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Insights in avian physiology 2022

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Colin Guy Scanes and Sandra G. Velleman

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Topic editors

Colin Guy Scanes — University of Wisconsin–Milwaukee, United States

Sandra G. Velleman — The Ohio State University, United States

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Nandor Nagy,
Semmelweis University, Hungary

*CORRESPONDENCE
C. G. Scanes,
cgscanes@icloud.com

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Editorial: Insights in avian physiology

C. G. Scanes[1]* and S. G. Velleman[2]

[1]Department of Biological Science, University of Wisconsin Milwaukee, Milwaukee, WI, United States, [2]Department of Animal Sciences, The Ohio State University, Columbus, OH, United States

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Editorial on the Research Topic
Insights in avian physiology

This Research Topic provides a compendium of eleven papers and reviews on avian physiology. Birds are successful in different climatic zones including polar, tropical, dry climates, moist subtropical, moist continental mid-Latitudes and highlands. It has been estimated that there are globally 18,043 avian species (95% confidence interval 15,845–20,470). This is in contrast to earlier estimates of 9,000–10,000 species (Barrowclough et al., 2016) and compares with 6,495 mammalian species (96 recently extinct, 6,399 extant) (Burgin et al., 2018). This Research Topic includes papers, opinion pieces and reviews on the physiology of birds.

Two papers cover the physiology of wild birds. In red-headed buntings subjected to simulated migration, there are shifts in reticulocytes (increased) and apoptosis of erythrocytes together with expression of stress oxidation related genes in blood (Bhardwaj et al.). Moreover, Kumar et al. review their and others studies on migration in Palearctic-Indian migratory buntings, the blackheaded bunting (*Emberiza melanocephala*) and redheaded bunting (Emberiza bruniceps).

Globally, there have been large increases in the production of poultry (chickens, turkeys, ducks and geese) and livestock for meat (Table 1: FAOSTAT, 2023). The development of poultry production can be broadly attributed to the following.

- Large genetic improvements,
- Nutrition to meet bird requirements,
- Vaccinations and health improvement,
- Efficient production systems.

Geneticists have been responsible for much of the large increases in the growth rate, particularly that of breast muscle, in young meat chickens or broilers (Siegel). They utilized the diverse storehouse of genes in different populations of chickens, the heritability of key traits and the short generation interval (Siegel). The shifts in growth rate and other production traits are based on the physiology of the poultry. There have been increases in the incidence and/or severity of myopathies (White striping, Wooden breast and Spaghetti breast) in broiler chickens (Bailey). There appears to be little genetic relationships between the myopathies and either growth or percentage breast muscle (Bailey). What is missing is more studies on reactive oxygen species and oxidative stress in the muscles and other organs in broiler type chickens.

TABLE 1 Shifts in global production of meats by species together with eggs between 1991 and 2021 (FAOSTAT, 2023).

Species	Global production in million metric tons	
	1991	2021
Meats		
Chicken	38.2	121.6
Pig	70.7	120.4
Cattle	54.2	74.4
Sheep	6.9	10.0
Goats	2.7	6.4
Ducks	1.4	6.2
Turkey	3.5	5.8
Geese	0.8	4.4
Eggs		
Chicken	38.2	86.4
Ducks and geese	3.05	6.17

Satellite cells are critical to muscle growth, itself the basis of meat production. Satellite cells are mono-nucleated stem cells that undergo asymmetric division generating two daughter cells:

- One playing a role in muscle growth and development.
- The other replenishing the satellite cell reservoir in the muscle.
- Satellite cells, as stem cells, can also develop into other cell types such as adipocytes (reviewed Velleman).

Velleman proposes that there are multiple populations of satellite cells. The corollary is that multiple populations of satellite cells have different properties and, therefore, functions. Among the different properties of such populations are the following:

- Growth potential,
- Signal transduction:
- Mechanistic target of rapamycin (mTOR) pathway,
- Wingless type mouse mammary tumor virus integration site family/planar cell polarity (Wnt/PCP) pathway,
- There are considerable implications to the presence of multiple forms of satellite cells including increase muscle growth and overcoming the problems of myopathies (reviewed Velleman).

Poultry provide a second food, namely, eggs (see Table 1). The Research Topic includes description of a new anterior pituitary hormone that appears related to reproduction. The pituitary gland expresses relaxin 3 (RLN3) in adult female but male chickens (Lv et al.). Expression of RLN3 *in vitro* by pituitary cells is increased by two releasing hormones, namely, gonadotropin releasing hormone and corticotropin releasing hormone and by estradiol (Lv et al.). Expression of two receptors for RLN3 have been examined in chickens with RXFP1 expressed in oviduct particularly infundibulum plus the brain but not the pituitary gland while RXFP3 is expressed in the kidneys, hypothalamus and spinal column but not the pituitary gland (Lv et al.). Lv et al. also propose that the avian RLN3 gene is a duplicated copy of the ancestral RLN3 gene.

Scanes reinterprets data from older studies on the timing of ovulation and oviposition in hens on ahemoral light cycles. He concludes that there is not a satisfactory model that fully accounts for the timing of ovulation/oviposition and now should be the time to develop one.

Environmental factors profoundly influence the physiology of birds. For instance, spectrum of light was demonstrated to influence reproduction in chickens with greater activation of the hypothalamo-pituitary axis in chickens photostimulated with red compared to green light (Rozenboim et al.). In addition, red but not green light increased expression of red opsin in the hypothalamus (Rozenboim et al.).

Reproductive development in female birds is accompanied by accumulation of calcium in bones. Bahry et al. examine the relationship between growth and nutrition using plasma concentrations of estradiol as an indicator of reproductive development together with bone mineralization.

There are other aspects of avian physiology included in the Research Topic. For example, Csillag et al. discuss the utility of chickens and songbirds as models for the typical failure symptoms associated with autism spectrum disorder (ASD). This intriguing concept provides a novel biomedical utilization of avian physiology. Pierzchala-Koziec and Scanes analyze the limited information on the neuropeptides and putative neuropeptides derived from three opioid genes, proenkephalin (PENK), prodynorphin (PDYN) and pronociceptin (PNOC). Data on the deduced structures of proenkephalin, prodynorphin, and pronociceptin in high vertebrates were employed to evaluate avian neuropeptides and putative peptides together with evolutionary considerations.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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References

Barrowclough, G. F., Cracraft, J., Klicka, J., and Zink, R. M. (2016). How many kinds of birds are there and why does it matter? *PLoS ONE* 11, e0166307. doi:10.1371/journal.pone.0166307

Burgin, C. J., Colella, J. P., Kahn, P. L., and Upham, N. S. (2018). How many species of mammals are there? *J. Mammal.* 99, 1–14. doi:10.1093/jmammal/gyx147

FAOSTAT (2023). Crops and livestock products. Available at: https://www.fao.org/faostat/en/#data/QCL (Accessed May 28, 2023).

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EDITED BY
Kazuhisa Honda,
Kobe University, Japan

REVIEWED BY
Paul Mozdziak,
North Carolina State University,
United States

*CORRESPONDENCE
Sandra G. Velleman,
velleman.1@osu.edu

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Why breast muscle satellite cell heterogeneity is an issue of importance for the poultry industry: An opinion paper

Sandra G. Velleman*

Department of Animal Sciences, The Ohio State University, Columbus, OH, United States

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Introduction

Skeletal muscle development and growth is unique in that it is characterized by two populations of cells, the myoblasts and a mononucleated adult stem cell termed satellite cells. Satellite cells are stem cells due to their asymmetric division in which one daughter cell will continue myogenesis and the other will replenish the muscle precursor cell reservoir. Skeletal muscle growth in poultry occurs through the formation of myofibers or hyperplasia of myoblasts with myoblasts fusing from to multinucleated myotubes that mature into muscle fibers and muscle fiber bundles. At hatch, myofiber formation is complete (Smith, 1963) and subsequent growth is through the donation of nuclei into existing fibers from satellite cells resulting in the enlargement of fibers through hypertrophic growth (Moss and LeBlond, 1971). The repair and regeneration of muscle fibers from damage is also due to satellite cells.

Satellite cell heterogeneity

The satellite cell is a mononuclear cell closely positioned adjacent to the sarcolemma of the myofiber (Mauro, 1961). Satellite cells are located on the periphery of the myofibers which resulted in the name satellite cells. The satellite cell contains primarily nuclear material, including a small amount of cytoplasm and only a few organelles, such as a mitochondria or Golgi apparatus. At hatch, satellite cells compose 30% of the total myonuclei of muscle. At the end of the growth period, satellite cell numbers diminish to 1–5% of the total nuclei and their proliferative capacity decreases (Hawke and Garry, 2001). Once growth is complete, the satellite cells will become quiescent (Schultz et al., 1978) and reside in their niche environment until activated to regenerate damaged myofibers. In heavy-weight meat type chickens, satellite cell myogenic activity persists at a high level for the first 8 days posthatch and peaks on day 3 of posthatch age (Halevy et al., 2000).

Satellite cells are not a homogenous population of cells within a muscle. Although satellite cells are commonly referred to as one population of cells, there is heterogeneity of satellite cells within a muscle indicating that there is more than one population. The lack of homogeneity of satellite cells was initially reported by Schultz and Lipton (1982) who

showed that proliferation potential of satellite cells was age dependent. Satellite cell heterogeneity can be characterized by satellite cells from different muscle fiber types expressing the genes of the fiber type it originated from (Feldman and Stockdale, 1991; Lagord et al., 1998; Huang et al., 2006; Manzano et al., 2011), and differing rates of proliferation in satellite cells isolated from the same muscle (McFarland et al., 1995; Schultz, 1996). Heterogeneity of satellite cells exists in a single fiber-type muscle like the turkey and chicken pectoralis major muscle that contains homogenous Type IIb fibers. McFarland et al. (1995) isolated 73 different individual satellite cells from the same pectoralis major muscle of a 6 week old tom turkey. The isolated cells were expanded and studied for proliferation, differentiation, and growth factor responsiveness. A range of fast growing to slow growing satellite cells were obtained. The fastest growing satellite cell reached 65% confluency in 17 days whereas the slowest growing satellite cell took 30 days. Differentiation was affected in a similar manner to proliferation. Muscle mass accretion is a result of satellite cell-mediated growth and these functional differences in satellite cells will impact muscle growth and subsequent meat quality of the breast muscle. Furthermore, Xu et al. (2022a, b) showed satellite cells, based on their growth potential, have differences in their regulation of signal transductions pathways like mechanistic target of rapamycin (mTOR) and wingless type mouse mammary tumor virus integration site family/planar cell polarity (Wnt/PCP) pathways. Mechanistic target of rapamycin signal transduction is a key pathway involved in muscle fiber hypertrophy and Wnt/PCP is involved with satellite cell proliferation and differentiation, and lipid synthesis.

Importance of satellite cell biology for breast muscle growth and regeneration, and myopathies

For the commercial poultry industry, focus needs to be placed on how selection for increased breast muscle yield has altered the biology of satellite cells. These changes in satellite cell biology will alter the morphological structure of the muscle and are associated with myopathies that are detrimental to meat quality like Wooden Breast and Spaghetti Meat. Meat quality reflects both the cell biology and biochemistry of the breast muscle. The reason that attention needs to be focused on satellite cells is that they are critical to both posthatch myofiber growth and regeneration of the myofibers in response to injury. With regard to hypertrophic myofiber growth, cross sectional area of a breast muscle myofiber has increased 3 to 5 times resulting in giant fibers (Dransfield and Sosnicki, 1999). The presence of giant fibers limits endomysial and perimysial spacing between individual myofibers and muscle fiber bundles. The consequences of reducing connective tissue area include myofibers and muscle fiber bundles making contact initiating muscle injury and satellite cell mediated repair (Velleman et al., 2003), and reducing available space for capillary supply to the muscle which is required for satellite cell activity (Velleman, 2015). A regenerated muscle fiber should be identical to the originating fiber. For repair to occur, satellite cell activation from a quiescent state to enter the cell cycle is required for proliferation and differentiation to regenerate the damaged muscle fiber. A required element for satellite cell activation is the proximity of satellite cells to circulatory supply in the satellite cell niche. Christov et al. (2007) and Rhoads et al. (2009) demonstrated that direct communication between the satellite cells and vascular system is required. The regeneration of damaged muscle fibers requires satellite cells to be near capillaries. Satellite cells must be within 21 μm of capillaries in humans to actively regenerate a muscle fiber (Christov et al., 2007). In Wooden Breast-affected breast muscle, satellite cell repair and regeneration of necrotic muscle fibers has been negatively impacted leading to meat quality downgrades (Clark and Velleman, 2017; Velleman et al., 2018). The end result of satellite cell-mediated regeneration is the restoration of the damaged muscle fibers back to their original state which does not occur in the Wooden Breast myopathy. Wooden Breast affected muscle is characterized by a high percentage of smaller diameter myofibers with disorganized contractile sarcomeres (Clark and Velleman, 2017; Velleman et al., 2018). Despite the smaller diameter myofibers with a high degree of myofibril sarcomeric disorganization, satellite cell-mediated regeneration is activated as supported by the increased expression of myogenic transcriptional regulatory factors modulating satellite cell proliferation and differentiation (Velleman and Clark, 2015), but the repair process is still inhibited. Since selection for breast muscle yield in most meat-type commercial broiler lines has resulted in an increase in myofiber diameter through hypertrophic growth, it is likely that the decrease in circulatory supply to the muscle, which is necessary for satellite cell-mediated regeneration, is associated with suppression of myofiber regeneration. These findings suggest that selection for increased breast muscle growth has altered the populations of satellite cells found in the broiler breast muscle to ones with reduced regenerative capabilities. As part of selection for breast muscle mass accretion, the functional characteristics of the satellite cells for myofiber growth through hypertrophy and their activation to regenerate damaged myofibers needs to be assessed to maintain appropriate muscle structure and avoid conditions negatively impacting meat quality.

Since the satellite cells are a stem cell, they can also differentiate into cellular fates other than muscle especially fat. During proliferation, some daughter cells self-renew to maintain the satellite cell pool (Kuang et al., 2007), others follow a myogenic pathway (Kuang et al., 2007), while some will spontaneously convert to an adipogenic cellular lineage (Rossi et al., 2010). Growth selection has likely altered the breast muscle satellite cell population to one with appropriate stimuli that

coverts to an adipogenic cellular fate instead of terminally differentiating into muscle. It has been shown that satellite cells during their period of peak mitotic activity are responsive to extrinsic stimuli like nutrition (Velleman et al., 2014a) and temperature (Xu et al., 2021a). Feed restrictions during the peak period of mitotic activity, the first week after hatch, result in pectoralis major muscle satellite cells synthesizing lipid which becomes intramuscular fat depots in the breast muscle (Velleman et al., 2014b) that are similar to intramuscular marbling fat depots observed in bovine meat (Smith and Johnson, 2014). Increased intramuscular fat will negatively impact the protein to fat ratio in the breast muscle meat product. This change in the protein to fat ratio is converse to poultry breast meat being sold as a low-fat protein choice. Thermal stress especially heat also affects the cellular fate of satellite cells (Clark et al., 2017; Xu et al., 2021b). Satellite cells are most sensitive to temperature when they are proliferating and growth selection has increased the thermal sensitivity of satellite cells to synthesize lipid (Halevy, 2020; Xu et al., 2021b).

Although even within a uniform muscle fiber type like the poultry breast muscle, heterogeneity within the satellite cell populations composing a muscle like the breast muscle has likely been altered by selection for breast muscle yield. Satellite cells dynamically express cell surface receptors (Yin et al., 2013) involved with key processes, including but not limited to, cell migration, growth factor responsiveness, and proliferation. Thus, selection for breast muscle yield has likely altered the balance between the different populations of satellite cells which could result as already demonstrated by Xu et al. (2021b) in satellite cells from growth selected meat-type turkeys being more prone to transdifferentiate to an adipogenic fate or as in broilers a decreased potential of satellite cells to activate from a quiescent state to regenerate damaged myofibers (Velleman et al., 2018).

