the Dryad repository, please include both the 'For publication' link and 'For review' link at this stage.
-- If you are requesting an article processing charge waiver, you must select the relevant waiver option (if requesting a discretionary waiver, the form should have been uploaded at Step 3 'File upload' above).
-- If you have uploaded ESM files, please ensure you follow the guidance at https://royalsociety.org/journals/authors/author-guidelines/#supplementary-material to include a suitable title and informative caption. An example of appropriate titling and captioning may be found at https://figshare.com/articles/Table_S2_from_Is_there_a_trade-off_between_peak_performance_and_performance_breadth_across_temperatures_for_aerobic_scope_in_teleost_fishes_/3843624.

At Step 7 'Review & submit', you must view the PDF proof of the manuscript before you will be able to submit the revision. Note: if any parts of the electronic submission form have not been completed, these will be noted by red message boxes.

Author's Response to Decision Letter for (RSOS-202274.R0)

See Appendix B.

RSOS-202274.R1 (Revision)

Review form: Reviewer 1

Is the manuscript scientifically sound in its present form?
Yes

Are the interpretations and conclusions justified by the results?
Yes

Is the language acceptable?
Yes

Do you have any ethical concerns with this paper?
No

Have you any concerns about statistical analyses in this paper?
No

Recommendation?
Accept with minor revision (please list in comments)

Comments to the Author(s)
Personally I think your revisions have improved the paper greatly. I only have one minor suggestion before publishing. In every other case in the paper were the term "for example" was used, you provided an example. In one case you did not. See the Line 445 comment below.

Line 445 – This is a personal preference, but I do think it helps the readers if when the text says "although there are a few example…", especially those new to the an area, make the information more useful to them, that some specific examples, maybe in parenthesis are provided.

Decision letter (RSOS-202274.R1)

We hope you are keeping well at this difficult and unusual time. We continue to value your support of the journal in these challenging circumstances. If Royal Society Open Science can assist you at all, please don't hesitate to let us know at the email address below.

Dear Miss Assersohn,

On behalf of the Editors, we are pleased to inform you that your Manuscript RSOS-202274.R1 "Physiological factors influencing female fertility in birds" has been accepted for publication in Royal Society Open Science subject to minor revision in accordance with the referees' reports. Please find the referees' comments along with any feedback from the Editors below my signature.

We invite you to respond to the comments and revise your manuscript. Below the referees' and Editors' comments (where applicable) we provide additional requirements. Final acceptance of your manuscript is dependent on these requirements being met. We provide guidance below to help you prepare your revision.

Please submit your revised manuscript and required files (see below) no later than 7 days from today's (ie 05-Jul-2021) date. Note: the ScholarOne system will 'lock' if submission of the revision is attempted 7 or more days after the deadline. If you do not think you will be able to meet this deadline please contact the editorial office immediately.

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Thank you for submitting your manuscript to Royal Society Open Science and we look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Kind regards,
Royal Society Open Science Editorial Office
Royal Society Open Science
openscience@royalsociety.org

on behalf of Professor Kevin Padian (Subject Editor)
openscience@royalsociety.org

Associate Editor Comments to Author:

Thank you for your patience while we sought re-review. Unfortunately, only one of the original reviewers was available to assess your changes, and though a new reviewer had agreed to report, we regret that they were not able to do so in the end. With this in mind, we've opted to make a decision based on the feedback of the reviewer who did agree and report. Thank you for this contribution and we'll look forward to receiving the final version shortly.

Reviewer comments to Author:
Reviewer: 1
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===PREPARING YOUR REVISION IN SCHOLARONE===

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-- An individual file of each figure (EPS or print-quality PDF preferred [either format should be produced directly from original creation package], or original software format).
-- An editable file of each table (.doc, .docx, .xls, .xlsx, or .csv).
-- An editable file of all figure and table captions.
Note: you may upload the figure, table, and caption files in a single Zip folder.
-- Any electronic supplementary material (ESM).
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Author's Response to Decision Letter for (RSOS-202274.R1)

See Appendix C.

Decision letter (RSOS-202274.R2)

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Appendix A

ROYAL SOCIETY OPEN SCIENCE

Physiological factors influencing female fertility in birds

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Subject Category:	Organismal and Evolutionary Biology

Author-supplied statements

Relevant information will appear here if provided.

Ethics

Does your article include research that required ethical approval or permits?:
This article does not present research with ethical considerations

Statement (if applicable):
CUST_IF_YES_ETHICS :No data available.

Data

It is a condition of publication that data, code and materials supporting your paper are made publicly available. Does your paper present new data?:
Yes

Statement (if applicable):
The dataset supporting this article has been uploaded as part of the supplementary material.

Conflict of interest

I/We declare we have no competing interests

Statement (if applicable):
CUST_STATE_CONFLICT :No data available.

Authors' contributions

This paper has multiple authors and our individual contributions were as below

Statement (if applicable):
K.A carried out the data collection, data analysis, created the figures and drafted the manuscript; P.B critically revised the manuscript; N.H participated in data collection, assisted in data analysis and critically revised the manuscript. All authors gave final approval for publication and agree to be held accountable for the work performed therein.

Physiological factors influencing female fertility in birds

Katherine Assersohn[1*], Patricia Brekke[2], Nicola Hemmings[1]

[1]*Department of Animal and Plant Sciences, University of Sheffield, Sheffield, S10 2TN*

[2]*Institute of Zoology, Zoological Society of London, Regents Park, London, NW1 4RY*

Abstract

Fertility is fundamental to reproductive success, but not all copulation attempts result in a fertilised embryo. Fertilisation failure is especially costly for females, and while there is a growing appreciation for the considerable influence that female processes can have over fertilisation, we lack a clear understanding of the causes of variation in female fertility across taxa. Birds make a useful model system for fertility research, partly because their large eggs are easily studied outside of the female's body, but also because of the wealth of data available on the reproductive productivity of commercial birds. Here, we review the factors that contribute to female infertility in birds, providing evidence that female fertility traits are being understudied relative to male fertility traits, and that there is a bias in research effort towards the study of Galliformes and captive (relative to wild) populations. We then highlight and discuss the key physiological stages of the female reproductive cycle where fertility may be compromised, and make recommendations for future research. We particularly emphasise the need for studies to clearly differentiate between infertility and embryo mortality as causes of hatching failure, and for information about non-breeding individuals to be more routinely collected where possible. This review lays the groundwork for developing a clearer

understanding of the causes of female infertility, with important consequences for multiple fields including reproductive science, conservation and commercial breeding.

Key words: hatching failure, female fertility, reproduction, egg production, sperm storage, fertilisation

I. Introduction

In order to contribute genes to future generations and obtain evolutionary fitness, an individual must successfully reproduce. Fertility is fundamental to reproductive success, so we should expect fertility traits to be under strong selection to maximise reproductive output and minimise the wastage of gamete investment [1,2]. Despite this, fertility varies remarkably across individuals, species and populations [3,4], and some degree of infertility is ubiquitous across taxa. Gametic wastage is likely to be more costly for females than males [4], since they typically invest considerably more in gamete production [5]. These costs are also likely to be greater in taxa that produce large yolky ova, such as birds, but despite this, females are thought to have received comparatively less attention than males within avian fertility research (particularly in poultry science) [6].

Successful fertilisation occurs when a male pronucleus and a female pronucleus fuse to form a zygote (i.e. syngamy) [7]. Infertility can therefore be defined as the failure of syngamy, and any male or female process contributing to failed syngamy is a cause of infertility. Confusingly, the term infertility has been used interchangeably in the literature to describe both fertilisation failure and embryo mortality across taxa, though these two processes often have a very different mechanistic basis [8]. Differentiating between infertility and embryo mortality is often difficult, particularly if the embryo dies during the very early stages of development [9],

but the failure to distinguish between them presents an important barrier to addressing the underlying causes of reproductive failure.

Birds are well suited to the study of reproductive failure, primarily because – unlike mammals – they produce large, well-protected eggs which make them easy to examine externally before they degrade [9]. It is also possible to precisely determine whether a bird's egg failed because it was unfertilised or because the embryo died very early, using microscopic methods, but many studies still fail to make the distinction [8,10]. Avian reproduction science also benefits from a wealth of knowledge from commercial poultry research. Despite intensive, long-term selection for consistent and efficient egg production in commercially important species such as the domestic fowl (*Gallus gallus domesticus)* and turkey (*Meleagris gallopavo)*, hatching failure is still a pervasive issue in commercial breeds, and the reasons for this are not fully understood [9,11]. In the wild, hatching failure is also ubiquitous; on average 10% of eggs never hatch [2], and in some threatened and bottlenecked species more than 60-70% of eggs fail [12,13]. While there has been some attention paid to embryo mortality in birds, we still lack a clear understanding of the incidence of true infertility and the factors that contribute towards it [14]. The incidence of infertility relative to embryo mortality in wild populations has most likely been overestimated by many studies [9], while in captive birds, infertility may be more likely [8]. Understanding the mechanisms that cause infertility could therefore be particularly important for captive breeding programmes.

Here, we provide a thorough review of female-specific factors that lead to infertility in birds, revealing that female fertility traits are consistently understudied relative to males. We identify and explore key phases in the female reproductive cycle where fertility may be compromised, including egg production, oviductal sperm storage and transport, and syngamy (the fusion of

sperm and ovum), drawing particular attention to the relationships between senescence, environmental factors, and female reproductive function. Our aim is to develop a clearer understanding of the proximate causes of variation in female fertility and highlight key directions for future research.

II. How much do we know about female fertility traits in birds?

We conducted a systematic search of the avian fertility literature (see supporting information for methods), identifying 718 relevant papers on avian fertility traits, of which 42% considered both male and female fertility, 37% focused on male fertility only, and 20% focused on female fertility only. As expected, the number of avian fertility papers published each year is increasing, but since 1985, the number of published papers that focus on male fertility have increased at a faster rate than the number of papers focused on female fertility (Fig 1A) (x^2 = 15, df = 2, p<0.001). By April 2020, published papers focusing on male avian fertility outnumbered those on female avian fertility by a factor of 1.84, indicating a strong bias in research effort against the study of female fertility, a gap that appears to be widening over time. However, studies that considered fertility traits in both males and females were almost as numerous as those that considered males only, perhaps indicating that many researchers are taking a more holistic approach. This may also reflect the inherent difficulties involved in disentangling the effects of male and female factors on fertilisation success, since they are likely to be non-independent processes exhibiting complex interactions [15]. Across all years (from 1921 to 2020), 79% of articles exclusively investigated captive populations, with only 16% investigating wild populations and 5% investigating both captive and wild populations. This suggests there has been a substantial bias in research effort towards the study of fertility in captive populations rather than wild populations. Furthermore, of the captive species studied, 88% focussed exclusively on the order Galliformes, with 54% focussed exclusively on a single

species: the domestic chicken (*Gallus gallus domesticus)*. This indicates a further taxonomic bias within the avian fertility literature toward gallinaceous birds of commercial importance. The bias in research effort towards male fertility appears consistent across both wild and captive populations (Fig 1B).

