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PERSPECTIVE

The time is ripe for functional genomics: Can epigenetic changes mediate reproductive timing?

Melanie J. Heckwolf[1] | **Britta S. Meyer**[2]

[1]Gurdon Institute, University of Cambridge, Cambridge, UK

[2]Max Planck Research Group Behavioural Genomics, Max Planck Institute for Evolutionary Biology, Plön, Germany

Correspondence
Melanie J. Heckwolf, Gurdon Institute, University of Cambridge, Cambridge, UK.
Email: mh2118@cam.ac.uk

[Correction added on 31-January-2022, after first online publication: The copyright line waschanged.]

Populations are under strong selection to match reproductive timing with favourable environmental conditions. This becomes particularly important and challenging with increasing interannual environmental variability. Adjusting reproductive timing requires the ability to sense and interpret relevant environmental cues, while responding flexibly to their interannual variation. For instance, in seasonal species, reproductive timing is often dependent on photoperiod and temperature. Although many genes influencing the timing of reproduction have been identified, far less attention has been paid to the gene-regulatory cascades orchestrating these complex gene-environment interactions. In a From the Cover article in this issue of *Molecular Ecology*, Lindner, Laine, et al. (2021) addressed this knowledge gap by investigating the role of DNA methylation in mediating reproductive timing in the seasonally breeding great tit (*Parus major*). Using a clever blood sampling design, they investigated genome-wide DNA methylation changes following individual female birds across multiple reproductive stages. This approach revealed 10 candidate genes with a strong correlation between promoter methylation and reproductive status. Some of these genes are known to be involved in reproductive timing (e.g., *MYLK-like* or *NR5A1*), yet for others this function was previously unknown (Figure 1). Interestingly, *NR5A1* is a key transcription factor, which may affect other genes that are part of the same regulatory network. The findings of Lindner, Laine, et al. (2021) provide a strong case for studying DNA methylation to uncover how gene-environment interactions influence important life-history traits, such as reproductive timing.

Reproductive timing is a key life-history trait that influences reproductive success through a match between reproduction and favourable environmental conditions for offspring. In seasonal and temperate-breeding bird species a variety of proximate factors were found to start the onset of gonadal maturation and thus reproduction, which act in hierarchical order. A primary environmental factor is day length, followed by food abundance and temperature (Dawson, 2008). Previous research has focused on identifying the genes that mediate intraspecific differences in reproductive timing (Liedvogel et al., 2009) and associating phenotypic trait variation, including gene expression, with early or late reproduction (Laine et al., 2019). However, far less attention has been paid to the gene-regulatory cascades mediating the interplay between environment and genome. Epigenetic modifications, such as DNA methylation or histone modifications, may play an important role in these gene-environment interactions, as they cannot only be modulated by the environment, but also regulate the expression of genes (Buchberger et al., 2019). Thereby, they have the potential to translate environmental influences into gene-regulatory signals, which can ultimately fine-tune phenotypic variation in ecologically relevant traits. To understand these processes, we need to investigate molecular changes in an ecologically relevant context, that is, in wild populations of