Discussion

Heavy weight fast growing meat-type poultry is characterized by excessive myofiber hypertrophic growth mediated by the satellite cells due to growth selection occurring during the posthatch period of satellite cell-mediated muscle mass accretion. The large diameter myofibers reduce connective tissue spacing in the endomysial and perimysial areas resulting in muscle fibers and fiber bundles making contact and initiating fiber degeneration (Wilson et al., 1990; Velleman et al., 2003). The juxtaposed position of the muscle fibers and bundles also limits spacing for circulatory supply which is necessary for satellite cell activity to repair muscle fiber damage. Additionally, as shown in previous studies satellite cells from modern heavy weight fast growing meat-type broilers have reduced regeneration and repair of damaged myofibers (Velleman et al., 2018), and in both broilers and turkeys the satellite cells are more prone to transdifferentiate to an adipogenic lineage rather than follow a muscle terminal differentiation cellular fate (Clark and Velleman, 2017; Xu et al., 2021b). Since satellite cells are critical to the development, growth, and regeneration of muscle, management strategies must evolve to include the cellular biology of satellite cells including population heterogeneity and their effect on the morphological structure of the breast muscle. Strategies encompassing satellite cell biology will likely significantly decrease meat quality downgrades from breast muscle myopathies.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

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References

Christov, C., Chrétien, F., Abou-Khalil, R., Bassez, G., Vallet, G., Authier, F-J., et al. (2007). Muscle satellite cells and endothelial cells: Close neighbors and privileged partners. *Mol. Biol. Cell.* 18, 1397–1409. doi:10.1091/mbc.e06-08-0693

Clark, D. L., Strasburg, G. M., Reed, K. M., and Velleman, S. G. (2017). Influence of temperature and growth selection on Turkey pectoralis major muscle satellite cell adipogenic gene expression and lipid accumulation. *Poult. Sci.* 96, 1015–1027. doi:10.3382/ps/pew374

Clark, D. L., and Velleman, S. G. (2017). Spatial influence on breast muscle morphological structure, myofiber size, and gene expression associated with the wooden breast myopathy in broilers. *Poult. Sci.* 95, 2930–2945. doi:10.3382/ps/pew243

Dransfield, E., and Sosnicki, A. A. (1999). Relationship between muscle growth and poultry meat quality. *Poult. Sci.* 78, 743–746. doi:10.1093/ps/78.5.743

Feldman, J. L., and Stockdale, F. E. (1991). Skeletal muscle satellite cell diversity: Satellite cells form fibers of different types in cell culture. *Dev. Biol.* 143, 320–334. doi:10.1016/0012-1606(91)90083-f

Halevy, O., Geyra, A., Barak, M., and Sklan, D. (2000). Early posthatch starvation decreases satellite cell proliferation and skeletal muscle growth in chicks. *J. Nutr.* 130, 858–864. doi:10.1093/jn/130.4.858

Halevy, O. (2020). Timing is everything—the high sensitivity of avian satellite cells to thermal conditions during embryonic and posthatch periods. *Front. Physiol.* 11, 235. doi:10.3389/fphys.2020.00235

Hawke, T. J., and Garry, D. J. (2001). Myogenic satellite cells: Physiology to molecular biology. *J. Appl. Physiol.* 91, 534–551. doi:10.1152/jappl.2001.91.2.534

Huang, Y., Dennis, R. G., and Barr, K. (2006). Cultured slow vs. Fast skeletal muscle cells differ in physiology and responsiveness to stimulation. *Am. J. Physiol. Cell. Physiol.* 291, C11–C17. doi:10.1152/ajpcell.00366.2005

Kuang, S., Kuroda, K., LeGrand, F., and Rudnicki, M. A. (2007). Asymmetric self-renewal and commitment of satellite stem cells in muscle. *Cell.* 129, 999–1010. doi:10.1016/j.cell.2007.03.044

Lagord, C., Soulet, L., Bonavaud, S., Bassaglia, Y., Rey, C., Barlovatz-Meimon, G., et al. (1998). Differential myogenicity of satellite cells isolated from extensor digitorum longus (EDL) and soleus rat muscles revealed *in vitro*. *Cell. Tissue Res.* 291, 455–468. doi:10.1007/s004410051015

McFarland, D. C., Gilkerson, K. K., Pesall, J. E., and Walker, J. S. (1995). Heterogeneity in growth characteristics of satellite cell populations. *Cytobios* 82, 21–27.

Manzano, R., Toivonen, J. M., Calvo, A. C., Miana-Mena, F. J., Zaragoza, P., Munoz, M. J., et al. (2011). Sex, fiber-type, and age dependent *in vitro* proliferation of mouse muscle satellite cells. *J. Cell. Biochem.* 112, 2825–2836. doi:10.1002/jcb.23197

Mauro, A. (1961). Satellite cell of skeletal muscle fibers. *J. Biophys. Biochem. Cytol.* 9, 493–495. doi:10.1083/jcb.9.2.493

Moss, F. P., and LeBlond, C. P. (1971). Satellite cells as the source of nuclei in muscles of growing rats. *Anat. Rec.* 170, 421–435. doi:10.1002/ar.1091700405

Rhoads, R. P., Johnson, R. M., Rathbone, C. R., Liu, X., Temm-Grove, C., Sheehan, S. M., et al. (2009). Satellite cell-mediated angiogenesis *in vitro* coincides with a functional hypoxia-inducible factor pathway. *Am. J. Physiol. Cell. Physiol.* 296, C1321–C1328. doi:10.1152/ajpcell.00391.2008

Rossi, C. A., Pozzobon, M., Ditadi, A., Archacka, K., Gastaldello, A., Sanna, M., et al. (2010). Clonal characterization of rat muscle satellite cells: Proliferation, metabolism and differentiation define an intrinsic heterogeneity. *PLoS One* 5, e8523. doi:10.1371/journal.pone.0008523

Schultz, E., Gibson, M. C., and Champion, I. (1978). Satellite cells are mitotically quiescent in mature mouse muscle: An EM and radioautographic study. *J. Exp. Zool.* 206, 451–456. doi:10.1002/jez.1402060314

Schultz, E., and Lipton, B. H. (1982). Skeletal muscle satellite cells: Changes in proliferation potential as a function of age. *Mech. Ageing Dev.* 20, 377–383. doi:10.1016/0047-6374(82)90105-1

Schultz, E. (1996). Satellite cell proliferative compartments in growing skeletal muscles. *Dev. Biol.* 175, 84–94. doi:10.1006/dbio.1996.0097

Smith, J. H. (1963). Relation to body size to muscle cell size and number in the chicken. *Poult. Sci.* 42, 619–623. Available at: https://twinwoodcattle.com/sites/default/files/publications/2018-11/Marbling%20-%20Management%20of%20cattle%20to%20maximize%20the%20deposition%20of%20intramuscular%20adipose%20tissue.pdf.

Smith, S. B., and Johnson, B. J. (2014). Marbling: Management of cattle to maximize the deposition of intramuscular adipose tissue. Available at: http://www.beefresearch.org.

Velleman, S. G., Anderson, J. W., Coy, C. S., and Nestor, K. E. (2003). Effect of selection for growth rate on muscle damage during Turkey breast muscle development. *Poult. Sci.* 82, 1069–1074. doi:10.1093/ps/82.7.1069

Velleman, S. G., and Clark, D. L. (2015). Histopathologic and myogenic gene expression changes associated with wooden breast in broiler breast muscles. *Avian Dis.* 59, 410–418. doi:10.1637/11097-042015-Reg.1

Velleman, S. G., Clark, D. L., and Tonniges, J. R. (2018). The effect of the wooden breast myopathy on sarcomere structure and organization. *Avian Dis.* 62, 28–35. doi:10.1637/11766-110217-Reg.1

Velleman, S. G., Coy, C. S., and Emmerson, D. A. (2014a). Effect of the timing of posthatch feed restrictions on broiler breast muscle development and muscle transcriptional regulatory factor gene expression. *Poult. Sci.* 93, 1484–1494. doi:10.3382/ps.2013-03813

Velleman, S. G., Coy, C. S., and Emmerson, D. A. (2014b). Effect of the timing of posthatch feed restrictions on the deposition of fat during broiler breast muscle development. *Poult. Sci.* 93, 2622–2627. doi:10.3382/ps.2014-04206

Velleman, S. G. (2015). Relationship of skeletal muscle development and growth to breast muscle myopathies: A review. *Avian Dis.* 59, 525–531. doi:10.1637/11223-063015-Review.1

Wilson, B. W., Nieberg, P. S., Buhr, R. J., Kelly, B. J., and Shultz, F. T. (1990). Turkey muscle growth and focal myopathy. *Poult. Sci.* 69, 1553–1562. doi:10.3382/ps.0691553

Xu, J., Strasburg, G. M., Reed, K. M., and Velleman, S. G. (2021b). Effect of temperature and selection for growth on intracellular lipid accumulation and adipogenic gene expression in Turkey pectoralis major muscle satellite cells. *Front. Physiol.* 12, 667814. doi:10.3389/fphys.2021.667814

Xu, J., Strasburg, G. M., Reed, K. M., and Velleman, S. G. (2021a). Response of Turkey pectoralis major muscle satellite cells to hot and cold thermal stress: Effect of growth selection on satellite cell proliferation and differentiation. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 252, 110823. doi:10.1016/j.cbpa.2020.110823

Xu, J., Strasburg, G. M., Reed, K. M., and Velleman, S. G. (2022b). Temperature and growth selection effects on proliferation, differentiation, and adipogenic potential of Turkey myogenic satellite cells through frizzled-7-mediated Wnt planar cell polarity pathway. *Front. Physiol.* 13, 892887. doi:10.3389/fphys.2022.892887

Xu, J., Strasburg, G. M., Reed, K. M., and Velleman, S. G. (2022a). Thermal stress affects proliferation and differentiation of Turkey satellite cells through the mTOR/S6K pathway in a growth-dependent manner. *PLoS One* 17 (1), e0262576. doi:10.1371/journal.pone.0262576

Yin, H., Price, F., and Rudnicki, M. (2013). Satellite cells and the muscle stem cell niche. *Physiol. Rev.* 93, 23–67. doi:10.1152/physrev.00043.2011

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EDITED BY
Sandra G. Velleman,
The Ohio State University, United States

REVIEWED BY
Colin Guy Scanes,
University of Arkansas, United States

*CORRESPONDENCE
Vinod Kumar,
drvkumar11@yahoo.com
Sanjay Kumar Bhardwaj,
drskumar7@yahoo.com

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Differential regulatory strategies for spring and autumn migrations in Palearctic-Indian songbird migrants

Vinod Kumar[1]*, Aakansha Sharma[2], Vatsala Tripathi[3] and Sanjay Kumar Bhardwaj[4]*

[1]IndoUS Center in Chronobiology, Department of Zoology, University of Delhi, Delhi, India, [2]IndoUS Center in Chronobiology, Department of Zoology, University of Lucknow, Lucknow, India, [3]Department of Zoology, Dyal Singh College, University of Delhi, Delhi, India, [4]Department of Zoology, CCS University, Meerut, India

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Introduction

Birds, like most if not all long-lived species, show cyclic behavior and physiology. They exhibit daily cycles in activity-rest, sleep-wake, feeding-starvation and high-low body temperature, and annual (seasonal) cycles in growth, migration, hibernation, reproduction and molt. These are the products of endogenous circadian (Latin: *circa* = about; *dies* = day) and circannual (Latin: *circa* = about; *annum* = year) timers interacting with reliable environmental *Zeitgebers* (*zeit* = time, *geber* = giver; or time cue), e.g., changes in day length (= photoperiod), temperature, and/or feeding resources. For seasonal cycles, for example, the circannual timer and photoperiodic regulation seem mutually inclusive mechanisms for seasonal LHSs comprising the annual cycle of a bird species (Misra et al., 2004). It remains poorly understood though if the photoperiod changes directly or with its consistent interaction (synchronization) control changes in seasonal behavioral and physiological phenotypes during the annual itinerary of a species. However, the most accepted view is that endogenous circannual rhythm set the temporal window of a seasonal life-history state (LHS) during the year, and the photoperiod change regulates the physiological preparedness for the appropriate biological response characteristics of a LHS (Kumar et al., 2010). This can be more challenging for millions of songbirds that follow a rigid seasonal schedule, with migrations placed before and after the reproduction. This brief article aims to highlight in particular, the differential adaptive strategies that migrants employ during non-migratory and migratory LHSs, and during spring and autumn migrant periods, largely based on our research on Palearctic-Indian migratory buntings—the blackheaded bunting (*Emberiza melanocephala*) and redheaded bunting (*Emberiza bruniceps*).

Photoperiod changes can reproduce the annual itinerary of a latitudinal avian migrant

A migrant's annual itinerary includes molt, autumn migration, overwintering period, vernal migration and reproductive seasonal LHSs. These are identifiable by striking differences in birds' behavioral and physiological phenotypes. For instance, the migrants maintain lean body mass except in the migratory period, and small reproductively inactive gonads except in the breeding season. With the transition from non-migrant to the migrant period, the birds show hyperphagia (increased foraging), fat accumulation in adipose and liver tissues to serve as flight fuel, and muscular hypertrophy for an enhanced flight endurance. There is also a drastic change in the behavior and physiology as exhibited in phase inversion of the 24-h pattern of activity behavior (from daytime to predominantly night-time activity) and olfaction and vision sensory systems linked with migration (Rastogi et al., 2011).

The timely departure gives time to explore feeding resources on the way and nesting resources at breeding grounds that enhance reproductive success. Post vernal equinox period, the overwintering birds begin to prepare for their northward migratory travel in response to increasing spring photoperiods (≥12 h daily light); the converse is true for the post autumn equinox period when birds respond to a decreasing photoperiod despite it being still close to the threshold for photoperiodic induction in spring. The ambient temperature also plays a crucial role in the development of the migration phenotype, as evidenced by transcriptional response to temperature in the development of spring migratory phenotype in captive redheaded buntings (Sur et al., 2020). The decision for migratory departure is, therefore, the outcome of the integration of environmental photoperiod and temperature with the migratory context (to breeding grounds in spring and to wintering areas in autumn) and the physiological state (pre-reproductive state in spring vs. post-reproductive state in autumn).

Seasonal LHSs can be faithfully reproduced in captive migratory buntings. Under short days mimicking a winter photoperiod, buntings maintain normal body mass and reproductively immature (inactive) gonads as well as responsivity to the photoperiodic induction. On exposure to long days (≥12 h, equal to or longer than the threshold photoperiod), the cascade of photoperiod-induced processes culminates into the development of spring migratory (copious subcutaneous fat deposition and *Zugunruhe*—intense nocturnal restlessness in captives) and reproductive (mature gonads) phenotypes (Misra et al., 2004; Rani et al., 2005, 2006; Malik et al., 2014; Trivedi et al., 2014; Singh et al., 2015). Prolonged long-day exposure leads to spontaneous regression, and birds become lean in body mass and day active again with regressed gonads. Subsequent response to a shorter photoperiod mimicking the late autumn day length, induces the autumn migratory phenotype (Singh et al., 2015; Sharma et al., 2018a).

Neural and molecular correlates of migratory phenotype

The transition into a migratory phenotype requires changes at the regulatory (brain) and effector organ (liver, muscle) levels. Rastogi et al. (2011) showed that in parallel with daily behavioral cycles, there was a phase inversion in Fos-immunoreactivity (a marker of the neuronal activity) of olfactory and visual sensory circuits involved in orientation and navigation. The dorsomedial part of the mediobasal hypothalamus (not suprachiasmatic nucleus) seems to be the site of photoperiod-induced functional coupling of endogenous seasonal clock and behavioral outputs (e.g., *Zugunruhe*) relevant to the migration (Rastogi et al., 2013). At molecular level, a large number of hypothalamic cytoskeletal and calcium signaling (important for neurogenesis and maintenance of synaptic connections) genes were also upregulated in redheaded buntings exhibiting the photoperiod-induced migratory phenotype (Sharma et al., 2018b). The liver was also enriched with genes associated with intracellular protein transport, calcium ion transport and small GTPase-mediated signal transduction pathways during the migrant period (Sharma et al., 2018b). Interestingly, there was a molecular switch in genes associated with energy utilization being highly expressed during the day and night during non-migratory and migratory LHSs, respectively (Sharma et al., 2022).