The observed bias in research effort against female only papers may reflect that, relative to ova, it is easy to collect sperm in a way that is non-invasive and repeatable. Sperm traits have also been extensively studied in birds, and sperm biotechnology has been developed considerably in poultry over the last century [16]. There may also be a degree of positive feedback, with ease of collection and study of sperm yielding greater advances in methodology, which in turn yields further research. The bias in research effort may also be a consequence of the historical view that sperm are the 'active' participants in fertilisation: seeking, binding to and penetrating the somewhat 'passive' egg [17]. Cultural biases have also been suggested to drive a male-orientated research focus across other taxa including mammals [18]. While this view has been challenged in recent years (especially with regards to postcopulatory processes such as cryptic female choice [19]), the gap between the number of male and female fertility papers suggests that female fertility traits in birds are still under-studied, and the role of the female in determining reproductive success is therefore underappreciated. The following sections explore the physiological mechanisms that may contribute to variation in female fertility in birds, and suggest new hypotheses and future directions that may help fill the gaps in our current understanding of avian female fertility.

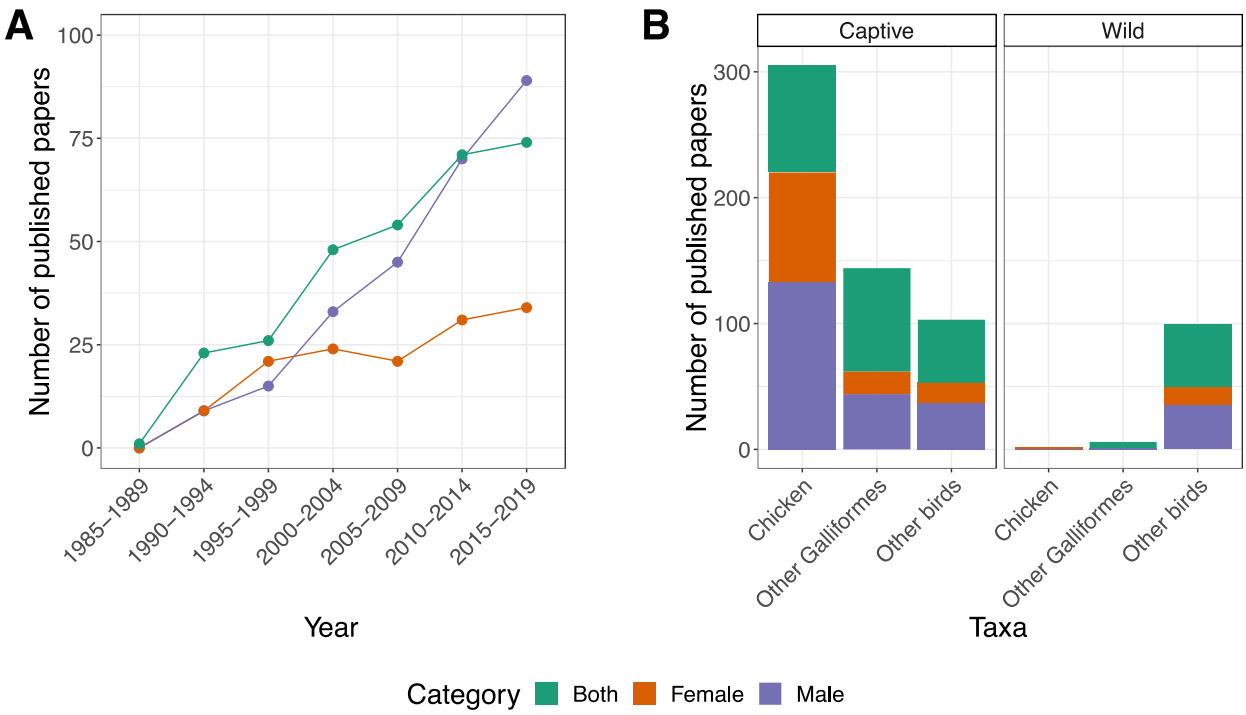

Figure 1: A) The number of papers published between 1985 and 2019 on avian fertility in males only (purple), females only (orange) and both males and females (green). B) The number of published papers on avian fertility published between 1921 and 2020 (all years) either focusing exclusively on the domestic chicken (*Gallus gallus domesticus*), other Galliformes, or other (non-Galliform) bird orders, for both captive (left) and wild (right) populations.

III. Physiological mechanisms of female infertility in birds

(1) Producing eggs

In birds, infertility is typically measured as the number of unfertilised eggs, but this makes the assumption that a female is already able to produce an ovum that can be fertilised. Female fertility is the product of not only fertilisation rate, but also the number of eggs produced that are capable of being fertilised. The process of egg formation – from follicular development through to release of the ovum during ovulation - is a metabolically demanding process [20,21]

and problems occurring during these early stages of reproduction are a costly and important cause of infertility.

a) The female reproductive system

Reproductively active females of most bird species have one functional oviduct in which there is usually just one ovary (Fig 2.). The right oviduct regresses early in development via hormonally controlled apoptosis (caused by the release of anti-Müllerian hormone), while the left oviduct is protected from regression by elevated concentrations of oestrogen (which inhibits the anti-Müllerian hormone receptor) [22]. The mature avian ovary contains multiple maturing follicles each at a different stage of development [23] (Fig 2.). Mature follicles consist of a large, protein rich yolky oocyte and a small germinal disc (which contains the genetic material), surrounded by a granulosa cell layer, multicellular theca layer and an epithelial layer (Fig 3.) [24,25]. At the vegetal pole of the egg, the epithelial layer becomes thin, forming a region known as the stigma that acts as the point of rupture during ovulation [25]. During the later stages of follicular growth, a glycoprotein structure known as the perivitelline layer forms between the granulosa cells and the oocyte (Fig 3.). The perivitelline layer functions to bind with sperm during fertilisation and initiate the acrosome reaction [26]. In birds, the mechanisms underpinning follicular development, maintenance and selection have yet to be well-defined, although the implications of these processes are likely to be significant for fertility [27]. Follicular selection (i.e. the selection of one white follicle to rapidly uptake yolk, undergo further differentiation and eventually ovulate as a mature yellow follicle) is thought to be mediated by cAMP (cyclic adenosine monophosphate) signaling, which acts through G protein coupled receptors to upregulate the expression of multiple genetic factors important for follicular development [22,27–29]. The unselected white follicles are maintained in an undifferentiated/arrested yet viable state within the ovary until

the next follicular selection [25]. This is thought to be regulated in part by the β-arrestin protein, which desensitises G protein couple receptors (and thus inhibits cAMP signalling), and depresses granulosa cell differentiation [27]. Understanding the mechanisms governing follicular recruitment and maintenance is considered a primary challenge in avian reproductive research [25,30].

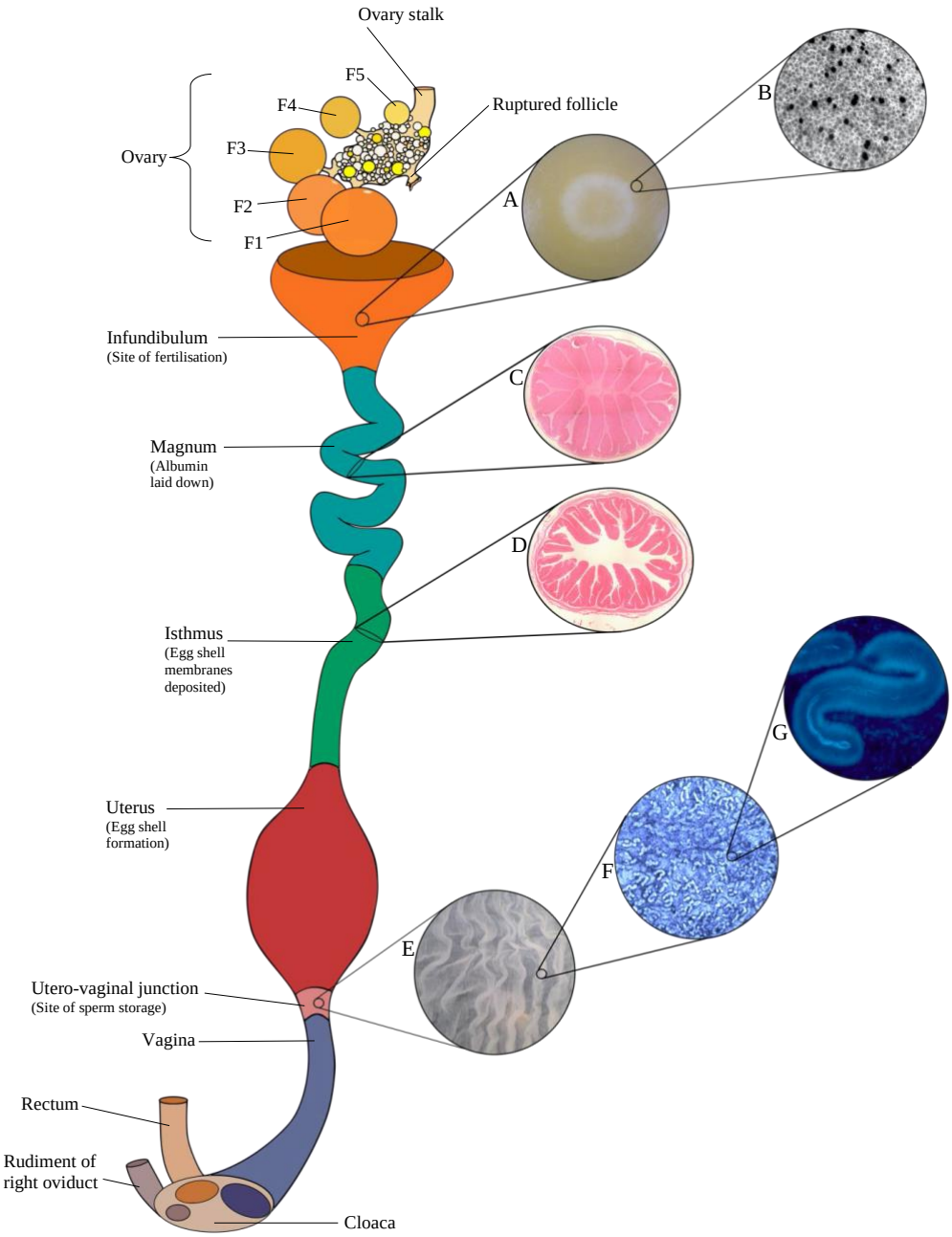

Figure 2: Schematic representation of the avian oviduct and ovary (not to scale). Note that the avian ovary consists of multiple follicles at different stages of development. The largest

yellow follicles are labelled F1-5 where F1 is the largest follicle and will be the next to rupture. **A:** The germinal disc of a fertilised ovum (from a zebra finch (*Taeniopygia guttata*)). Note the clear outer ring and paler center of the germinal disc which indicates embryonic development. **B:** Sperm penetration holes visible on the inner perivitelline layer of an ovum after fertilisation (from a bullfinch (*Pyrrhula pyrrhula*)). **C:** A cross section of the magnum (from a helmeted guineafowl (*Numida meleagris)).* Sperm is transported through the magnum prior to fertilisation, but this region functions mainly to produce the albumin which is laid down during egg development; **D**: A cross section of the isthmus (from Reeves pheasant (*Syrmaticus reevesii*)). Sperm is also transported through the isthmus prior to fertilisation, but this region functions mainly to produce and deposit shell membranes during egg development; **E**: The internal tissue lining and folds of the vagina and utero-vaginal junction region (from a bobwhite quail (*Colinus virginianus*)). The vagina is considered the primary site of sperm selection in the oviduct, and the utero-vaginal junction functions as the primary site of sperm storage, containing numerous sperm storage tubules; **F**: A single fold of the utero-vaginal junction (from a zebra finch (*Taeniopygia guttata*)) stained with Hoechst 33342 dye under a fluorescence microscope. The many small tubular structures are sperm storage tubules; **G**: A single sperm storage tubule (and visible trapped sperm) from a single fold of the utero-vaginal junction (from a Japanese quail (*Coturnix japonica*)), stained with Hoechst 33342 dye and viewed under a fluorescence microscope.