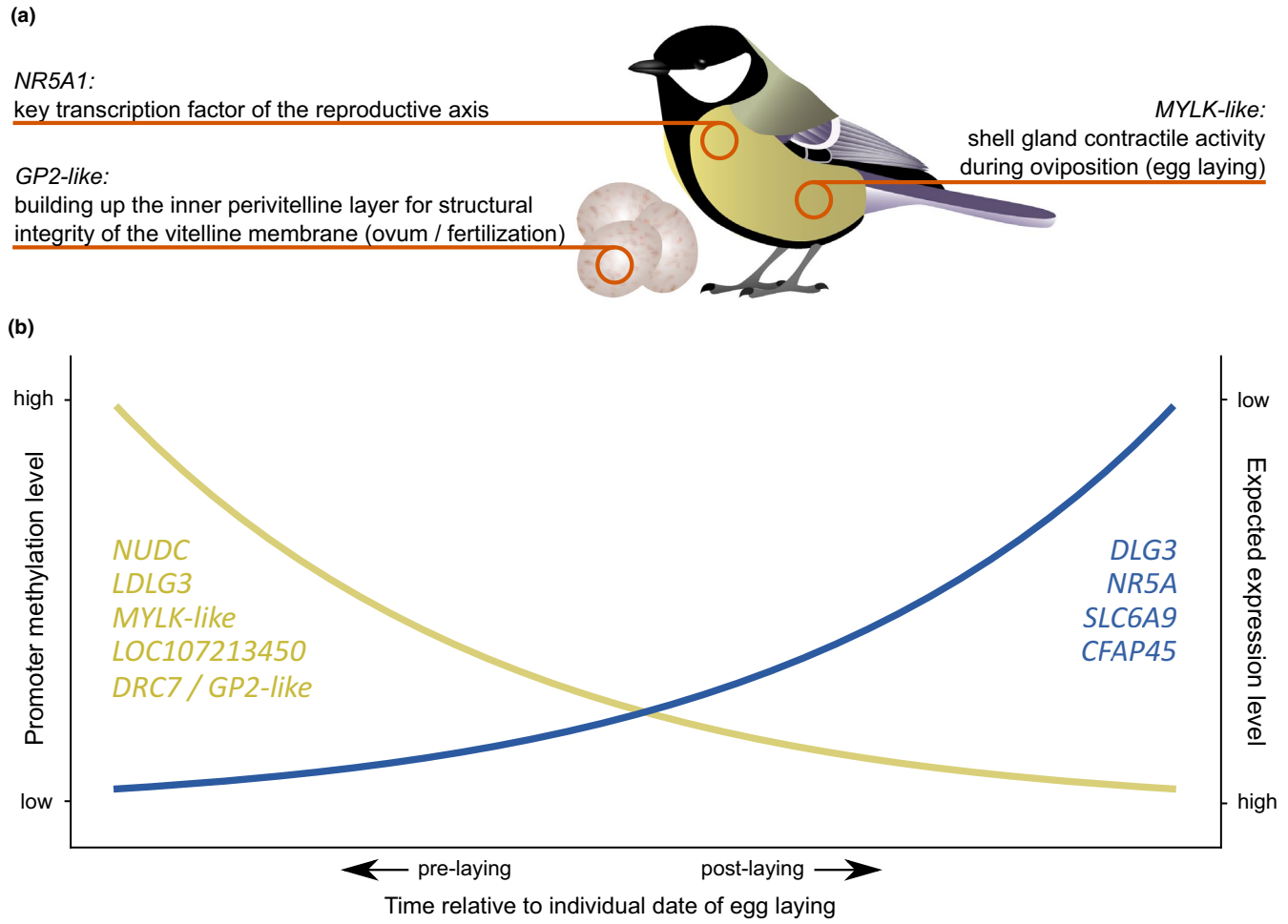

FIGURE 1 Candidate genes that mediate reproduction and egg laying in female great tits. Genes were derived from DNA methylation profiles of blood samples. Three of the 10 candidate genes with known reproductive function are described in (a). Promoter methylation levels and anticipated expression levels of the 10 candidate genes along the reproductive stages are schematically displayed in (b)

non-model organisms. However, this poses several challenges: (i) Genomic resources of non-model organisms are not always available or of poor quality; (ii) since noninvasive sampling is often a prerequisite, the tissue selection is complicated in wild species; and (iii) complex study designs require sophisticated analysis strategies. The study of Lindner, Laine, et al. (2021) stands out, because it offers great approaches to overcome these challenges: (i) The need for high-quality genomic resources—also for non-model organisms—in times of continuously dropping sequencing costs has fuelled great progress in molecular evolutionary-ecology. This can also be seen in the recent development of large sequencing initiatives, such as the Darwin Tree of Life or the Vertebrate Genome Project. Utilizing well-characterized genomic resources for a non-model species, the great tit, generated by Laine et al. (2016), Lindner, Laine, et al. (2021) have derived ecologically relevant conclusions from robust molecular analyses. More specifically, they revealed multiple candidate genes, whose role in reproduction previously has been unknown or underappreciated (Figure 1). (ii) Since noninvasive sampling is often a prerequisite to perform studies that answer fundamental eco-evolutionary questions, blood samples are a particularly valuable sample type. Importantly, Lindner, Verhagen, et al. (2021) investigated tissue-specific DNA methylation and revealed that temporal changes in methylation are similar across tissues in great tit females. Thus, DNA methylation changes in the blood provide a good indication of changes in, for instance, liver tissue. Recently, Husby (2020) advocated blood samples for ecological epigenetic studies as they allow repeated measurements of individuals over time and thus, across phenotypic states. Furthermore, blood samples may provide an optimal tissue for studying systemic responses and potentially an opportunity to explore the signal transduction cascade of environmental cues across tissues. Taking advantage of the properties of blood samples, Lindner, Laine, et al. (2021) were able to track individual female birds over time and therefore to determine the reproductive status relative to their specific laying date, including those sampling time points before egg laying. (iii) Lastly, to accommodate complex experimental designs, multiple environmental factors, and batch effects, which are common in ecoevolutionary studies, thorough statistical testing is needed. Yet, many studies exploring DNA methylation differences are restricted to simpler regression statistics or pairwise test designs. In contrast, Lindner, Laine, et al. (2021)

accommodated a complex experimental design, using a generalized linear mixed model and a weighted comethylation network analysis.