Differential strategies for spring and autumn migrations

The two seasonal migrations placed before and after the breeding season differ in several ways. First is the difference in context: spring (or vernal) migration is for the timely arrival of migrating birds at the breeding grounds, and so it is at a faster pace with fewer stopovers (hence longer flight bouts) (Newton, 2007; Nilsson et al., 2013). Hence, with relatively less opportunity to re-fuel, spring migrants need to acquire a copious amount of fat before they start nocturnal flight (Sharma et al., 2018a; Sharma et al., 2018b; Sharma and Kumar, 2019) (Figure 1). There can also be a sex-dependent difference in the migratory drive since males need to reach early in order to define their territories and build nests, which are key determinants of a successful reproduction (Tryjanowski and Yosef, 2002). Although reproductively immature at the time of departure, gonadal tissues grow over the course of the vernal migratory journey and secrete hormones facilitating an immediate onset of reproductive behavior upon arrival at the breeding ground (Wingfield et al., 1990). By contrast, the autumn migration is to escape from harsh winter conditions and to allow recovery and

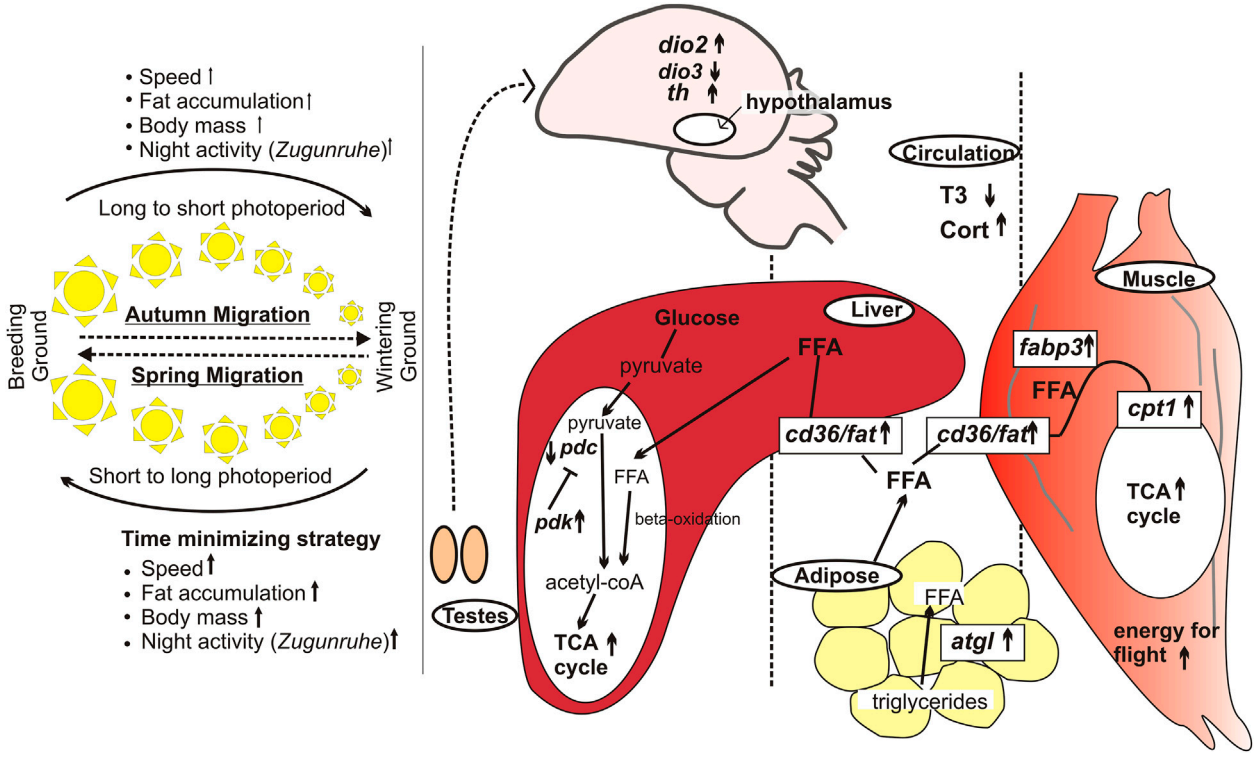

FIGURE 1
Differences in spring and autumn migrations, based on studies in captive migratory redheaded buntings. Left panel: Phenotypic changes associated with spring and autumn migrations. The thickness of arrows reflects differences in magnitude of the photostimulated response. The sun cartoons in the middle indicate the direction of photoperiod change. Right panel: Molecular changes in the hypothalamus, liver, muscle and adipose tissues in spring compared to the autumn migration. In spring, migrants show elevated plasma Cort and reduced T3 levels, and reciprocal switching of *dio2* and *dio3*, and increased *th* expression. The adipose triglycerides are converted into free fatty acids, which are up taken from circulation mediated by the enzymes and proteins encoded by *cd36/fat*, *fabp3* and *cpt1* genes. Hepatic *pdc* and *pdk* encoded enzymes regulate the fuel use. The arrow and the line ending with bar represent activation and inhibitory pathways, respectively. Upward arrows indicate upregulated gene expression in spring compared with that in the autumn migration (Sharma et al., 2018a; Sharma and Kumar 2019).

repair post-reproduction on wintering grounds with rich feeding resources. Secondly, birds during two seasonal migrations differ in the photoperiodic state: they are sensitive and refractory to long day at the beginning of spring and autumn migrant periods, respectively. Thirdly, the migrating birds also face differences in the direction of photoperiod (and perhaps temperature) change; for example, the birds experience increasing and decreasing photoperiods during the spring and autumn travels, respectively.

Hypothalamic control

Several recent studies have reported changes in hypothalamic gene expressions in migrant, compared to the non-migrant period. For example, the circadian clock gene (*per2*, *bmal1*) expressions showed alteration in their oscillatory waveform (24-h acrophase and amplitude) in redheaded buntings expressing photoperiod-induced transition from non-migratory to migratory LHS (Singh et al., 2015). Singh and Kumar (2017) also found increased amplitude and acrophase of *cry1* and *bmal1* (not *per2*) 24-h rhythms in the optic tectum and cerebellum of buntings expressing the *Zugunruhe*. Further, Sharma et al. (2018a) reported differential expression of thyroid hormone-responsive *dio2* and *dio3*, and light responsive *cry1*, *per2* and *adcyap1* genes between spring and autumn migrant periods. Consistent with the time-minimizing strategy and enhanced motivation, redheaded buntings almost doubled their *th* (tyrosine hydroxylase) expression during the photoperiod-induced spring migratory state (Sharma et al., 2018a) (Figure 1).

Metabolic support

As in the hypothalamus, *per2* and *bmal1* clock genes showed alteration in their oscillatory waveform in liver of buntings with transition from non-migratory to the migratory LHS (Singh et al., 2015; Singh et al., 2018). The metabolic machinery seems

modulated to support differential physiological requirements between spring and autumn migrant periods in buntings (Singh et al., 2018; Sharma and Kumar, 2019). Histological examination of the liver and adipose tissues revealed larger fat accumulation (shown by adipose cell area and hepatic fat droplets) during spring migratory state (Sharma and Kumar, 2019). There was an overall upregulation of fat utilization machinery during spring. Hepatic pyruvate dehydrogenase complex (PDC) and pyruvate dehydrogenase kinase (PDK) enzymes act antagonistically (PDK inactivates PDC) and regulate glycolytic cycle (conversion of pyruvate into acetyl-CoA) for the tricarboxylic acid cycle. A second substrate feeding to TCA cycle is by increased fatty acid oxidation. In simulated spring migrants, we found increased *pdk* and decreased *pdc* mRNA expressions in redheaded buntings (Sharma and Kumar, 2019). Increased fat utilization is evidenced by increased mRNA expression of genes coding for adipose tissue triglyceride lipase (*atgl*) in adipose tissues; and those coding for fatty acid translocase (*fat/cd36*), fatty acid binding protein (*fabp3*) and carnitine palmitoyl transferase 1 (*cpt1*) in the flight muscle. Enhanced conversion of triglycerides into free fatty acids can be up taken by flight muscles using FAT, FABP and CPT1 and be utilized by beta-oxidation for energy generation. Increased expressions of metabolic genes during migrant compared to non-migrant period, as well as during spring compared to autumn migrant period, suggest a robust seasonal metabolic plasticity in migratory buntings (Sharma and Kumar, 2019) (Figure 1).

Role of gonads

A key difference lies in the gonadal state between two seasonal migrations. Gonad is in the preparatory and regression phases of its annual cycle before migration in spring and autumn, respectively. A recent redheaded bunting study demonstrated that the removal of testes affected the hypothalamic transcriptome but the effect was much larger in simulated autumn migrant period. The castrated buntings expressed reduced *Zugunruhe* in autumn, compared to spring (Sharma et al., 2020). A large number of genes were also differentially expressed between intacts and castrates in autumn (62 genes), compared to the spring (37 genes). The differentially expressed genes in spring enriched G-protein coupled acetylcholine receptor signaling and signal transduction pathways, while those in autumn enriched largely the calcium signaling pathway (Sharma et al., 2020).

To sum up, the latitudinal migratory songbirds exhibit a significant seasonal difference in gene expressions between spring and autumn, concurrently with changes in behavior and physiology associated with migration. There are comprehensive changes in the olfaction, visual and hypothalamic neural circuits with the transition from non-migratory to migratory LHS. The songbird migrants utilize fat as major flight fuel supplied by adipose tissues *via* FFAs to support the migratory flight. The mechanisms underlying the generation and overall flux of energy involve oxidation of fatty acids in the liver and protein-mediated transport to the "working" muscles. The present discussion on neural and metabolic plasticities mediating differential seasonal responses provides molecular insights into the seasonal homeostasis at both regulatory and effector organ levels in long-lived species, possibly including humans.

Author contributions

VK conceived the idea and wrote the initial draft; AS, VT, and SB contributed to the writing. AS drew the figure. VK and AS produced the final version.

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Conflict of interest

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References

Kumar, V., Wingfield, J. C., Dawson, A., Ramenofsky, M., Rani, S., and Bartell, P. (2010). Biological clocks and regulation of seasonal reproduction and migration in birds. *Physiol. Biochem. Zool.* 83, 827–835. doi:10.1086/652243

Malik, S., Singh, S., Rani, S., and Kumar, V. (2014). Life at a different pace: Annual itineraries are conserved in seasonal songbirds. *J. Biosci.* 39, 485–491. doi:10.1007/s12038-014-9440-1

Misra, M., Rani, S., Singh, S., and Kumar, V. (2004). Regulation of seasonality in the migratory male blackheaded bunting (*Emberiza melanocephala*). *Reprod. Nutr. Dev.* 44, 341–352. doi:10.1051/rnd:2004039

Newton, I. (2007). *The migration ecology of birds*. London, UK: Academic Press.

Nilsson, C., Klaassen, R. H. G., and Alerstam, T. (2013). Differences in speed and duration of bird migration between spring and autumn. *Am. Nat.* 181, 837–845. doi:10.1086/670335

Rani, S., Malik, S., Trivedi, A. K., Singh, S., and Kumar, V. (2006). A circadian clock regulates migratory restlessness in the blackheaded bunting (*Emberiza melanocephala*). *Curr. Sci.* 91, 1093–1095. doi:10.2307/24093990

Rani, S., Singh, S., Misra, M., Malik, S., Singh, B. P., and Kumar, V. (2005). Daily light regulates seasonal responses in the migratory male redheaded bunting (*Emberiza bruniceps*). *J. Exp. Zool. A Comp. Exp. Biol.* 303, 541–550. doi:10.1002/jez.a.187

Rastogi, A., Kumari, Y., Rani, S., and Kumar, V. (2013). Neural correlates of migration: Activation of hypothalamic clock(s) in and out of migratory state in the blackheaded bunting (*Emberiza melanocephala*). *PLoS ONE* 8, e70065. doi:10.1371/journal.pone.0070065

Rastogi, A., Kumari, Y., Rani, S., and Kumar, V. (2011). Phase inversion of neural activity in the olfactory and visual systems of a night-migratory bird during migration. *Eur. J. Neurosci.* 34, 99–109. doi:10.1111/j.1460-9568.2011.07737.x

Sharma, A., Das, S., Komal, R., Malik, S., Rani, S., and Kumar, V. (2020). Seasonal reproductive state determines gene expression in the hypothalamus of a latitudinal migratory songbird during the spring and autumn migration. *Mol. Cell. Endocrinol.* 508, 110794. doi:10.1016/j.mce.2020.110794

Sharma, A., Das, S., Singh, D., Rani, S., and Kumar, V. (2022). Differences in transcription regulation of diurnal metabolic support to physiologically contrasting seasonal life-history states in migratory songbirds. *J. Ornithol.* 163, 199–212. doi:10.1007/s10336-021-01926-5

Sharma, A., and Kumar, V. (2019). Metabolic plasticity mediates differential responses to spring and autumn migrations: Evidence from gene expression patterns in migratory buntings. *Exp. Physiol.* 104, 1841–1857. doi:10.1113/EP087974

Sharma, A., Singh, D., Das, S., and Kumar, V. (2018b). Hypothalamic and liver transcriptome from two crucial life-history stages in a migratory songbird. *Exp. Physiol.* 103 (4), 559–569. doi:10.1113/EP086831

Sharma, A., Singh, D., Malik, S., Gupta, N. J., Rani, S., and Kumar, V. (2018a). Difference in control between spring and autumn migration in birds: Insight from seasonal changes in hypothalamic gene expression in captive buntings. *Proc. Biol. Sci.* 285, 20181531. doi:10.1098/rspb.2018.1531

Singh, D., and Kumar, V. (2017). Extra-hypothalamic brain clocks in songbirds: Photoperiodic state dependent clock gene oscillations in night-migratory blackheaded buntings, Emberiza melanocephala. *J. Photochem. Photobiol. B* 169, 13–20. doi:10.1016/j.jphotobiol.2017.02.016

Singh, D., Swarup, V., Le, H., and Kumar, V. (2018). Transcriptional signatures in liver reveal metabolic adaptations to seasons in migratory blackheaded buntings. *Front. Physiol.* 9, 1568. doi:10.3389/fphys.2018.01568

Singh, D., Trivedi, A. K., Rani, S., Panda, S., and Kumar, V. (2015). Circadian timing in central and peripheral tissues in a migratory songbird: Dependence on annual life history states. *FASEB J.* 29, 4248–4255. doi:10.1096/fj.15-275339

Sur, S., Chaturvedi, K., Sharma, A., Malik, S., Rani, S., and Kumar, V. (2020). Ambient temperature affects multiple drivers of physiology and behaviour: Adaptation for timely departure of obligate spring migrants. *J. Exp. Biol.* 223, jeb236109. doi:10.1242/jeb.236109

Trivedi, A. K., Kumar, J., Rani, S., and Kumar, V. (2014). Annual life history-dependent gene expression in the hypothalamus and liver of a migratory songbird: Insights into the molecular regulation of seasonal metabolism. *J. Biol. Rhythms* 29, 332–345. doi:10.1177/0748730414549766

Tryjanowski, P., and Yosef, R. (2002). Differences between the spring and autumn migration of the red-backed shrike *Lanius collurio*: Record from the eilat stopover (Israel). *Acta Ornithol.* 37, 85–90. doi:10.3161/068.037.0204

Wingfield, J. C., Schwabl, H., and Mattocks, P. W., Jr. (1990). "Endocrine mechanisms of migration," in *Bird migration, physiology and ecophysiology*. Editor E. Gwinner (Berlin, Germany: Springer), 232–256.