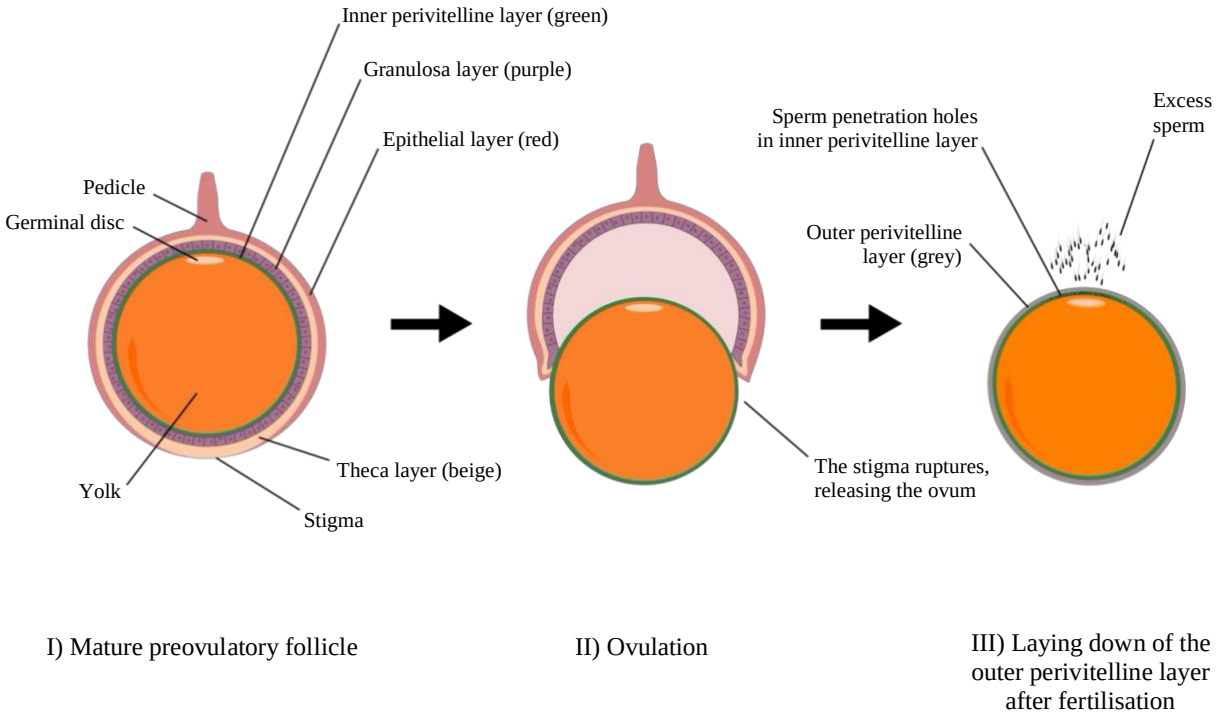

Figure 3: Schematic representation of a mature avian follicle. **I)** An avian follicle prior to ovulation; **II)** The ovum and follicle during ovulation, whereby a mature follicle ruptures at the stigma region, releasing the ovum. Sperm present in the infundibulum will begin to move towards the ovum in preparation for fertilisation, where they will penetrate the inner perivitelline layer (green)**; III)** The ovum after fertilisation, the outer perivitelline layer (grey) has been laid down (which blocks further sperm entry). The inner perivitelline layer (green) has an abundance of sperm penetration holes around the germinal disc region where sperm have penetrated during fertilisation (see Fig. 2B).

b) Ovulation

Ovulation is a complex process under fine hormonal control [22]. It occurs when the largest mature yellow follicle (labelled F1 in Fig 2.) ruptures at the stigma region (Fig 3.) [25], releasing the ovum which is then captured by the infundibulum – the site of fertilisation. Unlike

mammals, the granulosa layer provides the main source of gonadal steroids [29], and ovulation is initiated by the production of testosterone in the granulosa cells, which stimulates the release of granulosa cell progesterone. Progesterone then creates a positive feedback response in the hypothalamus which stimulates an increase in the secretion of gonadotropin-releasing hormone, and consequently causes a surge of pituitary luteinising hormone [28,31,32]. Clock genes expressed within granulosa cells after follicle selection are also thought to provide a degree of circadian control over the timing of ovulation [28,33]. Proper regression of the post-ovulatory follicle is thought to be required for managing the timing of ovulation and egg-laying [22], and typically one ovum is released per day.

In broiler breeder hens, which have been selected for rapid growth at the expense of fertility, double-yolk eggs are fairly common, and occur more frequently during the onset of egg production [22,34]. Double-yolk eggs are associated with a greater incidence of embryo mortality (at all stages of development) and are also more likely to be infertile [34], possibly because ova are ovulated early and in an immature state. Ovulation order of double-yolk eggs also affects the likelihood of fertilisation: in duck (*Anas platyrhynchos domesticus)* eggs, the first yolk captured by the infundibulum has a higher probability of being fertilised [35]. This may explain why double-yolk eggs commonly contain only one fertilised ovum [34]. Age, nutrition (e.g. feed restriction) and changes in photostimulation are all thought to play a role in the production of double-yolk eggs, the occurrence of which can also be increased via selection [35], indicating a genetic component. In addition to double-yolks, the presence of multiple germinal discs on a single yolk has been reported [36]. However, the cause and incidence of such (and other) ovum abnormalities are unknown, as well as the implications they might have for fertility and embryo development. Chromosomal abnormalities, such as whole genome triploidy, can significantly or completely impair fertility in some affected individuals, and some

triploid embryos are non-viable and die after a few days of incubation [37]. Triploidy is usually (but not always) maternally derived, and is thought to arise from diploid gametes produced as a result of chromosomal nondisjunction (where homologous chromosomes fail to separate during meiosis) [38,39]. Reports of triploid birds in the wild are rare, possibly because of the reduced survival of triploid embryo's, although the true rate of incidence in wild populations is unknown.

c) The female endocrine system

The proper functioning of the avian endocrine system is vital for egg production. In seasonally breeding species, photoperiodic cues are received by deep brain photoreceptors that stimulates activity of the hypothalamic-pituitary-gonadal (HPG) axis. The HPG axis is a tightly regulated system that, among other things, regulates physiological processes associated with reproduction [25]. Specifically, following an increase in photoperiod, the mediobasal hypothalamus is stimulated to produce local thyroid hormone which regulates the release of gonadotrophin releasing hormone. This in turn stimulates the pituitary to produce gonadotropins that initiates seasonal gonadal growth and activity [25,40]. Following breeding, the HPG axis is promptly 'switched off', resulting in a significant regression of the gonads [25]. In male Japanese quail (*Coturnix japonica)*, lesions of the mediobasal hypothalamus can inhibit the photoperiodic response and gonadal growth [41], but whether such lesions affect seasonal gonad development in females is unclear.

Endocrine disorders such as cystic hyperplasia, cystic ovaries and hypothyroidism are a clinical issue in captive birds [42–46], and there is extensive experimental evidence showing that hormonal disruption can significantly influence egg production. For example, in domestic fowl, administering luteinising hormone 8.5 hours after ovulation causes follicular degeneration

(atresia) of the next follicle within the follicular hierarchy [47]. An increase in progesterone at the wrong time may induce a spike in luteinising hormone, which triggers premature ovulation [44,48]. Inhibition of luteinising hormone (e.g. via serotonin injections) [48] can lead to anovulation and disruption of thyroid hormone function [49], and in pigeons (*Columba livia)*, administering synthetic gonadotropin-releasing hormone can reduce luteinising hormone concentrations, depressing egg production [50]. Ovulation was prevented in domestic fowl treated with the testosterone antagonist flutamide, which blocks the production of pre-ovulatory hormones [51]. Treatment with the inhibin A protein can increase the proliferation of granulosa cells and increase the secretion of granulosa steroid production, whilst decreased expression of the inhibin α subunit (which has been observed in cystic follicles in pigs [52]) is associated with follicle atresia in chickens [29]. Counterintuitively, injections of follicle stimulating hormone can decrease egg production in zebra finches (*Taeniopygia guttata)*, possibly because of a negative feedback effect with endogenous secretion of follicle stimulating hormone [53], although timing of treatment is also likely to be important since the function of follicle stimulating hormone is known to vary with follicle size under normal conditions [25]. Inappropriate levels of the anti-Müllerian hormone have been shown to disrupt normal reproductive development in some species [54,55], and experimentally inhibiting oestrogen synthesis in chicken embryos increases the expression of the anti-Müllerian hormone receptor, resulting in masculinisation of the reproductive tract [56]. The anti-Müllerian hormone is thought to play a vital role in follicular development within the ovary, and elevated levels have been associated with periods of restricted fertility in hens [57]. Elucidating the full significance of the anti-Müllerian hormone for avian female fertility is an active area of research [30,57].