Since Lindner, Laine, et al. (2021) highlight potential implications of promoter DNA methylation in reproductive timing, future research could broaden their focus on the signal transduction cascade from cue sensing and interpretation to the translation into a phenotypic response. For instance, it would be important to determine if there is a link between DNA methylation and the environmental cues initiating reproduction. In an attempt to do so, Lindner, Laine, et al. (2021) surprisingly found that natural temperature and photoperiod patterns of a cold and warm year did not result in a shift in the laying date. One reason for this could be the lack of seasonal variation in other proximate factors, such as food availability, as birds were fed ad libitum. On the other hand, this suggests that the temperature and photoperiod ranges in which great tit populations can adjust their reproductive timing might be limited. To assess species' resilience to interannual cue variability, these range limits should be investigated and the fitness consequences of mismatched laying dates monitored. Interestingly, Lindner, Laine, et al. (2021) also showed substantial interindividual variation in laying dates of approximately one month, which could be a means to reduce the population-wide risk of reproductive mismatches.

In addition to these research avenues focusing on sensing and processing environmental information, further research should address the effect of DNA methylation on the phenotype. The conclusions of Lindner, Laine, et al. (2021) on the relevance of promoter methylation are based on the assumption that methylation changes affect gene expression (Figure 1). While DNA methylation is known to play an important role in the regulation of gene expression, demonstrating this relationship is often not straightforward (Buchberger et al., 2019). More specifically, correlations between promoter methylation and gene expression are often weak on a genome-wide scale (Watson et al., 2021) or even absent for some candidate genes when compared over time (Lindner, Verhagen, et al., 2021). Furthermore, it has recently been discussed whether transcription factors (TFs) might act not only as readers but also as effectors of DNA methylation (Zhu et al., 2016). And indeed, it has been shown that changes in expression can occur prior to DNA methylation changes, implying that DNA demethylation is not always required for gene activation and may occasionally be a downstream consequence of TF binding (Pacis et al., 2019). Therefore, it will be important to establish whether observed promoter methylation changes are indeed the cause or rather a consequence of reproduction initiation.

Moreover, DNA methylation is only one of multiple epigenetic mechanisms that can impact gene expression. Therefore, investigating other mechanisms, such as histone modifications, may help to identify the driver(s) of the residual expression variation. One particularly useful tool in this context is assay for transposase-accessible chromatin using sequencing (ATACseq), as it maps chromatin accessibility across the genome and thereby identifies regions with gene-regulatory potential. Combined with (whole genome) bisulphite or RNA sequencing, these functional genomic assays will vastly improve our understanding of genotype-phenotype relationships, not only in ecoevolutionary contexts.

To date, most studies that investigate the role of epigenetic modifications in ecoevolutionary processes are descriptive and correlative. While such results are highly valuable, the field will have to move beyond that whenever feasible. Among other approaches, manipulative experiments can provide unparalleled insights into the ecoevolutionary relevance of epigenetic modifications. For instance, DNA methylation inhibiting reagents could be applied to investigate causal relationships between a relevant phenotype and the correlated DNA methylation pattern (Gore et al., 2018). Alternatively, global DNA methylation patterns can be manipulated by inducing a mutation in a gene responsible for adding (e.g., *dnmt3bb.1*) or removing (e.g., *tet2*) DNA methylation (Gore et al., 2018). In more targeted approaches, applications based on CRISPR-Cas or RNA interference technology can provide a tool for precise (epi)genome or transcriptome manipulation (Nakamura et al., 2021; Walton et al., 2020). While these technologies are not applicable in every context, they might be of interest for a few candidate genes after careful consideration of any ethical and resource issues, as well as biological limitations (offspring numbers, accessibility of cell type of interest, availability of a representative model, etc.). If feasible, experimental validation of field observations will undoubtedly provide unprecedented insights into the functional genomics of complex life-history and behavioural traits.

ORCID

Melanie J. Heckwolf https://orcid.org/0000-0002-7507-8895

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