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EDITED BY
Paul Siegel,
Virginia Tech, United States

REVIEWED BY
Andrzej Sechman,
University of Agriculture in Krakow, Poland
Gregoy Y. Bedecarrats,
University of Guelph, Canada

*CORRESPONDENCE
Colin G. Scanes,
cgscanes@icloud.com

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Discontinuities in understanding follicular development, the ovulatory cycle and the oviposition cycles in the hen: Advances, opportunities, slow downs and complete stops

Colin G. Scanes*

Department of Biological Science, University of Wisconsin Milwaukee, Milwaukee, WI, United States

There has been considerable progress in understanding follicular development, the ovulatory cycle and the oviposition cycles in the hen. In particular, there have been tremendous advances in understanding follicular development and recruitment of follicles to the hierarchy of large yellow follicles. There is a need to continue to follow the earlier literature while employing present techniques. Early work allowed an understanding of the ovulation and oviposition cycles. Models for ovulation were developed. However, while these have no passed the test of time, there is no present model that fully accounts to the cycles. Earlier work employed ahemoral light cycles to examine ovulation and oviposition cycles. Recent work has demonstrated that clock genes are expressed in the ovary. The control of incubation by prolactin has been largely elucidated in turkeys. There is evidence that other endocrine glands influence female reproduction in birds including the adrenal cortex, thyroid and pineal. However, there is much that remains to be fully understood.

KEYWORDS

ovulation cycle, oviposition, follicular development, ahemoral, hen reproduction

Introduction

My interest in the physiology of reproduction in birds dawned with my being a member of the team that developed a radioimmunoassay for chicken luteinizing hormone (LH) (Follett et al., 1972); this being the first assay for a non-mammalian hormone. This and other such assays were used, for instance, to determine changes in plasma concentrations of LH along with progesterone during the ovulatory cycle of chickens (Furr et al., 1973) and the circadian basis of photoperiodic induction of LH release in a wild bird (Follett et al., 1974). Brian Follett went on to an exemplary research career deducing much of the mechanism of photoperiodism in birds.

It is appropriate, 50 years later, to consider what has been learned on the ovulatory or oviposition cycles of chickens and turkeys and what questions remain needing to be

addressed. Among the unique features of female reproduction in poultry and other birds are the following:

- A hierarchy of yellow (yolk filled) follicles with a new follicle recruited on a daily basis.
- Ovulation of clutches of eggs with the time of ovulation occurring later in the day as the sequence progresses
- The ovum passing through the oviduct where it acquires albumen (egg white), membranes and a calcareous shell. Once this is complete, the egg is released from the oviduct in the process of oviposition (egg laying). This will not be covered except where is impacts ovulation, oviposition and their timing.
- Broodiness and the incubation of eggs.

Development of follicles

There is a hierarchy of yellow (yolk filled) follicles with follicles increasing in size to a maximum diameter of 2.5 cm due to their filling with yolk. There is also maturation of the granular and thecal cells. The largest follicle will be the first to be ovulated and then the next largest. A new follicle recruited on a daily basis.

Alan Johnson's laboratory have performed a series of studies on the recruitment and development of the ovarian follicles. Not only does this provide a comprehensive account of follicular development but also the studies themselves were exquisite. The recruitment of small (pre-hierarchal) follicles involves follicle stimulating hormone (FSH) and other factors such as growth factors increasing FSH receptors by granulosa cells from pre-hierarchal follicles. In turn, there are increases in the following:

1) Formation of cyclic adenosine monophosphate (cAMP).
2) Expression of steroidogenic acute regulatory protein (STAR).
3) Production of progesterone by granulosa cells (Kim and Johnson, 2018).

Effects of growth factors include the following:

Bone morphogenetic protein 4 (BMP4) increased expression of the FSH receptor in undifferentiated granulosa cells from pre-hierarchal follicles (Kim et al., 2013). Similarly, (BMP6 increased responsiveness to FSH by granulosa cells from pre-hierarchal follicles (Ocón-Grove et al., 2012). Conversely, BMP2 prevented FSH receptor expression by either transforming growth factor β (TGF β) or FSH by undifferentiated granulosa cells from pre-hierarchal follicles (Haugen and Johnson, 2010).

Transforming growth factor β1 (TGFβ1) increased expression of vascular endothelial growth factor A (VEGF) and its receptor, VEGF receptors (VEGFR) in granulosa cells from prehierarchal follicles (Kim et al., 2016). In turn, VEGF and VEGFR induce angiogenesis and consequently facilitate follicular growth and the deposition of yolk precursors.

BMP6 also increased the expression of anti-Müllerian hormone (AMH) by granulosa cells from pre-hierarchal follicles (Ocón-Grove et al., 2012). In the presence of FSH, BMP4 increased AMH expression by undifferentiated granulosa cells from pre-hierarchal follicle (Kim et al., 2013). Moreover, the effect of BMP4 was blocked in the presence of TGFα or noggin (Kim et al., 2013).

Ovulation and oviposition cycles

For every ovum to be ovulated, there is a surge in circulating concentrations of LH and progesterone. A very few pre-ovulatory surges in circulating concentrations of LH and progesterone were not associated with an egg laid in turkeys (Liu et al., 2001a; Liu et al., 2001b). The interval between LH/progesterone surges is increased late in reproductive period (Liu et al., 2002). A positive feedback loop exists with LH stimulating production of progesterone by granulosa cells particularly those in the largest follicle and progesterone increasing LH release.

Progesterone induces the pre-ovulatory LH surge (Rothchild and Fraps, 1949; Wilson and Sharp, 1975). The effect of progesterone requires the presence of estrogen. Progesterone only provokes a LH surge when ovariectomized hens received estradiol administration of daily prior to challenge (Wilson and Sharp, 1976).

Timing of oviposition and ovulation

The interval between ovipositions is 24–27 h [chickens: (Attwood, 1929) also see Table 1; turkeys: 26.8 h: (Liu et al., 2001a); 26 h: (Brady et al., 2019)]. The interval varies with the length of a sequence in the laying hen (Attwood, 1929) with greater intervals with the shorter sequences (Table 1). Assuming that the interval between oviposition and ovulation of the next ovum in the sequence remains constant at about 30 min (Warren and Scott, 1935), the duration the ovum spends in the oviduct is markedly less in long sequences (Table 1) tending to less 24 h.

The interval between oviposition and the next oviposition reflects the time an ovum spends in the oviduct together with the approximately the time between oviposition and the next ovulation (Table 1) [calculated from data in Biellier and Ostmann (1960)]. The interval between ovipositions is greater in short sequences (Table 2) and is longer with long ahemoral light cycles (Table 1) (Morris, 1973; other data calculated from data in Biellier and Ostmann, 1960). With ahemoral light cycles of progressively greater than 24 h, the timing of oviposition is earlier; migrating from during the photophase to the end of the scotophase (calculated from data in Biellier and Ostmann, 1960)

TABLE 1 Effect of sequence length on interval between ovipositions [from or calculated from Attwood (1929)].

Sequence length (number of eggs in a sequence)	Interval between ovipositions (h)	Duration in oviduct[a] (h)
2	28.0	26.4
3	26.8	27.5
4	25.9	25.4
5	25.6	25.1
8	24.6	24.1
11	24.7	24.2

[a]Calculated assuming interval between oviposition and ovulation of 30 min (Warren and Scott, 1935).

TABLE 2 Effect of ahemeral light/dark cycles on interval between ovipositions (based on or calculated from data in Biellier and Ostmann, 1960; Morris, 1973).

Light (photophase)/dark (scotophase) cycles	Length of "day"[a] (h)	Interval between ovipositions (h)	Duration of ovum in the oviduct[b] (h)
Biellier and Ostmann (1960)			
10.5L:10.5D	21	26.4	25.9
11L:11D	22	27.5	27.0
11.5L:11.5D	23	26.1	25.6
12L:12D	24	26.4	25.9
19L:19D	38	32.3	31.8
21L:21D	42	34.9	34.4
Morris (1973)			
14L:10D	24	24.9	24.4
14L:13D	27	27.1	26.6
14L:16D	30	29.0	28.5

[a]Photophase plus scotophase.
[b]Calculated assuming interval between oviposition and ovulation of 30 min (Warren and Scott, 1935).

(Figure 1; Tables 2, 3). It is unclear how the light/dark cycle influences the duration that an ovum spends in the oviduct?

In laying hens, ovulation occurs about 30 min following oviposition of the previously ovulated ovum (Warren and Scott, 1935; Melek et al., 1973). There is some difference between the interval oviposition and ovulation irrespective of the photoperiod; this being 24 min for laying hens on a 14L:10D photoperiod and 36 min for hens on ahemeral light schedule (14L:13D) (Melek et al., 1973). What is not clear is the mechanisms for the cross talk between the ovary and oviduct?

The corollary to ovulation occurring 30 min after oviposition of the previous ovum in a sequence is that the surge in circulating concentrations of LH and progesterone occurs about 6 h before oviposition of the previous ovum. What is not clear is how the ovum/pituitary anticipate oviposition of the previous ovum in the sequence? What is the cross talk between the ovary and/or its hypothalamic pituitary control (ovulation) and the oviduct (oviposition of the previously ovulated ovum)?

Laying hens have been selected for reduced intervals between ovipositions under continuous lighting (24L:0D) (Gow et al., 1985). These hens also exhibited reduced intervals between surges in circulating concentrations of LH (Gow et al., 1985). The timing of the surge in circulating concentrations of LH and progesterone occurs at a specific time of day. For instance, in the domestic duck, the first pre-ovulatory surge in circulating concentrations of LH and progesterone in a sequence occurs at the beginning of the scotophase on a 16L:8D photoperiod and 2 h into the scotophase on a 11L:13D photoperiod (Wilson et al., 1982). The first LH surge of a sequence occurs at the beginning of the scotophase in hens on a 14L:10D photoperiod (Johnson and van Tienhoven, 1984). The pre-ovulatory LH surge in chickens occurs at the beginning of the scotophase (Wilson et al., 1985). If the timing of the scotophase is advanced, there is some increase in circulating concentrations of LH, albeit not a full LH surges (Wilson et al., 1985).

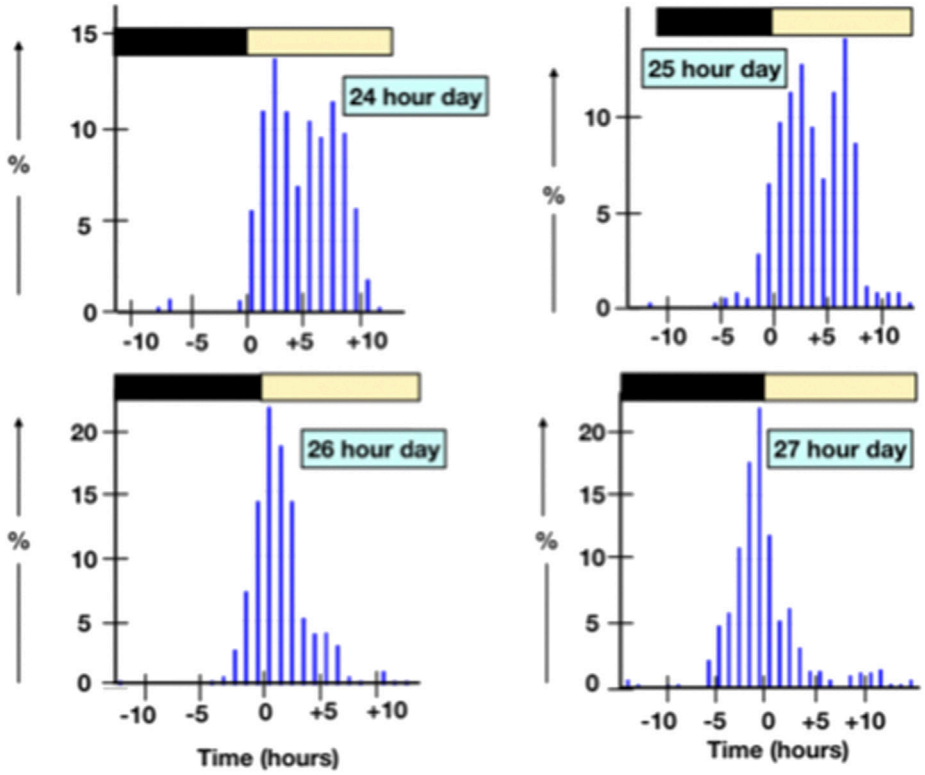

FIGURE 1
Shifts in the time of oviposition with ahemoral light cycles.

TABLE 3 Effect of ahemeral light/dark cycles on the timing of ovipositions (calculated from data in Biellier and Ostmann, 1960.

Light (photophase)/dark (scotophase) cycles	% Ovipositions during photophase	Earliest time for oviposition[a,b]	Time for latest of ovipositions[a,c]	Mean time of oviposition[a,b]
10.5L:10.5D	67.3	+5 to +6	−8 to −7	+7.9
11.L:11.D	86.8	+4 to +5	−9 to −8	+8.3
11.5L:11.5D	95.5	+2 to +3	+10 to +11	+4.8
12L:12D	98.4	0 to +1	+9 to +10	+4.9
12.5L:12.5D	88.1	−1 to 0	+7 to +8	+4.6
13L:13D	74.1	−2 to −3	+3 to +4	+2.2
13.5L:13.5D	35.9	−3 to −4	+3 to +4	−0.7

[a]Time for earliest ovipositions (>5% of total).
[b]Time in hours from onset of the photophase.
[c]Time for latest ovipositions (<5% of total).

Model for the ovulatory cycle

Both oviposition and ovulation occur only during a period of 8–10 h during the day in chickens and turkeys (on a photoperiod of 14L:10D) with the first ovulation in a sequence occurring 8–9 h after the subjective dusk and the last ovulation of a sequence occurring 18–19 h after the subjective dusk. This was called the open-period (Fraps, 1954; Fraps, 1965). The corollary is that the LH/progesterone surge for the first ovulation in a sequence occurs about 3 h into the scotophase. As the sequence progresses, the surge occurs later and later until the last surge occurs 12 h from the subjective dusk. A further corollary is that the LH/progesterone surge is limited to this open period. What was not clear was what was the open period? 1)

Was it the ability of the ovary to produce progesterone? There is not evidence for this. 2) Was it the ability of the hypothalamus/pituitary gland to respond to progesterone positive feedback? There is no evidence for this either. Occam's razor Was it some other mechanism?

An alternate model for the sequence of ovulations postulated two asynchronous cycles with ovulation only occurring when these were synchronized (Bastian and Zarrow, 1955). What was not clear is what was the physiological bases of each cycle? This would seem to be a non-testable hypothesis as the cycles are not defined.

Circadian genes

There are multiple circadian genes. For instance, BMAL1 is heterodimeric transcriptional protein and one of the master genes of the circadian clock (Menet et al., 2014). There is expression of Bmal1 together with other circadian genes in the ovary of the laying hen: cryptochrome circadian regulator (Cry1), Clock and period circadian regulator (Per 2). Moreover, there is evidence that the pre-ovulatory LH surge influences expression of circadian genes (Tischkau et al., 2011; Li et al., 2014). Expression of BMAL1 by chicken follicular granulosa cells has been reported with expression increased by vasoactive intestinal peptide (VIP) (Kim and Johnson, 2016). What are not clear include the following: 1) Are circadian genes expressed in the oviduct? 2) How are circadian genes in the ovary and, potentially also, the oviduct entrained following a shift in photoperiod and under ahemoral cycles? Are they entrained by the light dark cycle or the stage of the sequence and, if so, what is the mechanism of entraining expression of the clock genes? Is the expression of the clock genes influenced by phase advancing or phase delaying the LH/progesterone surge or the imposition of ahemeral lighting cycles?