The degree to which hormonal disorders naturally affect wild birds is largely unknown, but exposure of wild birds to environmental Endocrine-Disrupting Compounds (EDCs) has been shown to have a significant impact on fertility [58]. Over 90,000 anthropogenic chemicals are estimated to have been released into the environment; several hundred of these pollutants are confirmed to be EDCs but the majority remain untested for their effects on wildlife [59,60]. If passed onto developing embryos, EDCs can disrupt reproductive development and cause sterility [61]. Negative effects on reproductive success in birds have been observed even when they are exposed to very low and environmentally relevant concentrations of certain EDCs. For example, small amounts of crude oil is sufficient to depress egg yolk formation in seabirds [62,63]; exposure to flame-retardant additives at concentrations typically seen in the environment can depress reproductive success (including fertility) in American kestrels (*Falco sparverius)* [64], and in areas polluted with environmental oestrogens, severe reproductive tract abnormalities have been found in exposed females [63]. Toxic heavy metals are also known to act as EDCs and disrupt reproduction in exposed birds: a single dose of cadmium was enough to significantly reduce egg production in Japanese quail [65]; lead is known to accumulate in the ovaries of pheasants (*Phasianus colchicus)* [66], Japanese quail [67] and chickens [68] following exposure, which can reduce egg production and cause histopathological damage and developmental delays in the ovaries; and exposure to mercury (even at very low concentrations) can significantly reduce reproductive success in zebra finches [69]. EDCs have also been shown to disrupt mating behaviour in several seasonally-breeding birds [70] and are linked with population declines [71]. Understanding how EDCs influence reproductive physiology in wild birds is crucial, particularly for endangered birds where even small reductions in fertility can jeopardise species survival [72]. Relatively few long-term studies of wild birds have monitored the effects of EDCs on avian fertility, and in particular there is a lack of knowledge on the population-level effects of EDCs on fertility in wild birds [73,74].

Detecting the varied and often sublethal effects of EDCs is difficult: wild birds are likely exposed to many different types of EDC at one time [75,76], each with potentially complex and different effects [74]. The risks of EDC exposure to fertility is also likely to differ between species and across individual lifetimes [77]. Identifying the mechanisms by which EDCs affect fertility in wild birds therefore requires a combination of laboratory studies, long-term monitoring, and continued development of analytical methods [74].

d) Ovarian disorders and the immune system

In humans, ovarian disorders such as hormonal dysfunction, ovarian abnormalities (such as polycystic ovarian disease), premature menopause, and genetic defects [78], explain 30% of female infertility cases. Although less well studied in birds, ovarian disorders are known to reduce or stop egg production in poultry [79], and cystic ovarian disease is common in other bird species (e.g. cockatiels (*Nymphicus hollandicus)*, budgerigars (*Melopsittacus undulatus)*, and pheasants [80]) [44]. Coelomitis is another common clinical problem in domestic birds, causing inflammation of the ovaries (oophoritis) and ectopic ovulation [80]. The spontaneous development of ovarian cancers is extremely common in the laying hen [25], the incidence of which increases with age, occurring in 24% of hens aged >2 years [81], and 30-35% of hens by 3.5 years [22]. The increase in the incidence of ovarian cancers with age is thought to be, at least in part, a consequence of the accumulation of ovarian surface and DNA damage caused by ovulatory events over time [82]. Laying hens may therefore be at particular risk from ovarian cancers because of the selection for frequent ovulations in commercial breeds [25]. Progesterone can be effective at reducing the incidence of ovarian cancers, possibly because it limits the number of ovulations experienced [83]. Increased levels of progesterone have also been implicated in an increase in the number apoptotic events in the ovary, which may act to remove damaged cells [84]. Microbes present in the intestines and cloaca may be transported

to – and colonise – the ovaries [25]. Viral infections such as avian influenza, infectious bronchitus and avian hepatitis can cause the formation of chronic lesions within the oviduct, that may prevent the successful capture of ova following ovulation [85,86]. This can result in extensive damage to the oviduct [87], often leading to further bacterial infection due to the presence of yolk in the coelomic cavity (egg yolk peritonitis) [88]. Inflammation of the oviduct (such as salpingitis or metritis) caused by bacterial or viral infection can also result in oviductal impaction, egg abnormalities and infertility [44]. Little is known about the incidence of such ovarian disorders, or the degree to which they explain variation in female fertility in wild populations.

The proper functioning of the immune system is of great importance in the defense against bacterial, fungal and viral pathogens within the ovary. Toll-like receptors (TLRs) produced in the follicular tissue of domestic fowl are known to be involved in the recognition of pathogens, and play a key role in inducing an immune response in the ovary [25]. In particular, TLRs respond to pathogenic stimuli by producing avian β-defensins (antimicrobial peptides) and proinflammatory cytokines [89]. TLR signaling may also cause disruption to steroidogenesis, and result in apoptosis of undifferentiated granulosa cells, thereby providing a mechanism to prevent the selection of infected follicles into the preovulatory hierarchy [90]. The adaptive immune response then involves the migration of certain immunocompetent cells into the follicles, including MHC (major histocompatibility complex) antigen presenting cells, T cells, B cells and macrophages [25]. The distribution of immunocompetent cells in the oviduct increases during sexual maturation, but then decreases significantly thereafter with age [91]. In humans, ovarian autoimmunity has been associated with premature ovarian failure and infertility [92]. Less is known about the incidence and mechanisms of ovarian autoimmunity in birds (particularly wild populations), but antibodies that target ovarian tissue have been

identified and been associated with a decline in egg production with age in laying hens [91,93], and autoimmune thyroiditis is a clinical issue associated with obesity and fertility declines in chickens [46]. The frequency of immunocompetent cells present in the follicles of the hen ovary also decreases with age, suggesting a reduction in infection resistance in older hens that could have an associated impact on egg production [94]. The follicular reserve also depletes as birds age [95], inevitably resulting in changes to the HPG axis. This may occur via reduced secretion of gonadal steroids and peptides and/or reduced sensitivity of the hypothalamus to ovarian steroids, either because of diminished steroid stimulation or a general pattern of neural senescence [95]. Oestrogen has been associated with the upregulation of immunocompetent cells into maturing follicles, and so may be involved in the age-related decline of the immune response in the ovary [25,91,94].

e) Diet and stress

A wealth of experimental evidence in birds (mostly poultry) shows that diet strongly influences egg production and fertility. Striking the balance between optimum nutrient uptake (to maximise production) and nutrient toxicity and/or obesity is of primary concern to the poultry industry [96]. Modern broiler breeder hens are particularly sensitive to over-feeding during the weeks prior to laying, and even minor over-feeding can result in oviducal inflammation, prolapse and a reduction in egg production [97]. When broiler breeder hens are fed *ad libitum*, this can result in obesity and the onset of Erratic Oviposition and Defective Egg Syndrome (EODES), which is thought to be caused by excessive follicle development and the occurrence of multiple follicular hierarchies which disrupts ovulation [97]. Yolk formation is energetically demanding, and requires substantial changes in the body's metabolism of lipids [98]. Lipogenesis is responsive to both hormonal control as well as dietary changes, and overfed hens exhibit symptoms of lypotoxicity including ovarian abnormalities and follicular atresia

[98,99]. Feed restriction is a commonly used method for controlling obesity and the onset of EODES [97,98]. Over the last few decades, intense selection for greater body mass has resulted in an increase in food consumption during *ad libitum* feeding. Consequently, there has been an increase in the use and intensity of feed restriction regimes in broilers [96,98,99]. It is becoming increasingly difficult for poultry breeders to achieve a diet sufficient for growth and reproductive maintenance without over-feeding. Restricted feeding protocols also come with additional welfare issues, namely an increase in stress and social aggressiveness related to hunger [100].

Nutrients thought to be important for egg production and fertility in birds include: manganese; selenium; iodine; fluoride; sodium; zinc; copper; vitamin A; vitamin E; vitamin B_{12}; protein and linoleic acid [49,96,101–105]. An excess or deficiency in any of these can be disruptive. Nutritional deficiency and toxicity are common in captive birds but thought to be more rare in wild populations [101], although the dietary requirements and nutrient availability for wild populations are less well studied and may be impacted by environmental change and/or supplemental feeding. In particular, the nutritional needs of endangered populations with reduced natural habitat may be restricted, especially if they have been translocated to habitats with different food sources to those in their native range (but see Jamieson [101]).

It has long been known that stress also plays a vital role in the productivity of laying hens, and stressors may include fear (either of humans [106] or of novel social or physical environments [107]), insufficient space [108] and heat stress [109]. Heat stress reduces egg production by decreasing feed intake and causing nutritional deficiencies, but also by causing widespread disruption to the hormones important for ovulation [109]. In birds, temperatures above 30˚C can trigger heat stress [110], and Deng et al. [111] found that when laying hens were exposed

to 34˚C heat for 2 weeks, egg production decreased by 28.8%. Increasing environmental temperatures predicted under climate change is expected to have important repercussions for commercial egg production [110,112]. Evidence for thermally induced female fertility loss in non-commercial species is lacking [113], although in male zebra finches exposed to 30˚C and 40˚C heat there was an increase in the production of abnormal sperm [114]. Regarding wild populations; it is likely that small, isolated or endemic species are particularly vulnerable to heat-stress induced reductions in fertility, because lack of gene flow and genetic variation impose limitations on their ability to adapt to novel environmental stress. Species with limited ranges may also be at risk if they are unable to shift to cooler climates [113]. Early life/developmental conditions and stress may also heavily influence individual patterns of reproductive aging. For example, rates of reproductive senescence are higher in guillemots that invest more heavily in early life reproduction [115]; the effects of early life stress (in the form of predation pressure) increases the rate of reproductive senescence in barn swallows [116]; and female collared flycatchers from a low-competition natal environment experience higher reproductive rates early in life, but with a cost of earlier reproductive senescence [117]. We do not fully understand the mechanisms of fertility senescence in birds; more long-term longitudinal studies on age related changes in fertility traits are necessary, especially in wild (non-poultry) species and where environmental/developmental effects are incorporated [115,117–119].

f) Egg production: concluding remarks

The incidence of infertility resulting from egg production problems is unknown for most wild bird populations, making it difficult to determine how important it is as a driver of individual variation in fitness. Regardless, determining the incidence of egg production dysfunction is a logical step towards establishing the causes of reproductive failure. Future studies should

(where possible) attempt to collect data on failed breeding attempts (i.e. where copulation was successful, but no eggs were produced), particularly when the fertility status of the male breeding partner is known. This is likely to be somewhat easier for captive populations relative to long-term wild study populations, where data are not routinely collected on non-breeding individuals. Much of the avian fertility literature has a heavy focus on seasonally breeding species, where photoperiod provides a reliable cue eliciting an annual reproductive response. Generally, less is known about tropical/a-seasonal species and opportunistic breeders, where an individual's ability to respond rapidly to more unpredictable environmental cues is likely to significantly influence their fertility.

(2) Obtaining sperm

If ovulation proceeds normally, the ovum progresses into the infundibulum and a second glycoprotein layer is formed around it within approximately 15 minutes, preventing additional sperm from penetrating (Fig 3.). This short fertilisation window requires precise timing of insemination and/or the release of sperm from female storage to ensure sufficient sperm are in the infundibulum at ovulation [120]. Within the female reproductive tract, sperm are stored in blind ended tubular invaginations known as sperm storage tubules (SSTs) [121] found in the utero-vaginal junction (Fig 2.). While SSTs are considered the primary sperm storage site, it has been suggested that sperm may also be stored in the infundibulum. However, evidence for this is equivocal [122].

a) Copulation

Sperm may be prevented from reaching the site of fertilisation in several ways. Mechanical difficulties during mating resulting from physical injury (e.g. impaired vision or balance [44]) may prevent sperm from entering the reproductive tract. Clogged feathers (e.g. due to fecal

build-up, or heavy cloacal feathering) may also block access to the cloaca [45,123], though this may be more likely to occur in captive populations. In captive birds, failed copulation may also occur due to inappropriate husbandry, for example a lack of proper perching or nesting sites, aviary disturbances, a lack of flock stimulation or illness [45]. Immaturity and sexual inexperience may also result in failed mating in young birds [45]. If mating proceeds normally, then females could theoretically ensure sufficient sperm are available for fertilisation by copulating more frequently. Using an experimental approach that restricted inseminations by males, Török et al. [124] showed that multiple copulations were necessary to achieve a normal (unmanipulated) level of egg fertilisation success in wild collared flycatchers (*Ficedula ablbicollis)*, implying copulation frequency is important for fertility assurance [125]. However, multiple copulations could also reduce fertility if they damage the female reproductive tract. Copulation provides an opportunity for bacterial transfer [126,127], which can cause local inflammation in the vaginal wall, impairing sperm transport and reducing fertility [128]. In domestic turkey hens, inflammatory effects of repeated artificial insemination appear to be transient, with quick recovery [129], but the long-term consequences of repeated infections are unknown. The main defense against microbial infection in the oviduct is provided by the vaginal mucosa and associated mucin substances, although cilial action within the oviduct may also play a role in the removal of microbes [25]. Similarly to the ovary, TLRs and avian β-defensins are also expressed within the rest of the oviduct as well as other antimicrobial defensins such as gallin and cathelicidin [25].

Timing of insemination relative to ovulation is important for ensuring sperm are available for fertilisation at the right time. Fertility is increased in turkeys when sperm are artificially inseminated immediately before ovulation [130], possibly because sperm are lost passively from storage at a constant rate post-insemination [131]. In contrast, inseminations performed

immediately before or after egg-laying (egg-release from the cloaca) reduce fertility, possibly because egg-laying contractions impede the ability of sperm to move through the oviduct and/or the passage of sperm is blocked by the egg [23,25]. In chickens, sperm storage is up to 40 times more efficient when insemination occurs more than 4 hours after egg-laying [128]. Similar evidence of low sperm uptake during and just after egg-laying has also been observed for natural copulations [132].

b) Sperm transport to storage

Ensuring sufficient sperm are available for fertilisation has clear benefits for the female, but the mechanisms that might facilitate this are at odds with those facilitating female sperm selection. The vagina is considered the main sperm selection site in the avian oviduct [121,133], and only 1% of inseminated sperm make it through to the sperm storage tubules. Domestic fowl, for example, eject the sperm of undesirable males following forced copulations [134], thereby reducing the number of sperm available for fertilisation. The vaginal fluid of female barn swallows (*Hirundo rustica)* has also been shown to reduce sperm performance by varying degrees depending on female quality [135]. Huang et al. [136] found that vagina mucosal tissue of chickens produces exosomes (membrane vesicles enriched in transmembrane proteins) that significantly reduce sperm viability (possibly because they contain cytotoxic factors) and therefore may play a role in sperm selection. During egg production, vaginal pH and immunological activity also varies [23,137], and immune-competent cells appear to be expressed in the vagina [130]. Van Krey et al. [138] found that infertile females express antibody-producing plasma cells in their reproductive tract, and Higaki et al. [139] showed that the number of leukocytes present in the vagina increases following copulation. Localised immune responses are predicted to participate in non-random sperm selection [122], and the likelihood of an anti-sperm response may depend on male genotype [140]. For example, Løvlie

et al. [141] show that post-copulatory sperm selection in female red junglefowl (*Gallus* gallus) is biased towards males dissimilar at the MHC. Sperm are rapidly coated with immunoglobulin cells produced by the vaginal mucosa, and immunoglobulin IgA and IgG are thought to be at least partially responsible for the massive reduction in sperm viability during transport through the vagina [23]. Determining how female anti-sperm responses vary across individuals is a crucial step towards understanding the importance of the immune response for avian female fertility. Immune response strength likely depends on complex phenotypic trade-offs between fertility and infection resistance [142], similar to the trade-off females face between fertility and sperm quality during sperm selection. If sperm selection and/or immune response mechanisms are too effective, insufficient sperm may reach the site of fertilisation. Sperm selection and transport in the vagina may therefore be considered a balance between selecting high quality sperm, avoiding infection, and ensuring sufficient sperm remain available for fertilisation.

c) Sperm storage tubule function

Once sperm are in storage, the proper functioning of the SSTs is likely to be crucial to fertility, and a decline in sperm storage ability has been associated with fertility senescence in birds [143]. The number of sperm stored is strongly associated with the number that reach the ovum [144], and chickens selected for high fertility have significantly greater numbers of SSTs [130]. The mechanisms controlling sperm acceptance, storage and release are assumed to be under fine temporal control based on hormonal changes [145]. Fertilisation failure is therefore likely if hormonal imbalances result in a mismatch between the timing of ovulation and arrival of sperm in the infundibulum [122,145]. The significant variation in fertile periods across species has been attributed to differences in SST number and therefore sperm storage capacity [122],

but intra- and inter- specific variation in the structure and function sperm storage tubules has not yet been quantified.

The mechanisms by which sperm are maintained in a viable state in the SSTs are not fully understood, but it is thought that numerous compounds are produced to maintain a suitable environment for long-term sperm survival [122,146]. Recent work provides some experimental evidence to support this. For example, lactic acid – now known to be produced in SST cells in response to hypoxic conditions – can induce a reduction in sperm flagellar movement and so may contribute to the quiescence of Japanese quail sperm [25,147]. Additionally, Huang et al. [148] identified a number of fatty acids in the UVJ mucosa of domestic fowl, which have also been shown to depress rooster sperm motility, and *in vitro* sperm survival was found to be higher in the presence of oleic and linoleic acid. They also found that SST cells express lipid receptors, which may enable lipid droplets to accumulate and be used by resident sperm to maintain structural integrity. SST cells in turkeys are also known to shed microvillus vesicles that interact with sperm, transferring metabolic substrates that may be capable of temporarily inhibiting fertilising ability, protecting from oxidative stress, and transporting fluid from the SST cells into the SST lumen [149]. Whilst evidence suggests that these compounds play a key part in sperm maintenance, there are likely a number of other important compounds expressed in SSTs that have yet to be discovered [25]. The degree to which individuals and species vary in their ability to maintain sperm in storage is not clear, but this may play an important role in determining female fertility.

One factor that is essential for sperm survival in storage is the suppression of the female immune response, which if triggered can have highly detrimental effects on sperm. In domestic fowl, repeated artificial inseminations were associated with a complete lack of stored sperm

and a 57% decrease in fertility [150,151]. This has been attributed to an influx of lymphocytes and antigen-presenting cells into SSTs that likely impair sperm survivability, but also prevent sperm from entering storage [150]. A significant decrease in the expression of oestrogen receptors in the sperm storage tubules was also observed following infection, likely impairing the hormonal control of sperm storage tubule function [151]. Das et al. [152] demonstrated enhanced local expression of transforming growth factor β (TGFb) within sperm storage tubules, which suppresses the anti-sperm immune response by depressing the activity of lymphocytes.

As the rate of sperm release from storage increases, the duration of fertility will decline unless more sperm are inseminated [23]. Older hens tend to release sperm faster, possibly due to a decline in hormone production needed to regulate ovulation and sperm release [153]. This may partially explain why older hens have shorter fertile periods [23]. The mechanisms of sperm release are not fully understood, but SSTs have been shown to possess a constricted 'gate-like' entrance, that may act as a physical (and/or selective) barrier preventing sperm from leaving [154]. Constriction is likely to be hormonally triggered, since progesterone has been shown to induce contractions of the SSTs, and images taken using electron microscopy show sperm leaving the SSTs after intravenous injection with progesterone [155]. Furthermore, the specific membrane progestin receptor mPRα has been shown to be expressed within SSTs of Japenese Quail [155]. Additionally, in a comprehensive study, Hiyama et al. [156] demonstrated that heat shock protein 70 (HSP70) - a widespread and highly conserved molecular chaperone – is expressed in the utero-vaginal junction and its expression increases prior to ovulation. They also found that HSP70 binds to sperm and stimulates flagellar movement *in vitro*, and injection of an HSP70 antibody significantly reduces fertilisation success *in vivo*. Hiyama et al. speculate that HSP70 expression in the UVJ may be stimulated by progesterone; the progesterone surge

experienced prior to ovulation may therefore function in part to allow sperm to be released from storage at the right time whilst also ensuring sperm regain function. Imbalances in circulating progesterone levels can be triggered by conditions such as nutrient toxicity (e.g. excess fluoride [105]) and heat stress [157], and future work should explore if such imbalances reduce fertility by disrupting sperm release from storage.

d) Sperm transport to the site of fertilisation

Once released from storage, sperm are thought to travel passively through the uterus, isthmus and magnum (Fig.2) [122], as evidenced by the fact that dead sperm inseminated beyond the utero-vaginal junction reach the infundibulum in as great numbers as live sperm [158]. Past the utero-vaginal junction the reproductive tract is apparently free of immunoglobulins, and anti-peristaltic activity is thought to aid in the passive and rapid transport of sperm to the infundibulum, [159] where they remain until fertilisation [122].

(3) Achieving fertilisation

Following successful ovulation, the avian ovum is captured by the infundibulum where it encounters sperm. Successful fertilisation involves the initiation of multiple events in sequence: sperm-egg binding, acrosomal exocytosis, sperm penetration through the perivitelline layer and fusion of the male and female pronuclei in the germinal disc. The mechanisms of sperm-egg interactions in birds are not well understood, but the roles of several important molecules have been discovered.

a) The inner perivitelline layer

The inner perivitelline layer (IPVL), which is homologous to the zona pellucida (ZP) in mammals, is composed of a mesh of fibre that forms a 3-dimensional extracellular matrix.

Unlike in mammals, the IPVL of birds does not inhibit polyspermy [26], and in fact a degree of physiological polyspermy is required for normal development in birds [120]. The IPVL contains at least six ZP glycoproteins [14] (there has been significant confusion in the literature regarding the nomenclature of ZP proteins [160], here we provide the common aliases in parentheses), most notably ZP1 (ZPB1) and ZP3 (ZPC) which are major components of the IPVL and play a key role in the binding of sperm and initiation of the acrosome reaction [14,26]. Additionally, ZP2 (ZPA) has been found in the IPVL, but to a lesser extent, and accumulates primarily in the germinal disc region in chickens [161], but not turkeys. Interestingly, it is ZP4 (ZPB or ZPB2) that accumulates in the germinal disc region in the turkey [6], suggesting differences in sperm binding mechanisms may occur across species. Other minor constituents of the IPVL include ZPAX (ZPX1) and ZPD (UMOD), where ZPD has been found to be important for initiation of the acrosome reaction [26]. Acrosin, located in the sperm plasma membrane, was discovered to be a complimentary molecule that supports the binding of sperm to the ZP proteins in quail PVL [14].