Neuroendocrine control of female reproduction

A series of papers from J. P. Advis's laboratory provided evidence for noradrenergic, neuropeptide Y (NPY) and dopaminergic effects on gonadotropin releasing hormone (GnRH) release from the median eminence (Contijoch et al., 1990; Contijoch et al., 1992; Contijoch et al., 1993). Norepinephrine stimulated GnRH release from the median eminence from laying hen (Contijoch et al., 1990). Similarly, neuropeptide Y increased *in vitro* GnRH release from hen median eminence (Contijoch et al., 1993). Basal GnRH release from the median eminence of hens subjected to feed withdrawal was increased but decreased in the presence of dopamine (Contijoch et al., 1992). Unfortunately, this group did not appear to have continued research on the reproductive physiology of hen. There is further evidence for both dopaminergic and adrenergic control of preovulatory LH surge with the surge blocked in the presence of the dopamine agonist (apomorphine) or an α adrenergic antagonist (phenoxybenzamine) (Knight et al., 1982). What is still unclear whether additional neuropeptides are involved in the control of the pre-ovulatory surge?

Recent progress in dissecting the hypothalami-pituitary—ovarian axis

Gene expression in the hypothalamus, pituitary and ovary was compared between during the pre-ovulatory LH/progesterone in a study of turkey hens in Tom Porters's laboratory. The changes appear to be not those expected. For instance, there is reduced expression of GnRH in the hypothalamus (Brady et al., 2019). Moreover, there is decreased expression of GnRH receptors and increased expression of GnIH receptors in the pituitary gland (Brady et al., 2019). There was also shifts in expression in the preovulatory surge in the ovary such as decreases in LHR in the granulosa of F1 follicle (Brady et al., 2019). What is not clear is how the role for each component of the hypothalami-pituitary—ovarian axis controlling ovulation?

Broodiness and the incubation of eggs

Our knowledge of the endocrine control of incubation (sitting on eggs) and brooding (care of chicks/poults). Much of this stems for the work of Mohamed El Halawani. Circulating concentrations of prolactin are markedly increased in turkeys during incubation with a decline when the birds are deprived of access to nests (El Halawani et al., 1980). This increase in prolactin is controlled by hypothalamic peptide, vasoactive intestinal peptide (VIP). Immunization of turkeys against VIP prevents the increase in circulating concentrations of prolactin in turkeys (El Halawani et al., 1996; El Halawani et al., 2000). VIP increases prolactin expression and release in turkeys with the effect blocked by dopamine (Sun and El Halawani, 1995; Al Kahtane et al., 2005). Hypothalamic expression of vasoactive intestinal peptides is high in incubating turkeys (Rozenboim et al., 1993). Similarly, there is increased VIP receptor expression in the anterior pituitary glands in turkeys during incubation (Chaiseha et al., 2004). Administration of a dopamine antagonist prevented brooding behavior in poults (Thayananuphat et al., 2011). Unfortunately, Mohamed El Halawai has now retired and is inactive.

Other endocrine inputs

The relationship between adrenal cortical hormones and the ovulation cycle

There is evidence from early studies that adrenal cortical hormones influence can influence ovulation. Ovulation was blocked by administration of the glucocorticoid, dexamethasone, 14 h prior to ovulation (Soliman and Huston, 1974). The effect of dexamethasone was overcome by the administration of adrenocorticotropic hormone (ACTH) (Soliman and Huston, 1974). This is consistent with dexamethasone suppressing adrenocorticotropic hormone (ACTH) release either acting directly at the level of the anterior pituitary gland or indirectly by depressing release of corticotropin releasing hormone and/or the releasing hormones for ACTH, namely arginine vasotocin (AVT) from the hypothalamus. Premature ovulation was induced by the following in order of potency: Deoxycorticosterone, progesterone, and, at very high dose, corticosterone (Etches and Cunningham, 1976). Moreover, each agent induced premature oviposition (Etches and Cunningham, 1976; Wilson and Sharp, 1976). In contrast, ovulation was inhibited by either the synthetic glucocorticoid, dexamethasone (Rzasa et al., 1983) or corticosterone (Williams et al., 1985).

The relationship between thyroid hormones and reproduction

There is evidence for relationships between thyroid hormones and egg laying in poultry with, for instance, plasma concentrations of triiodothyronine decreased during the LH/progesterone surge (Brady et al., 2021). There are decreases in the plasma concentrations of thyroid hormones, triiodothyronine (T_3) and thyroxine (T_4) prior to the onset of ovulation during sexual maturation (Sechman et al., 2000). Moreover, T_3 has been demonstrated to depress plasma concentrations of both LH and estradiol, to induce follicular atresia and to bind to thyroid hormone receptor in ovarian follicles (Sechman et al., 2009; reviewed: Sechman, 2013). What is not clear is whether the shifts in thyroid hormones can be advanced or delayed experimentally?

The relationship between gonadotropin inhibitory hormone and reproduction

The RFamide peptide, gonadotropin inhibitory hormone (GnIH) depresses both release of LH and FSH and expression of the common alpha and FSH beta gonadotrophin subunit *in vitro* (Ciccone et al., 2004). There are also direct effects of GnIH on the ovary. There is expression of both GnIH and its receptor (GnIHR) in the chicken ovary (Maddineni et al., 2005). Expression of GnIH declines during sexual maturation (Maddineni et al., 2005).

The relationship between melatonin and reproduction

Melatonin administration increased ovarian expression of both melatonin receptors type 1A (MTNR1A) and melatonin receptors type 1B (MTNR1B) (Hao et al., 2020). There have been studies on the effects on light spectrum on ovarian expression of MTNR1A, MTNR1B and melatonin receptors type 1C (MTNR1C) (Li et al., 2015). Expression of MTNR1A and MTNR1C was greater in hens on monochromatic red (660 nm) than green (560 nm) and blue (480 nm) light in small yellow follicles, F5 and F2 follicles (Li et al., 2015). However, there were no effects on ovarian expression of MTNR1B (Li et al., 2015). A role for gonadal receptors melatonin receptors in seasonal breeding has been proposed in starlings (McGuire et al., 2011).

There is a positive correlation between plasma concentration of melatonin and both hypothalamic concentrations of GnIH and GnIHR during reproductive development in chickens (Zhang et al., 2017).

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Al Kahtane, A., Kannan, M., Kang, S. W., El Halawani, M. E., and Al KahtAne, A. (2005). Regulation of prolactin gene expression by vasoactive intestinal peptide and dopamine in the Turkey: Role of Ca signalling. *J. Neuroendocrinol.* 17, 649–655. doi:10.1111/j.1365-2826.2005.01352.x

Attwood, H. (1929). A study of the time factor in egg production. *Poult. Sci.* 8, 137–140.

Bastian, J. W., and Zarrow, M. X. (1955). A new hypothesis for the asynchronous ovulatory cycle of the domestic hen (*Gallus domesticus*). *Poult. Sci.* 34, 776–788. doi:10.3382/ps.0340776

Biellier, H. V., and Ostmann, O. W. (1960). Effect of varying day-length on time of oviposition in domestic fowl. *U. Mo. Agr. Exp. Sta. Res. Bull.* 747.

Brady, K., Long, J. A., Liu, H. C., and Porter, T. E. (2021). Characterization of hypothalamo-pituitary-thyroid axis gene expression in the hypothalamus, pituitary gland, and ovarian follicles of Turkey hens during the preovulatory surge and in hens with low and high egg production. *Poult. Sci.* 100, 100928. doi:10.1016/j.psj.2020.12.026

Brady, K., Porter, T. E., Liu, H.-C., and Long, J. A. (2019). Characterization of gene expression in the hypothalamo-pituitary-gonadal axis during the preovulatory surge in the Turkey hen. *Poult. Sci.* 98, 7041–7049. doi:10.3382/ps/pez437

Chaiseha, Y., Youngren, O. M., and El Halawani, M. E. (2004). Expression of vasoactive intestinal peptide receptor messenger RNA in the hypothalamus and pituitary throughout the Turkey reproductive cycle. *Biol. Reprod.* 70, 593–599. doi:10.1095/biolreprod.103.022715

Ciccone, N. A., Dunn, I. C., Boswell, T., Tsutsui, K., Ubuka, T., Ukena, K., et al. (2004). Gonadotrophin inhibitory hormone depresses gonadotrophin alpha and follicle-stimulating hormone beta subunit expression in the pituitary of the domestic chicken. *J. Neuroendocrinol.* 16, 999–1006. doi:10.1111/j.1365-2826.2005.01260.x

Contijoch, A. M., Gonzalez, G., Singh, H. N., Malamed, S., Troncoso, S., and Advis, J. P. (1992). Dopaminergic regulation of luteinizing hormone-releasing hormone release at the median eminence level: Immunocytochemical and physiological evidence in hens. *Neuroendocrinology* 55, 290–300. doi:10.1159/000126128

Contijoch, A. M., Johnson, A. L., and Advis, J. P. (1990). Norepinephrine-stimulated *in vitro* release of luteinizing hormone-releasing hormone (LHRH) from median eminence tissue is facilitated by inhibition of LHRH-degrading activity in hens. *Biol. Reprod.* 42, 222–230. doi:10.1095/biolreprod42.2.222

Contijoch, A. M., Malamed, S., McDonald, J. K., and Advis, J. P. (1993). Neuropeptide Y regulation of LHRH release in the median eminence: Immunocytochemical and physiological evidence in hens. *Neuroendocrinology* 57, 135–145. doi:10.1159/000126353

El Halawani, M. E., Burke, W. H., and Dennison, P. T. (1980). Effect of nest-deprivation on serum prolactin level in nesting female turkeys. *Biol. Reprod.* 23, 118–123. doi:10.1095/biolreprod23.1.118

El Halawani, M. E., Pitts, G. R., Sun, S., Silsby, J. L., and Sivanandan, V. (1996). Active immunization against vasoactive intestinal peptide prevents photo-induced prolactin secretion in turkeys. *Gen. Comp. Endocrinol.* 104, 76–83. doi:10.1006/gcen.1996.0143

El-Halawani, M. E., Whiting, S. E., Silsby, J. L., Pitts, G. R., and Chaiseha, Y. (2000). Active immunization with vasoactive intestinal peptide in Turkey hens. *Poult. Sci.* 79, 349–354. doi:10.1093/ps/79.3.349

Etches, R. J., and Cunningham, F. J. (1976). The effect of pregnenolone, progesterone, deoxycorticosterone or corticosterone on the time of ovulation and oviposition in the hen. *Br. Poult. Sci.* 17, 637–642. doi:10.1080/00071667608416320

Follett, B. K., Mattocks, P. W., and Farner, D. S. (1974). Circadian function in the photoperiodic induction of gonadotropin secretion in the white-crowned sparrow, Zonotrichia leucophrys gambelii. *Proc. Natl. Acad. Sci. U. S. A.* 71, 1666–1669. doi:10.1073/pnas.71.5.1666

Follett, B. K., Scanes, C. G., and Cunningham, F. J. (1972). A radioimmunoassay for avian luteinizing hormone. *J. Endocrinol.* 52, 359–378. doi:10.1677/joe.0.0520359

Fraps, R. M. (1954). Neural basis of diurnal periodicity in release of ovulation-inducing hormone in fowl. *Proc. Nat. Acad. Sci.* 40, 348–356. doi:10.1073/pnas.40.5.348

Fraps, R. M. (1965). Twenty-four-Hour periodicity in the mechanism of pituitary gonadotrophin release for follicular maturation and ovulation in the chicken. *Endocrinology* 77, 5–18. doi:10.1210/endo-77-1-5

Furr, B. J. A., Bonney, B. C., England, R. J., and Cunningham, F. J. (1973). Luteinizing hormone and progesterone in peripheral blood during the ovulatory cycle of the hen, *Gallus domesticus*. *J. Endocrinol.* 57, 159–169. doi:10.1677/joe.0.0570159

Gow, C. B., Sharp, P. J., Carter, N. B., Scaramuzzi, R. J., Sheldon, B. L., Yoo, B. H., et al. (1985). Effects of selection for reduced oviposition interval on plasma concentrations of luteinising hormone during the ovulatory cycle in hens on a 24 h lighting cycle. *Br. Poult. Sci.* 26, 441–451. doi:10.1080/00071668508416834

Hao, E.-y., Chen, H., Wang, D.-H., Huang, C.-x., Tong, Y.-g., Chen, Y.-f., et al. (2020). Melatonin regulates the ovarian function and enhances follicle growth in aging laying hens via activating the mammalian target of rapamycin pathway. *Poult. Sci.* 99, 2185–2195. doi:10.1016/j.psj.2019.11.040

Haugen, M. J., and Johnson, A. L. (2010). Bone morphogenetic protein 2 inhibits FSH responsiveness in hen granulosa cells. *Reproduction* 140, 551–558. doi:10.1530/REP-10-0211

Johnson, P. A., van Tienhoven, A., and vAn Tienhoven, A. (1984). Investigations of the significance of the crepuscular LH peak in the ovulatory cycle of the hen (*Gallus domesticus*). *J. Endocrinol.* 100, 307–313. doi:10.1677/joe.0.1000307

Kim, D., and Johnson, A. L. (2018). Differentiation of the granulosa layer from hen prehierarchal follicles associated with follicle-stimulating hormone receptor signaling. *Mol. Reprod. Dev.* 85, 729–737. doi:10.1002/mrd.23042

Kim, D., and Johnson, A. L. (2016). Vasoactive intestinal peptide promotes differentiation and clock gene expression in granulosa cells from prehierarchal follicles. *Mol. Reprod. Dev.* 83, 455–463. doi:10.1002/mrd.22641

Kim, D., Lee, J., and Johnson, A. L. (2016). Vascular endothelial growth factor and angiopoietins during hen ovarian follicle development. *Gen. Comp. Endocrinol.* 232, 25–31. doi:10.1016/j.ygcen.2015.11.017

Kim, D., Ocón-Grove, O., and Johnson, A. L. (2013). Bone morphogenetic protein 4 supports the initial differentiation of hen (*Gallus gallus*) granulosa cells. *Biol. Reprod.* 88, 161. doi:10.1095/biolreprod.113.109694

Knight, P. G., Wilson, S. C., Gladwell, R. Y., and Cunningham, F. J. (1982). Evidence for the involvement of central catecholaminergic mechanisms in mediating the preovulatory surge of luteinizing hormone in the domestic hen. *J. Endocrinol.* 94, 295–304. doi:10.1677/joe.0.0940295

Li, D. Y., Wu, N., Tu, J. B., Hu, Y. D., Yang, M. Y., Yin, H. D., et al. (2015). Expression patterns of melatonin receptors in chicken ovarian follicles affected by monochromatic light. *Genet. Mol. Res.* 14, 10072–10080. doi:10.4238/2015.August.21.14

Li, L., Zhang, Z., Peng, J., Wang, Y., and Zhu, Q. (2014). Cooperation of luteinizing hormone signaling pathways in preovulatory avian follicles regulates circadian clock expression in granulosa cell. *Mol. Cell. Biochem.* 394, 31–41. doi:10.1007/s11010-014-2078-3

Liu, H.-K., Long, D. W., and Bacon, W. L. (2001b). Concentration change patterns of luteinizing hormone and progesterone and distribution of hierarchical follicles in normal and arrested laying Turkey hens. *Poult. Sci.* 80, 1509–1518. doi:10.1093/ps/80.10.1509

Liu, H.-K., Long, D. W., and Bacon, W. L. (2002). Interval between preovulatory surges of luteinizing hormone increases late in the reproductive period in Turkey hens. *Biol. Reprod.* 66, 1068–1075. doi:10.1095/biolreprod66.4.1068

Liu, H.-K., Long, D. W., and Bacon, W. L. (2001a). Preovulatory luteinizing hormone surge interval in old and young laying Turkey hens early in the egg production period. *Poult. Sci.* 80, 1364–1370. doi:10.1093/ps/80.9.1364

Maddineni, S., Metzger, S., Ocón, O., Hendricks, G., and Ramachandran, R. (2005). Adiponectin gene is expressed in multiple tissues in the chicken: food deprivation influences adiponectin messenger ribonucleic acid. *Endocrinology* 146, 4250–4256. doi:10.1210/en.2005-0254

McGuire, N. L., Kangas, K., and Bentley, G. E. (2011). Effects of melatonin on peripheral reproductive function: Regulation of testicular GnIH and testosterone. *Endocrinology* 152, 3461–3470. doi:10.1210/en.2011-1053

Melek, O., Morris, T. R., and Jennings, R. C. (1973). The time factor in egg formation for hens exposed to ahemeral light-dark cycles. *Br. Poult. Sci.* 14, 493–498. doi:10.1080/00071667308416056

Menet, J. S., Pescatore, S., and Rosbash, M. (2014). CLOCK:BMAL1 is a pioneer-like transcription factor. *Genes Dev.* 28, 8–13. doi:10.1101/gad.228536.113

Morris, T. R. (1973). The effects of ahemeral light and dark cycles on egg production in the fowl. *Poult. Sci.* 52, 423–445. doi:10.3382/ps.0520423

Ocón-Grove, O. M., Poole, D. H., and Johnson, A. L. (2012). Bone morphogenetic protein 6 promotes FSH receptor and anti-Müllerian hormone mRNA expression in granulosa cells from hen prehierarchal follicles. *Reproduction* 143, 825–833. doi:10.1530/REP-11-0271

Rothchild, I., and Fraps, R. M. (1949). The induction of ovulating hormone release from the pituitary of the domestic hen by means of progesterone. *Endocrinology* 44, 141–149. doi:10.1210/endo-44-2-141

Rozenboim, I., Silsby, J. L., Tabibzadeh, C., Pitts, G. R., Youngren, O. M., and El Halawani, M. E. (1993). Hypothalamic and posterior pituitary content of vasoactive intestinal peptide and gonadotropin-releasing hormones I and II in the Turkey hen. *Biol. Reprod.* 49, 622–626. doi:10.1095/biolreprod49.3.622

Rzasa, J., Williams, J., and Etches, R. J. (1983). A study of the ovulation-inhibiting effects of dexamethasone in the domestic hen. *Gen. Comp. Endocrinol.* 52, 311–314. doi:10.1016/0016-6480(83)90126-0

Sechman, A., Paczoska-Eliasiewicz, H., Rzasa, J., and Hrabia, A. (2000). Simultaneous determination of plasma ovarian and thyroid hormones during sexual maturation of the hen (*Gallus domesticus*). *Folia Biol.* 48, 7–12.