In mammals, a variety of protein coding genes associated with gamete cell surfaces have been discovered [162–164]. This includes Juno and Izumo – the only known interacting pair of sperm-egg adhesion proteins. Izumo is a sperm protein [165], and Juno is the more recently discovered egg Izumo receptor [166]. In mice, Juno and Izumo knockouts result in sterility, and Juno is vital in preventing polyspermy; its rapid loss from the egg surface membrane following fertilisation causes the blocking of the zona pellucida to further sperm entry [166]. Reproductive proteins are known to evolve rapidly compared to many other gene classes, and both Juno and Izumo have been found to be under positive selection in mammals [167]. No such interacting proteins have been discovered in birds: comparisons of the genomic regions containing Juno (and surrounding loci) in mice and humans with that of the chicken shows that

they are generally syntenic (the gene order is conserved), however Juno loci are absent in the chicken [25]. A key step for avian fertility research will be to identify avian Juno and Izumo equivalents. Also essential for sperm-egg fusion is the ubiquitously expressed membrane protein CD9: female (but not male) CD9 knockout mice are infertile [164] - CD9 was the first identified gene with female specific fertility effects [168]. There is one known homologue of CD9 in the chicken (ID: AB032767) though to our knowledge no studies have explored the involvement of this (or any other gene) on sperm-egg fusion in birds [25].

b) Sperm-egg interaction and syngamy

Diagrammatic representations of fertilisation often depict the ovum oriented such that the germinal disc faces towards the reproductive tract (and oncoming sperm). However, we suggest that the animal pole – where the germinal disc is located - faces towards the ovary during ovulation and fertilisation (Fig 3.), because the ovum is in that orientation whilst inside the follicle [25], and to our knowledge there is no known mechanism by which it would turn to face the opposite direction after ovulation. If true, the consequence is that sperm would have to travel around the ovum to reach the tiny germinal disc target. Sperm are known to bind to the germinal disc region in higher concentrations than elsewhere on the ovum [161], suggesting there must be an underlying mechanism by which sperm locate and/or preferentially bind to this region. Such a mechanism has yet to be discovered, but Nishio et al. [161] suggest that sperm may locate the germinal disc region via egg chemo-attractants, and/or by utilising site-specific egg coat receptors. The PVL glycoproteins ZP2 and ZP4 are promising receptor candidates, since ZP2 and ZP4 are concentrated primarily in the germinal disc region of chicken and turkey PVL respectively, but their sperm binding properties have yet to be investigated [6,14,161]. Recently, a number of new PVL proteins have been identified which

appear to vary across species (see Damaziak et al., [169]), but their function in fertilisation has not yet been determined.

Once bound with the PVL, the sperm acrosomal contents (including proteases and endopeptidases) are released during the acrosomal reaction and locally degrade the PVL, forming a hole via which sperm can penetrate the ovum [14,25]. Sperm penetration holes are visible on the PVL *in vitro* (Fig. 2 B) and can be used as a reliable proxy for the number of sperm that reach the ovum [170,171]. Following the acrosomal reaction, the inner acrosomal membrane of sperm becomes exposed, binds to the ovum and the male pronucleus is released. While multiple sperm can penetrate the PVL in birds, only one male pronucleus typically fuses with the female pronucleus in the germinal disc – though the exact mechanisms of avian syngamy remain unknown [25]. Supernumerary male pronuclei are degraded by DNAses in the germinal disc and PVL of mature oocytes [172]. During or immediately after fertilisation, a granular continuous layer is laid down around the ovum, followed by the outer perivitelline layer (OPVL), which blocks further sperm entry (Fig 3.) [25]. The OPVL is multi-layered and composed of proteins secreted by the infundibular mucosa [173]. Macrophages present within the infundibulum are thought to function in the phagocytosis of superfluous sperm (i.e. those that did not participate in fertilisation) [174].

c) Variation in egg quality

In addition to proteins, the consistency and structure of the IPVL differs markedly between species. For example, Damaziak et al. [169] observed that cockatiels have a more densely and irregularly arranged IPVL than that of three other species studied (pigeons, grey partridges (*Perdix perdix*) and pheasants). They also found that the pigeon PVL is markedly different in structure; its numerous sublayers are more homogenous, less porous and unusually loose in

arrangement compared with the other species. Pigeon PVL is also composed of flat sheets rather than the cylindrical fibers which are observed in the PVL of all the other species. It is unknown how this variation in structure affects the integrity of the PVL, its interaction with sperm, or whether this variation corresponds to post-copulatory sexual selection intensity and/or sperm traits. Damaziak et al. suggest that interspecific variation in PVL structure may be related to differences in the function of the PVL during embryo development, which may vary depending on whether the species is precocial or superaltricial. The germinal disc region is also known to show subtle intra- and inter-specific variation in terms of morphology, and also in terms of the location, size and number of sperm penetration holes [175]. It is currently unknown how variation in PVL structure affects fertility, but fertilisation rates are positively correlated with the number of sperm that penetrate the PVL [176]. There is known to be variation in how readily sperm can bind to the PVL [133,177], and it seems logical that intra- and inter-specific differences in PVL structure may affect how easy it is for sperm to bind to and penetrate the PVL. In taxa where polyspermy is lethal to the egg (such as in mammals), egg 'fertilisability' is known to vary according to the risk of polyspermy [178]. Consequently, females and males will be locked in an apparent cycle of co-evolutionary conflict where females are selected for greater 'egg defensiveness' (resistance to sperm) and males selected to counter this with greater fertilising ability and competitiveness [18]. Currently, egg defensiveness has been most largely explored in sea urchins and in mice [19,178], with virtually nothing known in birds. Since polyspermy is a normal and important part of fertilisation in birds, this suggests that mechanisms of polyspermy avoidance are unlikely to be important other than to prevent excessive sperm penetration that might damage the integrity of the ovum. Theoretically, females might be expected to evolve mechanisms of resistance to sperm for other reasons, for example to alleviate the costs of hybridisation, avoid incompatible sperm, or as a mechanism of selection for high quality sperm [19,179]. Indeed, evidence

suggests that the strength of positive selection on gamete-recognition genes is similar between birds and mammals, suggesting that in the absence of polyspermy avoidance, there must be some other adaptive mechanism to explain the rapid evolution of avian gamete-recognition genes [179]. Recently, Hurley et al. [170] found significant variation in PVL sperm numbers between breeding pairs of estrildid finches, as well as variation in PVL sperm numbers across the laying order. It was unclear however, if this variation was male or female mediated. Exploring the degree of variation in egg quality, egg defensiveness and sperm selection at the gametic level is challenging but important for elucidating the full role of the avian ovum for fertility.

Whether other aspects of ovum quality, such as the integrity of DNA in the female pronucleus, influence the likelihood of successful sperm-ovum fusion remains unknown. In mammals, heritable mutations of ZP2 and ZP3 are known to cause infertility [180], and antibodies raised against ZP proteins can depress ovarian function [6]. Investigating the incidence of similar ovum abnormalities and immunological activity, and the degree to which they might affect avian fertility, may be a fruitful avenue for future research.

IV. Conclusions and future directions

It is clear that females can exert far more control over fertilisation than has historically been assumed, but if and how females influence whether their ova are successfully fertilised is often ignored in favour of male processes (such as sperm quality and quantity) [6]. Here we have quantitatively demonstrated that there is a bias in research effort towards the study of male fertility rather than female fertililty in birds. We also show that the vast majority of avian fertility research has focussed on captive populations, with a further taxonomic bias towards gallinaceous birds and the domestic chicken in particular. We have also highlighted

key advances and gaps in knowledge on the role of female physiological processes in determining fertilisation success. In particular, the field would benefit from more studies investigating variation in fertility in non-poultry species (i.e. that have not undergone intense artificial selection for high productivity), and wild populations. Within wild birds, more attention to a-seasonal/tropical species, and opportunistic breeders would also be valuable. Although we acknowledge that detailed study of variation in female fertility may be difficult in wild populations, because information about non-breeders is not always easy to collect, we nonetheless urge that such efforts are made, particularly in non-poultry species and managed and/or experimental populations where male processes can be controlled for. If studies are unable to monitor failed breeders (i.e. those that did not produce any eggs following a successful copulation), it would be useful to acknowledge that infertility may be underestimated.

When investigating reproductive failure, the fact that infertility and embryo mortality are fundamentally distinct processes needs to be explicitly stated. Specifically, that infertility is used to describe failed fertilisation, and any process contributing to failed fertilisation is a mechanism of infertility (rather than embryo mortality). Similarly, if an egg fails to hatch but was fertilised, then the cause of hatching failure must be referred to as embryo mortality, even if development arrested after only a few cell divisions. If fertility status cannot be unequivocally determined using the appropriate techniques (see Birkhead et al. [10]), then the mechanisms of hatching failure cannot be conclusively known. Very early embryo mortality is likely to be mistaken for infertility when using traditional methods (e.g. candling or macroscopic examination), which may result in an overestimation of infertility [9]. Moving forward, a clearer estimation of the incidence of infertility in a given population will require a

combination of both careful monitoring (to identify failed breeders) as well as an accurately determined fertilisation status for unhatched eggs.

The female reproductive tract typically offers a hostile environment for sperm, providing considerable potential for female processes to influence sperm survival and transport to the ovum. While the processes of sperm selection, storage, release and transport within the reproductive tract has received increasing research attention over the past few decades, we still lack fundamental understanding of the underlying mechanisms, and the degree of intra- and inter-specific variation in these processes, with the vast majority of work having focussed on a very limited number of domestic species. Many of the female-mediated processes required for high fertility also deteriorate to some degree with age, making fertility problems more likely in older birds. This may have particularly important consequences for captive and managed threatened populations, where individuals may reproduce to an older age than their wild counterparts, due to reduced predation and competition pressure, and high accessibility of food and other resources. The field of avian reproductive science will also benefit from better understanding the impact of other factors on female fertility, such as stress, hormonal and physiological disorders (particularly in wild birds); environmental pollutants, intra- and inter- individual variation in egg production, egg quality, sperm selection, and the female immune response within the oviduct (including the ovaries).

The causes and maintenance of variation in fertility is a key question in evolutionary biology, and one in which the role of the female is often sidelined. Our hope is that this review challenges the field of avian reproductive science and evolutionary biology to consider female processes to a greater degree when investigating the causes of depressed fertility in birds. Using birds as a model system for the study of female fertility across taxa presents

several advantages and will provide insights not only in the field of reproductive biology, but also for fields such as conservation and commercial animal breeding as well.

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Appendix B

We thank the editor and reviewers for their time and efforts spent reviewing our manuscript, and we thoroughly appreciate the comments and suggestions that have been provided. The reviewers offered us much food for thought, and we have extensively revised the manuscript based on their suggestions. We feel that the review is much improved as a consequence of this process. The changes we have made are detailed on a point-by-point basis, with each reviewer comment followed by our response in bold type. We also provide a version of the manuscript with all the changes highlighted to facilitate further review, and a clean version with all changes made but not highlighted. We hope you now consider our manuscript suitable for publication in Open Science.