Sechman, A., Pawłowska, K., and Rzasa, J. (2009). Influence of triiodothyronine (T(3)) on secretion of steroids and thyroid hormone receptor expression in chicken ovarian follicles. *Domest. Anim. Endocrinol.* 37, 61–73. doi:10.1016/j.domaniend.2009.03.001

Sechman, A. (2013). The role of thyroid hormones in regulation of chicken ovarian steroidogenesis. *Gen. Comp. Endocrinol.* 90, 68–75. Epub 2013 Apr 28. doi:10.1016/j.ygcen.2013.04.012

Soliman, K. F. A., and Huston, T. M. (1974). Involvement of the adrenal gland in ovulation of the fowl. *Poult. Sci.* 53, 1664–1667. doi:10.3382/ps.0531664

Sun, S., and El Halawani, M. E. (1995). Protein kinase-C mediates chicken vasoactive intestinal peptide stimulated prolactin secretion and gene expression in Turkey primary pituitary cells. *Gen. Comp. Endocrinol.* 99, 289–297. doi:10.1006/gcen.1995.1112

Thayananuphat, A., Youngren, O. M., Kang, S. W., Bakken, T., Kosonsiriluk, S., Chaiseha, Y., et al. (2011). Dopamine and mesotocin neurotransmission during the transition from incubation to brooding in the Turkey. *Horm. Behav.* 60, 327–335. doi:10.1016/j.yhbeh.2011.06.009

Tischkau, S. A., Howell, R. E., Hickok, J. R., Krager, S. L., and Bahr, J. M. (2011). The luteinizing hormone surge regulates circadian clock gene expression in the chicken ovary. *Chronobiol. Int.* 28, 10–20. doi:10.3109/07420528.2010.530363

Warren, D. C., and Scott, H. M. (1935). The time factor in egg formation. *Poult. Sci.* 14, 195–207. doi:10.3382/ps.0140195

Williams, J. B., Etches, R. J., and Rzasa, J. (1985). Induction of a pause in laying by corticosterone infusion or dietary alterations: Effects on the reproductive system, food consumption and body weight. *Br. Poult. Sci.* 26, 25–34. doi:10.1080/00071668508416783

Wilson, S. C., Cunningham, F. J., and Morris, T. R. (1982). Diurnal changes in the plasma concentrations of corticosterone, luteinizing hormone and progesterone during sexual develop- ment and the ovulatory cycle of Khaki Campbell ducks. *J. Endocrinol.* 93, 267–277. doi:10.1677/joe.0.0930267

Wilson, S. C., Jennings, R. C., and Cunningham, F. J. (1985). Effects of an advance of darkness on the ovulatory cycle of the hen. *Br. Poult. Sci.* 26, 83–96. doi:10.1080/00071668508416790

Wilson, S. C., and Sharp, P. J. (1975). Changes in plasma concentrations of luteinizing hormone after injection of progesterone at various times during the ovulatory cycle of the domestic hen (*Gallus domesticus*). *J. Endocrinol.* 67, 59–70. PMID: 1194828. doi:10.1677/joe.0.0670059

Wilson, S. C., and Sharp, P. J. (1976). Induction of luteinizing hormone release by gonadal steroids in the ovariectomized domestic hen. *J. Endocrinol.* 71, 87–98. doi:10.1677/joe.0.0710087

Zhang, L., Chen, F., Cao, J., Dong, Y., Wang, Z., Hu, M., et al. (2017). Green light inhibits GnRH-I expression by stimulating the melatonin-GnIH pathway in the chick brain. *J. Neuroendocrinol.* 29. doi:10.1111/jne.12468

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Sandra G. Velleman,
The Ohio State University, United States

REVIEWED BY
Rie Henriksen,
Linköping University, Sweden
Paola Sgadò,
University of Trento, Italy

*CORRESPONDENCE
András Csillag,
csillag.andras@med.semmelweis-univ.hu

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Avian models for brain mechanisms underlying altered social behavior in autism

András Csillag*, Ágota Ádám and Gergely Zachar

Department of Anatomy, Histology, and Embryology, Faculty of Medicine, Semmelweis University, Budapest, Hungary

The current review is an update on experimental approaches in which birds serve as model species for the investigation of typical failure symptoms associated with autism spectrum disorder (ASD). The discussion is focused on deficiencies of social behavior, from social interactions of domestic chicks, based on visual and auditory cues, to vocal communication in songbirds. Two groups of pathogenetic/risk factors are discussed: 1) non-genetic (environmental/epigenetic) factors, exemplified by embryonic exposure to valproic acid (VPA), and 2) genetic factors, represented by a list of candidate genes and signaling pathways of diagnostic or predictive value in ASD patients. Given the similarities of birds as experimental models to humans (visual orientation, vocal learning, social cohesions), avian models usefully contribute toward the elucidation of the neural systems and developmental factors underlying ASD, improving the applicability of preclinical results obtained on laboratory rodents. Furthermore, they may predict potential susceptibility factors worthy of investigation (both by animal studies and by monitoring human babies at risk), with potential therapeutic consequence.

KEYWORDS

social behavior, songbird, ASD, dopamin, valproic acid, genes

Introduction

With many problems in biomedicine, the key to success is finding a suitable model system/species. Experimental models are essential for evidence-based studies, especially those of the interventional type (which would be impractical or ethically unacceptable to carry out in humans). They also help finding the appropriate level of explanation, blissfully avoiding the traps of Scylla and Charybdis, extreme reductionism (*cf.* the problem described as "the janitor's dream" by Calvin, 1998) or undue generalization. Model systems, however, may fail at times. Ever since, there has been an increasing demand for alternative model species (Bolker, 2012; Yartsev, 2017) and phylogenetic comparisons to improve the poor applicability of preclinical results obtained mostly on laboratory rodents (Perrin, 2014).

Autism Spectrum Disorder (ASD) is one of the most common neurodevelopmental disorders associated with altered social behavior. The variable and multifaceted character of the disease necessitates reliable animal models and multilateral approaches. Birds as

evolutionary alternatives to mammals, with a wide behavioral repertoire, may well represent a special window of observation. Animal models may help discern genetic vs. non-genetic (epigenetic, environmental) factors in the etiology of ASD (Ergaz et al., 2016). Experimental intervention may help reveal the pathogenetic causes during gestation, and whether such risk factors can be antagonized or reversed.

The current summary is not intended to give a comprehensive account of available animal models for different autism related syndromes (*cf.* previous thorough reviews, Ergaz et al., 2016; Nicolini and Fahnestock, 2018). Here, we are focusing on those applied on avian species, elaborating on the valproic acid model (an example for non-genetic interventions), and on a set of genetic modifications, in which birds have been used as experimental subjects. Avian models have helped in the past to solve problems such as neural plasticity in early learning (McCabe and Horn, 1994; Rose, 2000), adult neurogenesis (Goldman and Nottebohm, 1983; Nottebohm, 2005), thanks to the existence of common patterns in evolution. In many ways, birds are better models of human behavior than mammals. They are highly visual beings, display vocal communication, even vocal learning, and often live in pairs or flocks, exposed to multiple social signals.

Newly hatched domestic chicks have often been used as models in studies of behavioral neuroscience (Bolhuis and Honey, 1998; Rose, 2000; Zachar et al., 2008), because they can display complex behaviors, not confounded by earlier experience (Rose, 2000). Just like newborn humans, chicks have a predisposition to prefer the proximity of conspecifics (for review see Di Giorgio et al., 2017). Whether such predispositions are affected by autism is a matter of debate (Elsabbagh et al., 2013; Jones and Klin, 2013; Sgadò et al., 2018; Zachar et al., 2019), however, the social bonds based on innate stimulus preferences are certainly impaired by ASD. The social interactions of domestic chicks are based on visual and auditory cues (Koshiba et al., 2013), i.e., traits that are more human-like than the olfactory-biased sociability of most mammals (Brennan and Kendrick, 2006).

There are striking behavioral similarities between children with ASD and domestic chicks with socio-sensory deprivation (Koshiba et al., 2016), further supporting feasibility of the avian model.

Chicks react to social isolation by displaying behaviors aimed at reuniting with conspecifics (Gallup and Suarez, 1980), and they prefer larger groups of siblings over smaller ones (Zachar et al., 2017). The drive to reinstatement can be evaluated by measurement of distress vocalization (Marx et al., 2001; Takeuchi et al., 1996; Yazaki et al., 1999; Montevecchi et al., 1973; Zsedényi et al., 2014). Such innate gregariousness of naïve domestic chicks likely relies on the social brain network (SBN, Goodson, 2005), and affiliation to siblings is likely processed similarly to other social behaviors (Mayer et al., 2017). The mesolimbic dopaminergic reward system is amply interconnected and overlapping with SBN forming the phylogenetically conservative social decision-making network (O'Connell and Hofmann, 2011) (Figure 1). Therefore, the separation-reinstatement paradigm of the young domestic chick can be an appropriate laboratory model of sociability [e.g., the test for group preference (Zachar et al., 2017) and other tests of belongingness or aggregation, see Nishigori et al., 2013].

Song learning and singing in oscine birds is often paralleled with the human language (affected by ASD). Remarkably, however, no studies known to us have been reported on effect of embryonic VPA treatment on the vocal behavior of songbirds (either learned singing or innate calls).

Relevant VPA based (or other environmental intervention-based) models should also consider the importance of innate calls. Parental care requires intense cooperation through coordination and synchronization of behavior, and an intense communication between the parents. Zebra finches change their vocal communication over pair formation and during nest building and incubation (Gill et al., 2015). They reduce the amount of distance calls and courtship singing, while increasing the frequency of the low amplitude calls specifically used for short distance communication between the pairs (D'Amelio et al., 2017). Furthermore, the acoustic interactions between the two members of the pair become more synchronized (Gill et al., 2015). Coordinated duetting between the parents may function as negotiations over the parental effort, at least during the egg incubation phase (Boucaud et al., 2016, 2017). Compared to widely studied courtship songs, little is known about the short distance calls (Ter Maat et al., 2014). The neural substrate responsible for these calls overlaps, at least partially, with the brain's song system (Gobes et al., 2009; Giret et al., 2015), however the different social function implies the involvement of different regions and/or genes. The elaborate male song is a learned behavior, and, in this sense, it is more like human speech than are innate calls. The shorter calls are suitable for individual recognition (Elie and Theunissen, 2018) and for promoting cooperation between individuals, another facet of similarity with human language. The latter is often neglected in the scientific literature on birdsong. Moreover, female zebra finches are also capable of cooperative vocalization. They possess a less developed (but otherwise homologous) neural network for processing song than that of males (Shaughnessy et al., 2019). Such, more specific, songbird models might provide novel insight into cooperative vocalization in normal or pathological states.

Embryonic treatment with VPA

A well-established ASD model for laboratory rodents is prenatal exposure to valproic acid (VPA), a known antiepileptic substance (Rodier et al., 1996, for comprehensive reviews see Roullet et al., 2013; Nicolini and Fahnestock, 2018)

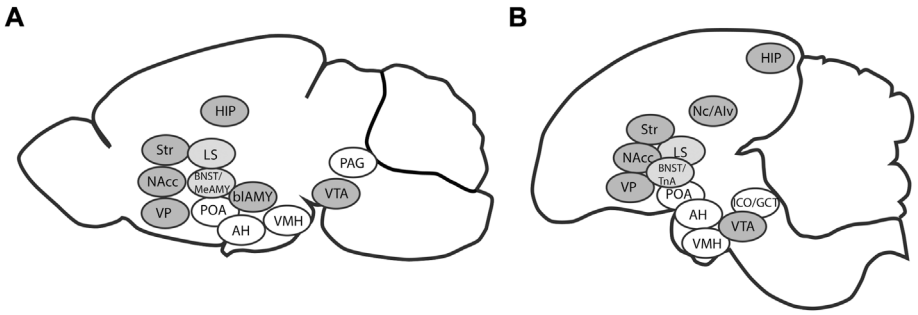

FIGURE 1
Brain regions constituting the social brain network (white), the mesolimbic dopaminergic reward system (dark grey) and their overlap (light grey) in mammals **(A)** and in birds **(B)**. The two interconnected networks are collectively designated as the social decision-making network. AH: anterior hypothalamus, blAMY: basolateral amygdala (avian homologue Nc/AIv: caudal nidopallium/ventral intermediate arcopallium), BNST: bed nucleus of the stria terminalis, HIP: hippocampus, LS: lateral septum, MeAMY: medial extended amygdala (avian homologue TnA: nucleus taeniae), NAcc: nucleus accumbens, PAG: periaqueductal gray (avian homologue ICO/GCT: intercollicular nucleus/midbrain central gray), POA: preoptic area, Str: striatum, VMH: ventromedial hypothalamus, VP: ventral pallidum, VTA: ventral tegmental area. Based on O'Connell and Hofmann, 2011.

and mood stabilizing agent (Cipriani et al., 2013). The key factor in the action of VPA seems to be inhibition of histone deacetylase (Göttlicher et al., 2001), affecting gene expression and transcription during CNS development.

VPA has been used in birds first as a teratogenic agent. Domestic chicken eggs were injected with VPA at critical times of development to study malformations (Barnes et al., 1996; Whitsel et al., 2002; Hsieh et al., 2013), or modifications of gene expression (Akhtar et al., 2015), e.g., reduction of PAX6 (Zosen et al., 2022). The reported distinct and dose-dependent effects of VPA on brain development potentially reflect on behavioral measures (Barnes et al., 1996; Zosen et al., 2022).