Response to reviewer 1

Reviewer comment:
I would not consider this to be a comprehensive review. But based on the objectives listed at the beginning of the paper, I am not sure it is meant to be. There are several other papers on the topic in poultry in particular that are not included in this review.
Author reply:
We would like to thank Reviewer 1 for taking the time to read and comment on our paper – many of the suggestions made have been very useful, and have facilitated changes which we feel have significantly improved the manuscript. With respect to the reviewer's point about the comprehensiveness of the review: our intention for this review was to integrate the most valuable and key insights from a broad range of fields. In this way we hope this review taps into a broader readership, encouraging greater cross-utility between different fields such as behavioural ecology, evolutionary biology, conservation science etc. We have therefore not been able to cite every relevant fertility paper across all these fields, however we do believe the review to be extensive and thorough, covering the most important aspects of female physiological function and fertility. To ensure our intentions for the review are not misunderstood, we have added a clarification to the introduction which describes its aims and scopes (lines 66-69). We have also included a number of extra citations in the text, including those that you have suggested. We would also like to mention that the quantitative analysis section was comprehensive, including every relevant fertility paper from a systematic Web of Science search, and these papers can all be found in the supplementary data provided.

Reviewer comment:
In the sperm:egg interaction section of the review, I know of several papers that are not cited. There is at least one theory of thought that i did not think was included and that is the interaction of the yolk with the IPVL in the non-germinal disc areas of the egg. I provided the authors some of those references but it has been a while since I have reviewed the literature on that topic and i would not consider this to be a comprehensive list.
Author reply:
Thank you for pointing this out, we have now included this in lines 633-639.

Reviewer comment:

Line 35 – 37: This is not an objective or definitive statement. Surely a review of the literature such as the current manuscript would be able to more better define the differences in male and female work. While one paper may state this, it does not necessarily make it true.

Author reply:

We have added additional lines here (lines 32-35) to further support the hypothesis that females have received less attention in avian fertility research. The intention for this line is to introduce the hypothesis that female fertility has been understudied, setting up the reader for the quantitative analysis which later provides evidence to support this.

Reviewer comment:

Line 54 – 56: Reproductive traits are only focused on in either the breeds used for table egg production or in some of the female broiler and turkey lines. Otherwise, the selection focus is on economically important traits (weight, weight gain, feed conversion, etc.) which are often inversely correlated with reproductive efficiency.

Author reply:

While it is certainly true that not all lines are selected for egg production traits, the focus of this point was to draw attention to the pervasive issues associated with egg production even in the layer lines where egg production has been specifically selected for. We later make the point that in some lines, economically important traits have been selected for at the expense of fertility (see lines 430 and 684). However, we have also altered this sentence (now line 53 and 55) to include the caveat that selection for efficient egg production has only been focussed on in certain lines.

Reviewer comment:

Line 83 – 84: The wording here makes it sound like scientists are ignoring the female fertility research but in reality it is more likely that male and male management factors are more significant factors that with correction influences the number of fertilized eggs more. Suggest rewriting this this sentence

Author reply:

We have included here some additional evidence that the pattern of a greater focus on male fertility is consistent across both wild populations, and captive populations – even when excluding the domestic chicken (which makes up over half of all the captive studies). This suggests that even beyond the studies made in poultry, male fertility is still focussed on more heavily. We have, however, altered this sentence here to avoid using the term 'bias' as per your suggestion, rewording it to the following: "there is a deficit of papers focusing on females (compared with males) within avian fertility research" on lines 86-87. We have also included a further statement about the practical advantages of studying male fertility traits for commercial species (lines 106 – 110), and reworded other lines of the review in keeping with the change in terminology.

Reviewer comment:

Line 92 – 93: Again, is bias the correct word to use here? More efforts on captive populations are probably overwhelmed by those studies that are carried out with poultry.

Author reply:

As above, we have altered the wording used here. We ensure that the new wording we have applied is consistent throughout the review when referring to this point.

Reviewer comment:
Line 100 – 108: While these points may be factors in the number of male to female fertility studies, it may just be the case, especially in poultry, that males mate with up to 10-15 females. So that management of the male with regards to fertility would affect the number of fertilised eggs more while having a larger economic impact.

Author reply:

It is definitely true that there are several practical advantages to studying sperm relative to ova, and we have further highlighted this within the text. However, the result we have found stands even when we remove domestic fowl (which make up half of all captive studies and is likely to be the main driver of any effects linked to commercial/economic impact) from our dataset. We have now included some additional evidence showing that even when removing domestic fowl, there are over twice as many papers focussed on males than on females. We also see this pattern mirrored in wild populations. We think this was an important point to raise. It suggests that even beyond any economic advantages to studying males for poultry science, this pattern is consistent across the entire field (see lines 100-104). That said, we do agree though that the historic economic and practical benefits for studying males in poultry has probably driven greater research, and this has likely contributed to the greater number of papers on males outside of poultry research as well, since we expect such advances to generate positive feedback in research effort. We had already made this point within the text, but have highlighted it further on lines 110-112.

Reviewer comment:
Line 140: Does the follicle or egg rupture?

Author reply:

It is the follicle that ruptures at the stigma region, releasing the ovum. To ensure this is clear we have adjusted the wording of this line (now line 160 – 162).

Reviewer comment:
Line 432 - 434: Bird size due to obesity as an impediment to successful mating?

Author reply:

Yes, thank you for pointing this out. This is now included (now line 450).

Reviewer comment:
Line 621: Another theory on the mechanism of preferential binding of sperm in the germinal disc region involved the discontinuous nature of the oolema which allow yolk to contact those areas of the IPVL. As a result of this, structural blocks from the yolk/IPVL interaction may prevent sperm from attaching. Bakst and Howarth 1977a; Robertson 1999. Also see Fertilization in Birds, 2000 Wishart and Horrocks in Fertilization in Protozoa and Metazoan Animals

Author reply:

Thank you, we have included a short statement regarding this hypothesis on line 633-639, including your suggested references in addition to some others.

Reviewer comment:
Line 701: How would it benefit? Up until this section the review has been backed by research studies. In these few sentences more opinion than fact are offered.

Author reply:
The previous sentence draws attention to our finding that far fewer studies have investigated fertility in non-poultry / wild species, which our earlier quantitative analysis finds evidence for. The intention here is to highlight that further studies in wild populations will rebalance our knowledge. But to highlight further the benefits to studying wild populations we have added an additional statement to expand on this (line 789 - 792)

Response to reviewer 2

Reviewer comment:
I have not followed the avian ovarian physiology literature in several years, but I am knowledgeable in the area of reproductive ecology and behavior. So, I feel I should be able to provide some insight from that perspective. My overall impression of this manuscript is very favorable. It is an important topic, and the authors are correct that fertility problems have been poorly defined historically and that female fertility is a vastly understudied area. The authors seem to cover the topic thoroughly, so it is my opinion that this manuscript would make an important and much-needed contribution to the field. With all that said, however, I am not convinced that Royal Society Open Science is the best venue for its publication, given the journal's own account of favoring papers that are of very broad, general interest. Indeed, I am not sure how broad and general the interest in physiological mechanisms of female bird fertility is. If the paper were on mechanisms of vertebrate or perhaps mammalian fertility, it might be of broader interest. But its current focus on female birds would seem to lend itself better to a bird journal, perhaps, or a journal more specifically oriented toward reproductive physiology, such as Biology of Reproduction.
Author reply:
We would like to thank Reviewer 2 for the time taken to review our manuscript, and their positive comments and support of the need for this work. The suggestions made by Reviewer 2 have facilitated productive discussion, and the changes we have made as a consequence have significantly improved the paper. While we appreciate that historically, mammalian fertility is considered to be of more general interest, birds are one of the most intensely studied taxa in terms of reproduction and fertility. Birds are also far more diverse and more populous than mammals, and are incredibly popular study organisms within a variety of scientific fields. Furthermore, we believe that this review encourages greater integration between disciplines, bringing together an extensive range of literature that will interest a broad readership. Insights from birds – an important model species for fertility – will be of great value across taxa and we think it would be a shame to limit its readership to ornithologists alone. Royal Society Open Science specifically encourages reviews that generate new avenues for future work, which we have provided extensively throughout the review, as well as providing constructive critiques of fields, which we also focus on heavily. Consequently, we believe that Royal Society Open Science is the perfect home for this review.

Reviewer comment:
As I indicated, however, I do feel that it is a worthwhile contribution, but some changes, as follows, would make it better. First, it is difficult to understand its overall organization. I

wonder if a table of contents would be appropriate. I found it difficult to understand how each section tied in with the one before it and the one after it and then how they all fit together to address the central question: what causes infertility in female birds? One possibility would be to organize the paper around the anatomy of figure 2, walking the reader through each part of egg formation and how fertility can be affected in that part. Additionally, somewhere around line 41, it would be extremely helpful to list as many mechanisms for failed syngamy as possible in order to set up the rest of the paper. The paper could then be better organized around these. Currently, it seems vaguely organized around aspects of Figure 2, but a more deliberate breakdown of the specific ways syngamy can fail, perhaps in order of timing, would greatly facilitate the organization of the paper and therefore understanding it. Currently the paper reads like a long list of what we know about reproduction with little direct tie-in to failed syngamy, especially in any sort of chronological or other logical order.

Author reply:

This is an excellent suggestion. We have consequently restructured the review to pull focus back onto the specific mechanisms of female infertility in birds. As you suggested, we have organised the paper to follow roughly the anatomy of Figure 2. The introduction and systematic analysis of the literature sections have remained as they were, but section III is now restructured with the title of this section and the title of the subheadings pulling more focus onto the mechanisms of fertilisation failure. We feel this has much improved the entire manuscript, with the organisation having a more succinct and understandable flow. We also were able to provide a broad list of the mechanisms of infertility within the introduction (line 72 – 74), as you suggested, and this sets the reader up nicely for the rest of the review since this list now mirrors the overall structure of section III. We also found that by restructuring the review we have naturally incorporated many of your other suggestions and comments. We decided not to include a contents table given that this does not seem fitting with the general style of Royal Society Open Science, but have provided a table of the new structure for section III below for your information:

III. What causes fertilisation failure in female birds?

- **1 Failure during egg formation**
  - **1.1 Hormonal factors**
  - **1.2 Disease and immune factors**
  - **1.3 Environmental factors**
    - **1.3.1 Diet**
    - **1.3.2 Stress**
    - **1.3.3 Pollution**
- **2 Failure during ovulation**
  - **2.1 Hormonal factors**
  - **2.2 Disease and immune factors**
- **3 Failure to obtain sperm**
  - **3.1 Copulation**
  - **3.2 Timing of insemination**
  - **3.3 Vaginal sperm selection**
- **4 Failure to maintain and transport sperm**
  - **4.1 Sperm storage tubule function**
  - **4.2 Sperm release and transport**

5 Failure of sperm-egg fusion

5.1 Sperm-egg interactions

5.2 Syngamy

Reviewer comment:

I think about fertility from an adaptive perspective. Given the presence of a multitude of life-history trade-offs (particularly the trade-offs between number of offspring and quality of offspring within a single brood and also the trade-off between number of current offspring and number of future offspring), perhaps the term "failure" is inappropriate when describing when fertilization does not occur. There may be times when it benefits the female from a fitness perspective to avoid fertilization, even if it means the wasted time and energy that went into production of an infertile ovum or egg. Given that, at least in wild, altricial birds, far more investment occurs in feeding young than in forming the egg for the young, a female suddenly faced with an unexpected challenge (such as unexpected energy demands, predatory risks, or poor quality mate) might benefit by selectively blocking fertilization to avoid costly stages later during nestling rearing when conditions might not merit the investment. For example, the female may be constrained to laying 4 eggs, but challenging food conditions might mean that, this particular bout, 3 eggs might be more adaptive, so she can at least raise 3 good offspring rather than 4 poor ones. In this case, one way to save energy would be to adaptively prevent fertilization of one of the ova, giving rise to 4 eggs but only 3 mouths to feed. Ultimately, I think use of the terms "fail" and "succeed" when it comes to syngamy or fertilization is loaded and assumes fertilization is always adaptive, when there might be situations in which it is not adaptive.