In relation to autism-related social deficits, administration of VPA *in ovo* caused impairment of social behavior (but not imprinting) in chicks (Nishigori et al., 2013). VPA alters the approach response to visual cues resembling to conspecifics such as simulated biological motion (Lorenzi et al., 2019; Matsushima et al., 2022) or face like stimuli (Adiletta et al., 2021), suggesting an early effect on social stimulus processing similar to autistic children. VPA abolished the innate visual predispositions of chicks to hen features, while imprinting remained unaffected (Sgadò et al., 2018). Similarly, in a study by Zachar et al. (2019), early learning (passive avoidance training) and color discrimination were not impaired by VPA treatment. At variance with the results of Sgadò et al. (2018), albeit at a later phase of post-embryonic development, VPA exposure did not affect the innate approach preference of birds for the larger over smaller group of conspecifics, or for companion birds with natural facial features over those with blurred features (Zachar et al., 2019). However, VPA did attenuate social exploration and the recognition of familiar conspecifics, by the end of the third week post-hatch, drawing attention to the importance of early social exploration in human ASD (Zachar et al., 2019). The corollary from these studies is that subtle alterations in innate predispositions and social exploration might well predict the future manifestation of ASD. Therefore, a standardized recording and monitoring of human babies at risk during the early postnatal period would be highly recommended practice.

The valproate model exemplifies the potential role of epigenetic/environmental factors in the pathogenesis of ASD. Other animal models, including those applied mainly in songbirds, are based upon genomic alterations.

Genomic alterations

Deficits in the acquisition of culturally transmitted social skills, including speech and language are important early indicators of ASD (Tager-Flusberg et al., 2005; Mody and Belliveau, 2013; Sperdin and Schaer, 2016). The elaboration of birdsong is often compared to the complexity of human speech (Aamodt et al., 2019). Songbirds may represent useful models for certain aspects of ASD both in terms of vocal communication and sociability. Several candidate genes, common to songbirds and humans, have been described to participate in the production and socially meaningful perception of song/speech (for an overview of zebra finch studies see Panaitof, 2012). Genomic interventions in altricial songbirds may help understand the etiology of some of the failure symptoms in ASD. By contrast, very few studies have tackled the genetic basis of social behavior of precocial birds. Of five candidate genes, TTRAP showed a correlation with social behavior of domestic chicks (Johnsson et al., 2018). Though none of those five genes were confirmed candidates in human autism (Satterstrom et al., 2020), TTRAP is associated with language-related regions (Pinel et al., 2012).

The following account is not intended to cover the ever-expanding plethora of genomic factors that are potentially linked to the pathogenesis of ASD. We merely attempted here to summarize the most promising lines of investigation, in which songbirds played an important part as experimental subjects.

FOXP1, FOXP2

The Forkhead Box transcription factors FOXP1 and FOXP2 were found to be linked to speech and language disorders (Lai et al., 2001), and are among the risk genes for autism (Satterstrom et al., 2020). Similarly distributed in the developing human and songbird language-related centers, they proved to be promising candidates for cross-species studies (Teramitsu et al., 2004). Knockdown of *FoxP2* in the basal ganglia song nucleus, Area X, was found to impair singing in zebra finches (Haesler et al., 2007). The importance of *FoxP2* in the regulation of singing has been supported by other suppression or overexpression studies (Murugan et al., 2013; Heston and White, 2015). In a more recent zebra finch study, *FoxP1* was found to be expressed mainly in striatal-projecting HVC neurons (forebrain mirror neurons). Knockdown of *FoxP1* expression in juvenile birds led to a selective learning deficit, affecting the ability to form memories essential for the cultural transmission of behavior (adult model song) (Garcia-Oscos et al., 2021).

Cntnap2

An important target of FOXP2, Contactin-associated protein-like 2 (*Cntnap2*) (Spiteri et al., 2007) has been identified as an autism susceptibility gene (Alarcón et al., 2008). This gene is considered a risk factor for language-related disorders, including ASD, language impairment, and stuttering (Arking et al., 2008; Li et al., 2010). A specific enrichment of the CNTNAP2 protein was found in the song nuclei of male zebra finches (Condro and White, 2014), pointing to a generalized role in vocal learning across vertebrate species.

FXS, FMRP

Fragile X syndrome (FXS) is the most common inherited form of ASD, characterized by hyperactivity, impulsivity, and anxiety, as well as by defective language development. Many FXS symptoms appear early in life, together with emerging autistic features (Hagerman et al., 2017). A trinucleotide repeat disorder, silencing of the gene leads to the loss of its product, Fragile X mental retardation 1 protein (FMRP). FMRP is an RNA-binding protein regulating the translation of numerous mRNAs instrumental in the development and maintenance of synapses. FXS animal models are based on the loss of neural plasticity and an imbalance between inhibitory and excitatory neuronal circuits, also mimicking certain clinical symptoms of ASD. FMRP is a promising target for therapeutic intervention. The gene and its product have been identified in the vocal control system of the zebra finch, recommended as a model for FXS-associated language disorders (Winograd et al., 2008). Curiously, however, despite obvious therapeutic advantages, experimental interventions on FXS or FMRP, in relation to ASD, have not yet been reported in avian species.

In addition to songbirds, FMRP has been located also in the brainstem auditory nuclei of domestic chicks, with a specific role in dendritic dynamics (Wang et al., 2014) and axonal growth (Wang et al., 2020). In this capacity, FMRP is just one of many genomic factors to regulate axonal pathfinding, some of which have been demonstrated in avian vocal learning-relevant regions, e.g., the SLIT-ROBO system (Wang et al., 2015).

ADNP

ADNP is an essential protein instrumental in brain development and neural plasticity, thereby determining a host of social and cognitive functions potentially malfunctioning in autism. Mutations in ADNP system have been found in human ASD cases (Helsmoortel et al., 2014; Satterstrom et al., 2020). An established experimental model, *Adnp*± mice develop impairments of cognitive and social behaviors (Vulih-Shultzman et al., 2007), resulting in Alzheimer's disease related symptoms, as well as autistic features (Malishkevich et al., 2015). Notably, male *Adnp*± mice are more seriously affected, mimicking a similar prevalence of failure symptoms in human subjects with ASD. The sex- and age-related expression of ADNP mRNA was reported in different areas (cerebellum, cerebrum, brainstem) of the zebra finch (Hacohen-Kleiman et al., 2015), with a distinct sexual dimorphism (young males expressing higher levels of ADNP than females, in agreement with the notion that only males perform courtship singing). The gene expression profile was largely confirmed in the domesticated canary, and ADNP mRNA was found to be enriched mainly in the mesopallium, harboring centers for sensory integration and higher auditory processing (Hacohen-Kleiman et al., 2020).

mTOR

Owing to its role in experience-dependent synaptic plasticity (Garza-Lombó and Gonsebatt, 2016), the Mechanistic Target of Rapamycin (mTOR) signaling cascade has been implicated as a factor in the etiology of ASD, based chiefly on mouse models (Chen et al., 2014; Kazdoba et al., 2016). In an elegant study on zebra finch (Ahmadiantehrani and London, 2017) mTOR signaling was activated in the auditory forebrain by memorization of tutor song in adult males but not in younger males (not old enough to copy song) or in females (who cannot sing). Both the inhibition and constitutive activation of mTOR during tutor experiences diminished copying of tutor song. Remarkably, constitutive mTOR activation lowered the 'social engagement' of juvenile zebra finches during tutor experiences, somewhat similarly to the situation found in humans with autism. The findings bear relevance for the role of the onset

of mTOR cascade in the encoding of early life experience to determine future behavior.

Glycogen synthase kinase-3 (GSK-3)

GSK-3 is a highly conserved serine/threonine protein kinase that plays a central role in a wide variety of cellular processes associated with cognition and behavior (Beurel et al., 2015). In a recent study on zebra finch, inhibition of the splice variant GSK-3β was found to attenuate social recognition and decision making (Moaraf et al., 2022). Interestingly, birds are "natural knockouts" for the *GSK-3α* gene (Alon et al., 2011), enabling selective investigation of the effects of *GSK-3β*. Although *GSK-3β* is not among the 102 key risk genes recently defined for autism, being the 150th among 18,000 observed genes (Satterstrom et al., 2020), the findings related to social recognition in songbirds may potentially indicate a future involvement and predict a novel line of investigation in this direction.

Novel aspects of convergent genomic regions

Based on a meta-analysis of convergence between avian and human accelerated genomic regions (AR), the important study by Cahill et al. (2021) casts light on the regulation of vocal learning in different clades of birds ("rediscovered" two to three times during avian evolution) and that of human speech. In addition to known AR such as FOXP2 (already discussed above), further novel candidate genes were 'mined' in this study. For example, NR2F1, a neurodevelopment regulating transcription factor with predicted function in vocalization behavior, proved to be the highest density AR hotspot specific to vocal learning birds, and it is also a SFARI class S gene for ASD (Abrahams et al., 2013).

In most of the cases described above the genomic risk factors had been identified first in humans, then confirmed in mammalian model systems, and avian experiments "followed suit" as logical sequels. Notably, however, in the last two paragraphs examples were given for a reverse order of events: avian studies taking the lead to predict potential susceptibility factors worthy of investigation.

Dual subject studies

The past decade witnessed a tendency for coupled/comparative studies, in which the results obtained from avian and human subjects were jointly analyzed. Most of these studies tackled different behavioral features of diagnostic or therapeutic significance of ASD (Koshiba et al., 2016; Kelley et al., 2017; Galizio et al., 2020; Shvarts et al., 2020). In addition, molecular neuroanatomical studies, carried out on multiple species, including man, have also been reported, e.g., for the comparative localization of FMRP (see above) in the auditory system (Wang et al., 2014). Dual subject reports further highlight the translational importance of investigation into mechanisms across species, in which birds have a fair and growing share.

Notion from comprehensive animal experiments will likely be extrapolated to normal and impaired regulation of social behaviors in humans. By model building of causal and therapeutic significance, avian experiments continue to contribute toward the elucidation of anatomically traceable neural systems and developmental factors underlying human autism spectrum disorder.

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Conflict of interest

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References

Aamodt, C. M., Farias-Virgens, M., and White, S. A. (2019). Birdsong as a window into language origins and evolutionary neuroscience. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 375, 20190060–20191789. doi:10.1098/RSTB.2019.0060

Abrahams, B. S., Arking, D. E., Campbell, D. B., Mefford, H. C., Morrow, E. M., Weiss, L. A., et al. (2013). SFARI gene 2.0: A community-driven knowledgebase for the autism spectrum disorders (ASDs). *Mol. Autism* 4, 36. doi:10.1186/2040-2392-4-36

Adiletta, A., Pedrana, S., Rosa-Salva, O., and Sgadò, P. (2021). Spontaneous visual preference for face-like stimuli is impaired in newly-hatched domestic chicks exposed to valproic acid during embryogenesis. *Front. Behav. Neurosci.* 15, 733140. doi:10.3389/fnbeh.2021.733140

Ahmadiantehrani, S., and London, S. E. (2017). Bidirectional manipulation of mTOR signaling disrupts socially mediated vocal learning in juvenile songbirds. *Proc. Natl. Acad. Sci. U. S. A.* 114, 9463–9468. doi:10.1073/pnas.1701829114

Akhtar, L., Khan, M. Y., and Minhas, L. A. (2015). The effect of prenatal administration of valproic acid on the survivability and day of hatching of chick embryo. *J. Pak. Med. Assoc.* 65 (2), 175–178.

Alarcón, M., Abrahams, B. S., Stone, J. L., Duvall, J. A., Perederiy, J. V., Bomar, J. M., et al. (2008). Linkage, association, and gene-expression analyses identify CNTNAP2 as an autism-susceptibility gene. *Am. J. Hum. Genet.* 82, 150–159. doi:10.1016/j.ajhg.2007.09.005

Alon, L. T., Pietrokovski, S., Barkan, S., Avrahami, L., Kaidanovich-Beilin, O., Woodgett, J. R., et al. (2011). Selective loss of glycogen synthase kinase-3α in birds reveals distinct roles for GSK-3 isozymes in tau phosphorylation. *FEBS Lett.* 585 (8), 1158–1162. doi:10.1016/j.febslet.2011.03.025

Arking, D. E., Cutler, D. J., Brune, C. W., Teslovich, T. M., West, K., Ikeda, M., et al. (2008). A common genetic variant in the neurexin superfamily member CNTNAP2 increases familial risk of autism. *Am. J. Hum. Genet.* 82, 160–164. doi:10.1016/j.ajhg.2007.09.015

Barnes, G. L., Jr, Marian, B. D., and Tuan, R. T. (1996). Valproic acid-induced somite teratogenesis in the chick embryo: Relationship with *pax-1* gene expression. *Teratology* 54, 93–102. doi:10.1002/(SICI)1096-9926(199606)54:2<93:AID-TERA5>3.0.CO;2-5

Beurel, E., Grieco, S. F., and Jope, R. S. (2015). Glycogen synthase kinase-3 (GSK3): Regulation, actions, and diseases. *Pharmacol. Ther.* 148, 114–131. doi:10.1016/j.pharmthera.2014.11.016

Bolhuis, J. J., and Honey, R. C. (1998). Imprinting, learning and development: From behaviour to brain and back. *Trends Neurosci.* 21, 306–311. doi:10.1016/S0166-2236(98)01258-2

Bolker, J. (2012). Model organisms: There's more to life than rats and flies. *Nature* 491 (7422), 31–33. doi:10.1038/491031a

Boucaud, I. C. A., Mariette, M. M., Villain, A. S., and Vignal, C. (2016). Vocal negotiation over parental care? Acoustic communication at the nest predicts partners' incubation share. *Biol. J. Linn. Soc.* 117, 322–336. doi:10.1111/bij.12705

Boucaud, I. C. A., Perez, E. C., Ramos, L. S., Griffith, S. C., and Vignal, C. (2017). Acoustic communication in zebra finches signals when mates will take turns with parental duties. *Behav. Ecol.* 28, 645–656. doi:10.1093/beheco/arw189

Brennan, P. A., and Kendrick, K. M. (2006). Mammalian social odours: Attraction and individual recognition. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 361, 2061–2078. doi:10.1098/rstb.2006.1931

Cahill, J. A., Armstrong, J., Deran, A., Khoury, C. J., Paten, B., Haussler, D., et al. (2021). Positive selection in noncoding genomic regions of vocal learning birds is associated with genes implicated in vocal learning and speech functions in humans. *Genome Res.* 31, 2035–2049. doi:10.1101/gr.275989.121

Calvin, W. H. (1998). Competing for consciousness: A darwinian mechanism at an appropriate level of explanation. *J. Conscious. Stud.* 5, 389–404.