Author reply:

This is an interesting point, however we believe that it goes somewhat beyond the scope of the current review to consider the adaptive potential of infertility. Here, we are solely concerned with physiological fertility problems encountered by female birds, making the term fertilisation 'failure' appropriate in this case. Furthermore, the potential for infertility to be adaptive is currently rather speculative, with (to our knowledge) no current research indicating that infertility itself could be advantageous, and no known mechanisms by which it could occur. We believe there is a strong argument that selective embryo mortality (i.e. rejection of the egg after laying) could be adaptive, and indeed this does occur in some species with some females choosing not to incubate certain eggs/clutches. However, we do not believe a block to fertilisation is likely to be either physiological feasible or adaptive. That is because, once ovulation has occurred, the female has no choice but to lay the resulting egg. A mechanical mechanism to block fertilisation of an ovum would therefore not ameliorate any costs of reproduction and so be unlikely to evolve. Theoretically, if a female could selectively fail to produce an egg in the first place, this could be adaptive since it avoids the initial heavy cost of egg production and laying. However, in many species, females lay eggs even when they do not have access to sperm, suggesting that control over egg production itself is limited. A final theoretical mechanism would be to avoid copulation itself, although this would not be possible for species where forced copulations are common. Females could alternatively physically reject sperm before it reaches the site of fertilisation, though this would not be adaptative for species that lay eggs even without the presence of sperm, and would not ameliorate the costs associated with copulation either. Given that, we do not believe

there is currently a strong argument for infertility to be adaptive, and so for the purposes of this review, we believe that the term fertilisation 'failure' is acceptable.

Reviewer comment:
Lines 408-410: Up to this point, the manuscript has mainly focused on what we know in domestic species, each time referring to how little is known in wild species. The reader is left wondering what IS known for wild species. So far, the manuscript mostly reads as a review in domestic species despite what seemed to be a focus early on on the importance of this topic to wild species.

Author reply:

We agree that it is surprising and somewhat frustrating that such little knowledge exists for wild populations. Indeed, it is partly our intention to highlight this within the review. We have included the most relevant and key insights gained from wild species, which admittedly is dwarfed by what is known in domestic birds. We agree however, that the inclusion of this information was not well balanced throughout the review under the previous structure. As per your recommendation, we have restructured the review, and one of our goals here was also to better highlight the findings for wild species within this new structure. Since the majority of research into fertility in wild species has focussed on the influence of environmental factors (such as heat stress/ climate change and pollution), we have drawn better attention to this by creating a separate 'environmental factors' subheading early in the review. We would also like to draw your attention to the analysis where we have further highlighted that there is a deficit of fertility papers on wild populations, and we also return to this point within the conclusions and future directions section.

Reviewer comment:
Lines 686-687: It is this possibility specifically that I think of above all else when it comes to possible ways for fertilization to fail, and yet apparently nothing is known. Nonetheless, if the authors could go into greater detail, even if just speculation, I think it would resolve a lot of wondering by their readers.

Author reply:

We agree that is somewhat surprising that so little is known about how ovum quality influences fertility in birds. To address your suggestion, we have included some more speculation as to the influence of ovum quality on fertility, specifically drawing more parallels with what is known in mammals, and suggesting similar mechanisms could occur in birds. We believe this section is now much improved due to this inclusion.

Reviewer comment:
In several places throughout the manuscript, the authors mention effects of aging on female fertility and mechanisms surrounding it. Although aging is relevant to an extent, it is unlikely to be particularly relevant in wild birds that never get very old. In fact, most wild birds may experience age-related increases in reproductive function during their short lives, and this may be due, in part, to priming by photostimulation (Sockman, K.W., Williams, T.D., Dawson, A., & Ball, G.F. (2004). Prior experience with photostimulation enhances photo-induced reproductive development in female European starlings: a possible basis for the age-related increase in avian reproductive performance. Biology of Reproduction 71, 979-

986.)

Author reply:

Thank you, yes this is an interesting point. While we do see an initial increase in reproductive success in some seasonally reproducing birds, the general and gradual decline in fertility with age is common across species. It is usually observed in birds as an increase in the length of inter-clutch intervals, smaller clutch sizes and a general increase in the number of failed reproductive attempts (Ellison & Ottinger, 2014). You are right in that this is primarily of concern to captive populations of both commercial and non-commercial birds which do tend to live longer than many wild species and also be pushed to be reproductively active for longer too. However, ageing in wild species has been shown to also be widespread across birds and mammals (e.g. Nussey et al., 2013; Robery et al., 2015), and it is also a very important consideration for some endangered species, (such as the whooping crane (Brown et al., 2019)) and for managed wild populations where management interventions (such as supplemental feeding) influences patterns of ageing. We have consequently included a short discussion here (line 340 -353), emphasising that fertility can initially increase with photo-experience. We have included your references here as well as the notable example of fertility senescence in whooping cranes, and the contradictory example of long-lived seabirds who experience apparently no reproductive decline throughout their lives.

Reviewer comment:

Lines 28-29: Delete.

Author reply:

Thank you, we have deleted this line.

Reviewer comment:

Line 40: Is this the most broadly used and accepted definition of infertility? If not, reword to say something like "We define infertility as . . . "

Author reply:

Thank you, we have corrected this.

Reviewer comment:

Line 87: It may be more technically difficult to study and understand female compared to male fertility.

Author reply:

We have further highlighted the technical and practical advantages to studying male fertility vs female fertility (lines 106-110).

Reviewer comment:

Lines 133-136: Out of curiosity, how is the left but not right protected?

Author reply:

This is a really interesting question which unfortunately doesn't appear to have a clear answer. Briefly - both the left and the right ovary begin developing together but then asymmetric development is observed after around 6 days of incubation. Development of the left ovary is promoted by asymmetrical gene expression and migration of primordial germ cells into the left gonad during embryogenesis. Expression of pituitary homeobox 2 (*PITX2*) within the left ovary suppresses oestrogen receptors in the right ovary, resulting in

elevated concentrations of oestrogen in the left ovary relative to the right. The anti-Müllerian hormone (AMH) (the expression of which is regulated by the transcription factor SF1) is then thought to initiate apoptosis of the right Müllerian duct, causing it to regress. As briefly mentioned in the manuscript, it is widely thought that the elevated concentrations of oestrogen in the left ovary suppress the anti-Müllerian hormone receptor, and this prevents the left ovary from regressing. Unfortunately, the molecular mechanisms underpinning this have not been fully defined, and while many papers do find evidence for apoptosis in the right ovary (e.g. Ukeshima 1996, Grzegozewska 2012, Shaikat et al., 2018 etc), there is at least one case where apoptosis was not evident (De Melo Bernardo et al., 2015). We believe including full details of the molecular mechanisms governing this process goes beyond the scope of this paper, however we have added a clarification here that this is still an area of active research.

Reviewer comment:
The paper's figures are beautifully made. Textbook quality.
Author reply:
Thank you for this comment!

Reviewer comment:
Lines 192-193: Although not directly tied to the topic of fertility, it might be worth mentioning the role this is thought to play in yolk steroid deposition (Schwabl, H. (1993). Yolk is a source of maternal testosterone for developing birds. Proceedings of the National Academy of Sciences USA 90, 11446-11450; Groothuis, T.G.G., Müller, W., Von Engelhardt, N., Carere, C., & Eising, C. (2005). Maternal hormones as a tool to adjust offspring phenotype in avian species. Neuroscience and Biobehavioral Reviews 29, 329-352).
Author reply:
Thank you for bringing this up, and it is certainly an interesting phenomenon, however here we primarily discuss the influence of gonadal steroids only as it pertains to maternal fertility (in this case ovulation), not of how maternal hormones later go on to influence embryo development. We have tried to steer away from a discussion of embryo development as much as possible given how common it is for researchers to confuse infertility and embryo mortality with each other. We believe a discussion of this might muddy the waters in terms of fertility, so have chosen to leave this out.

Reviewer comment:
Line 220: But see: Arlt, D., Bensch, S., Hansson, B., Hasselquist, D., & Westerdahl, H. (2004). Observation of a ZZW female in a natural population: implications for avian sex determination. Proceedings of the Royal Society of London B Biological Sciences 271, S249-S251. doi:10.1098/rsbl.2003.0155
Author reply:
Thank you, we have now included this reference (now line 718)

Reviewer comment:
Lines 457-458: Increased relative to what? To a time more distant from ovulation?
Author reply:
We have reworded and improved the clarity of this section which is now on lines 479 – 484.

Appendix C

We are thrilled at the editor's decision to accept our manuscript for publication. We would like to thank the editors for their time and efforts spent attempting to obtain a second reviewer to assess the changes we have made. We appreciate the decision to move forward based on the feedback from reviewer 1, and further thank reviewer 1 for the time taken to review our manuscript for a second time. We have addressed the additional suggestion made by reviewer 1 below and have attached both a clean and marked up version of the manuscript with the changes highlighted. Thank you again for accepting our paper for publication in Open Science.

Kind regards,
Katherine Assersohn, Patricia Brekke and Nicola Hemmings

Response to reviewer 1

Reviewer comment:
Personally I think your revisions have improved the paper greatly. I only have one minor suggestion before publishing. In every other case in the paper were the term "for example" was used, you provided an example. In one case you did not. See the Line 445 comment below.

Line 445 – This is a personal preference, but I do think it helps the readers if when the text says "although there are a few example…", especially those new to the an area, make the information more useful to them, that some specific examples, maybe in parenthesis are provided.

Author reply:
We agree the paper is much improved and this is largely thanks to your helpful feedback, which we are extremely grateful for. Thank you for the additional comment, we agree and have addressed this in the manuscript by adding several examples in parentheses as you suggest, and we have included two additional references.