Chen, J., Alberts, I., and Li, X. (2014). Dysregulation of the IGF-I/PI3K/AKT/mTOR signaling pathway in autism spectrum disorders. *Int. J. Dev. Neurosci.* 35, 35–41. doi:10.1016/j.ijdevneu.2014.03.006

Cipriani, A., Reid, K., Young, A. H., Macritchie, K., and Geddes, J. (2013). Valproic acid, valproate and divalproex in the maintenance treatment of bipolar disorder. *Cochrane Database Syst. Rev.* 10, CD003196. doi:10.1002/14651858.CD003196.pub2

Condro, M. C., and White, S. A. (2014). Distribution of language-related Cntnap2 protein in neural circuits critical for vocal learning. *J. Comp. Neurol.* 522, 169–185. doi:10.1002/cne.23394

D'Amelio, P. B., Trost, L., and Ter Maat, A. (2017). Vocal exchanges during pair formation and maintenance in the zebra finch (*Taeniopygia guttata*). *Front. Zool.* 14, 13. doi:10.1186/s12983-017-0197-x

Di Giorgio, E., Loveland, J. L., Mayer, U., Rosa-Salva, O., Versace, E., and Vallortigara, G. (2017). Filial responses as predisposed and learned preferences: Early attachment in chicks and babies. *Behav. Brain Res.* 325, 90–104. doi:10.1016/j.bbr.2016.09.018

Elie, J. E., and Theunissen, F. E. (2018). Zebra finches identify individuals using vocal signatures unique to each call type. *Nat. Commun.* 9, 4026. doi:10.1038/s41467-018-06394-9

Elsabbagh, M., Gliga, T., Pickles, A., Hudry, K., Charman, T., and Johnson, M. H., the BASIS Team (2013). The development of face orienting mechanisms in infants at-risk for autism. *Behav. Brain Res.* 251, 147–154. doi:10.1016/j.bbr.2012.07.030

Ergaz, Z., Weinstein-Fudim, L., and Ornoy, A. (2016). Genetic and non-genetic animal models for autism spectrum disorders (ASD). *Reprod. Toxicol.* 64, 116–140. doi:10.1016/j.reprotox.2016.04.024

Galizio, A., Higbee, T. S., and Odum, A. L. (2020). Choice for reinforced behavioral variability in children with autism spectrum disorder. *J. Exp. Anal. Behav.* 113, 495–514. doi:10.1002/jeab.591

Gallup, G. G., and Suarez, S. D. (1980). An ethological analysis of open-field behaviour in chickens. *Anim. Behav.* 28, 368–378. doi:10.1016/s0003-3472(80)80045-5

Garcia-Oscos, F., Koch, T. M. I., Pancholi, H., Trusel, M., Daliparthi, V., Co, M., et al. (2021). Autism-linked gene FoxP1 selectively regulates the cultural transmission of learned vocalizations. *Sci. Adv.* 7, eabd2827. doi:10.1126/sciadv.abd2827

Garza-Lombó, C., and Gonsebatt, M. E. (2016). Mammalian target of rapamycin: Its role in early neural development and in adult and aged brain function. *Front. Cell. Neurosci.* 10, 157. doi:10.3389/fncel.2016.00157

Gill, L. F., Goymann, W., Ter Maat, A., and Gahr, M. (2015). Patterns of call communication between group-housed zebra finches change during the breeding cycle. *Elife* 4, e07770. doi:10.7554/eLife.07770

Giret, N., Menardy, F., and Del Negro, C. (2015). Sex differences in the representation of call stimuli in a songbird secondary auditory area. *Front. Behav. Neurosci.* 9, 290. doi:10.3389/fnbeh.2015.00290

Gobes, S. M., Ter Haar, S. M., Vignal, C., Vergne, A. L., Mathevon, N., and Bolhuis, J. J. (2009). Differential responsiveness in brain and behavior to sexually dimorphic long calls in male and female zebra finches. *J. Comp. Neurol.* 516, 312–320. doi:10.1002/cne.22113

Goldman, S. A., and Nottebohm, F. (1983). Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain. *Proc. Natl. Acad. Sci. U. S. A.* 80, 2390–2394. doi:10.1073/pnas.80.8.2390

Goodson, J. L. (2005). The vertebrate social behavior network: Evolutionary themes and variations. *Horm. Behav.* 48, 11–22. doi:10.1016/j.yhbeh.2005.02.003

Göttlicher, M., Minucci, S., Zhu, P., Krämer, O. H., Schimpf, A., Giavara, S., et al. (2001). Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *EMBO J.* 20, 6969–6978. doi:10.1093/emboj/20.24.6969

Hacohen-Kleiman, G., Barnea, A., and Gozes, I. (2015). Adnp: A major autism mutated gene is differentially distributed (age and gender) in the songbird brain. *Peptides* 72, 75–79. doi:10.1016/j.peptides.2015.04.008

Hacohen-Kleiman, G., Moaraf, S., Kapitansky, O., and Gozes, I. (2020). Sex-and region-dependent expression of the autism-linked ADNP correlates with social-and speech-related genes in the canary brain. *J. Mol. Neurosci.* 70, 1671–1683. doi:10.1007/s12031-020-01700-x

Haesler, S., Rochefort, C., Georgi, B., Licznerski, P., Osten, P., and Scharff, C. (2007). Incomplete and inaccurate vocal imitation after knockdown of FoxP2 in songbird basal ganglia nucleus Area X. *PLoS Biol.* 5, e321. doi:10.1371/journal.pbio.0050321

Hagerman, R., Berry-Kravis, E., Hazlett, H. C., Bailey, D. B., Jr, Moine, H., Kooy, R. F., et al. (2017). Fragile X syndrome. *Nat. Rev. Dis. Prim.* 3, 17065. doi:10.1038/nrdp.2017.65

Helsmoortel, C., Vulto-van Silfhout, A., Coe, B., Vandeweyer, G., Rooms, L., van den Ende, J., et al. (2014). A SWI/SNF-related autism syndrome caused by de novo mutations in ADNP. *Nat. Genet.* 46, 380–384. doi:10.1038/ng.2899

Heston, J. B., and White, S. A. (2015). Behavior-linked FoxP2 regulation enables zebra finch vocal learning. *J. Neurosci.* 35, 2885–2894. doi:10.1523/JNEUROSCI.3715-14.2015

Hsieh, C. L., Chen, K. C., Ding, C. Y., Tsai, W. J., Wu, J. F., and Peng, C. C. (2013). Valproic acid substantially downregulated genes folr1, IGF2R, RGS2, COL6A3, EDNRB, KLF6, and pax-3, N-acetylcysteine alleviated most of the induced gene alterations in chicken embryo model. *Rom. J. Morphol. Embryol.* 54 (4), 993–1004.

Johnsson, M., Henriksen, R., Fogelholm, J., Höglund, A., Jensen, P., and Wright, D. (2018). Genetics and genomics of social behavior in a chicken model. *Genetics* 209, 209–221. doi:10.1534/genetics.118.300810

Jones, W., and Klin, A. (2013). Attention to eyes is present but in decline in 2-6-month-old infants later diagnosed with autism. *Nature* 504 (7480), 427–431. doi:10.1038/nature12715

Kazdoba, T. M., Leach, P. T., and Crawley, J. N. (2016). Behavioral phenotypes of genetic mouse models of autism. *Genes. Brain Behav.* 15, 7–26. doi:10.1111/gbb.12256

Kelley, M. E., Nadler, C. B., Rey, C., Cowie, S., and Podlesnik, C. A. (2017). Noncontingent reinforcement competes with response performance. *J. Exp. Anal. Behav.* 107, 343–353. doi:10.1002/jeab.255

Koshiba, M., Karino, G., Mimura, K., Nakamura, S., Yui, K., Kunikata, T., et al. (2016). Psycho-cognitive intervention for ASD from cross-species behavioral analyses of infants, chicks and common marmosets. *CNS Neurol. Disord. Drug Targets* 15 (5), 578–586. doi:10.2174/1871527315666160413121613

Koshiba, M., Shirakawa, Y., Mimura, K., Senoo, A., Karino, G., and Nakamura, S. (2013). Familiarity perception call elicited under restricted sensory cues in peer-social interactions of the domestic chick. *PLoS ONE* 8, e58847. doi:10.1371/journal.pone.0058847

Lai, C. S., Fisher, S. E., Hurst, J. A., Vargha-Khadem, F., and Monaco, A. P. (2001). A forkhead-domain gene is mutated in a severe speech and language disorder. *Nature* 413, 519–523. doi:10.1038/35097076

Li, X., Hu, Z., He, Y., Xiong, Z., Long, Z., Peng, Y., et al. (2010). Association analysis of CNTNAP2 polymorphisms with autism in the Chinese Han population. *Psychiatr. Genet.* 20, 113–117. doi:10.1097/YPG.0b013e32833a216f

Lorenzi, E., Pross, A., Rosa-Salva, O., Versace, E., Sgadò, P., and Vallortigara, G. (2019). Embryonic exposure to valproic acid affects social predispositions for dynamic cues of animate motion in newly-hatched chicks. *Front. Physiol.* 10, 501. doi:10.3389/fphys.2019.00501

Malishkevich, A., Amram, N., Hacohen-Kleiman, G., Magen, I., Giladi, E., and Gozes, I. (2015). Activity-dependent neuroprotective protein (ADNP) exhibits striking sexual dichotomy impacting on autistic and Alzheimer's pathologies. *Transl. Psychiatry* 5, e501. doi:10.1038/tp.2014.138

Marx, G., Leppelt, J., and Ellendorff, F. (2001). Vocalisation in chicks (Gallus gallus dom.) during stepwise social isolation. *Appl. Anim. Behav. Sci.* 75, 61–74. doi:10.1016/S0168-1591(01)00180-0

Matsushima, T., Miura, M., Patzke, N., Toji, N., Wada, K., Ogura, Y., et al. (2022). Impaired epigenesis of imprinting predispositions causes autism-like behavioral phenotypes in domestic chicks. *bioRxiv* 19, 492744. doi:10.1101/2022.05.19.492744

Mayer, U., Rosa-Salva, O., and Vallortigara, G. (2017). First exposure to an alive conspecific activates septal and amygdaloid nuclei in visually naïve domestic chicks (Gallus gallus). *Behav. Brain Res.* 317, 71–81. doi:10.1016/j.bbr.2016.09.031

McCabe, B. J., and Horn, G. (1994). Learning-related changes in Fos-like immunoreactivity in the chick forebrain after imprinting. *Proc. Natl. Acad. Sci. U. S. A.* 91, 11417–11421. doi:10.1073/pnas.91.24.11417

Moaraf, S., Rippin, I., Terkel, J., Eldar-Finkelman, H., and Barnea, A. (2022). GSK-3β inhibition in birds affects social behavior and increases motor activity. *Front. Physiol.* 13, 881174. doi:10.3389/fphys.2022.881174

Mody, M., and Belliveau, J. W. (2013). Speech and language impairments in autism: Insights from behavior and neuroimaging. *N. Am. J. Med. Sci.* 5, 157–161. doi:10.7156/v5i3p157

Montevecchi, W. A., Gallup, G. G., and Dunlap, W. P. (1973). The peep vocalization in group reared chicks (*Gallus domesticus*): Its relation to fear. *Anim. Behav.* 21 (1), 116–123. doi:10.1016/s0003-3472(73)80049-1

Murugan, M., Harward, S., Scharff, C., and Mooney, R. (2013). Diminished FoxP2 levels affect dopaminergic modulation of corticostriatal signaling important to song variability. *Neuron* 80 (6), 1464–1476. doi:10.1016/j.neuron.2013.09.021

Nicolini, C., and Fahnestock, M. (2018). The valproic acid-induced rodent model of autism. *Exp. Neurol.* 299, 217–227. doi:10.1016/j.expneurol.2017.04.017

Nishigori, H., Kagami, K., Takahashi, A., Tezuka, Y., Sanbe, A., and Nishigori, H. (2013). Impaired social behavior in chicks exposed to sodium valproate during the last week of embryogenesis. *Psychopharmacol. Berl.* 227, 393–402. doi:10.1007/s00213-013-2979-y

Nottebohm, F. (2005). The neural basis of birdsong. *PLoS Biol.* 3 (5), e164. doi:10.1371/journal.pbio.0030164

O'Connell, L. A., and Hofmann, H. A. (2011). The vertebrate mesolimbic reward system and social behavior network: A comparative synthesis. *J. Comp. Neurol.* 519, 3599–3639. doi:10.1002/cne.22735

Panaitof, S. C. (2012). A songbird animal model for dissecting the genetic bases of autism spectrum disorder. *Dis. Markers* 33, 241–249. doi:10.3233/DMA-2012-0918

Perrin, S. (2014). Preclinical research: Make mouse studies work. *Nature* 507 (7493), 423–425. doi:10.1038/507423a

Pinel, P., Fauchereau, F., Moreno, A., Barbot, A., Lathrop, M., Zelenika, D., et al. (2012). Genetic variants of FOXP2 and KIAA0319/TTRAP/THEM2 locus are associated with altered brain activation in distinct language-related regions. *J. Neurosci.* 32, 817–825. doi:10.1523/JNEUROSCI.5996-10.2012

Rodier, P. M., Ingram, J. L., Tisdale, B., Nelson, S., and Romano, J. (1996). Embryological origin for autism: Developmental anomalies of the cranial nerve motor nuclei. *J. Comp. Neurol.* 370, 247–261. doi:10.1002/(SICI)1096-9861(19960624)370:2<247:AID-CNE8>3.0.CO;2-2

Rose, S. P. (2000). God's organism? The chick as a model system for memory studies. *Learn. Mem.* 7, 1–17. doi:10.1101/lm.7.1.1

Roullet, F. I., Lai, J. K., and Foster, J. A. (2013). *In utero* exposure to valproic acid and autism--a current review of clinical and animal studies. *Neurotoxicol. Teratol.* 36, 47–56. doi:10.1016/j.ntt.2013.01.004

Satterstrom, F. K., Kosmicki, J. A., Wang, J., Breen, M. S., De Rubeis, S., An, J. Y., et al. (2020). Large-scale exome sequencing study implicates both developmental and functional changes in the neurobiology of autism. *Cell.* 180, 568–584. doi:10.1016/j.cell.2019.12.036

Sgadò, P., Rosa-Salva, O., Versace, E., and Vallortigara, G. (2018). Embryonic exposure to valproic acid impairs social predispositions of newly hatched chicks. *Sci. Rep.* 8, 5919. doi:10.1038/s41598-018-24202-8

Shaughnessy, D. W., Hyson, R. L., Bertram, R., Wu, W., and Johnson, F. (2019). Female zebra finches do not sing yet share neural pathways necessary for singing in males. *J. Comp. Neurol.* 527, 843–855. doi:10.1002/cne.24569

Shvarts, S., Jimenez-Gomez, C., Bai, J. Y. H., Thomas, R. R., Oskam, J. J., and Podlesnik, C. A. (2020). Examining stimuli paired with alternative reinforcement to mitigate resurgence in children diagnosed with autism spectrum disorder and pigeons. *J. Exp. Anal. Behav.* 113, 214–231. doi:10.1002/jeab.575

Sperdin, H. F., and Schaer, M. (2016). Aberrant development of speech processing in young children with autism: New insights from neuroimaging biomarkers. *Front. Neurosci.* 10, 393. doi:10.3389/fnins.2016.00393

Spiteri, E., Konopka, G., Coppola, G., Bomar, J., Oldham, M., Ou, J., et al. (2007). Identification of the transcriptional targets of FOXP2, a gene linked to speech and language, in developing human brain. *Am. J. Hum. Genet.* 81, 1144–1157. doi:10.1086/522237

Tager-Flusberg, T., Paul, R., and Lord, C. E. (2005). "Language and communication in autism," in *Handbook of autism and pervasive developmental disorders, and behavior*. Editors R. P. F. Volkmar, A. Klin, and D. J. Cohen (New Jersey, United States: Wiley), 335–364.

Takeuchi, H., Yazaki, Y., Matsushima, T., and Aoki, K. (1996). Expression of Fos-like immunoreactivity in the brain of quail chick emitting the isolation-induced distress calls. *Neurosci. Lett.* 220 (3), 191–194. doi:10.1016/s0304-3940(96)13256-0

Ter Maat, A., Trost, L., Sagunsky, H., Seltmann, S., and Gahr, M. (2014). Zebra finch mates use their forebrain song system in unlearned call communication. *PLoS One* 9, e109334. doi:10.1371/journal.pone.0109334

Teramitsu, I., Kudo, L. C., London, S. E., Geschwind, D. H., and White, S. A. (2004). Parallel FoxP1 and FoxP2 expression in songbird and human brain predicts functional interaction. *J. Neurosci.* 24, 3152–3163. doi:10.1523/JNEUROSCI.5589-03.2004

Vulih-Shultzman, I., Pinhasov, A., Mandel, S., Grigoriadis, N., Touloumi, O., Pittel, Z., et al. (2007). Activity-dependent neuroprotective protein snippet NAP reduces tau hyperphosphorylation and enhances learning in a novel transgenic mouse model. *J. Pharmacol. Exp. Ther.* 323, 438–449. doi:10.1124/jpet.107.129551

Wang, R., Chen, C. C., Hara, E., Rivas, M. V., Roulhac, P. L., Howard, J. T., et al. (2015). Convergent differential regulation of SLIT-ROBO axon guidance genes in the brains of vocal learners. *J. Comp. Neurol.* 523, 892–906. doi:10.1002/cne.23719

Wang, X., Kohl, A., Yu, X., Zorio, D. A. R., Klar, A., Sela-Donenfeld, D., et al. (2020). Temporal-specific roles of fragile X mental retardation protein in the development of the hindbrain auditory circuit. *Development* 147, dev188797. doi:10.1242/dev.188797

Wang, Y., Sakano, H., Beebe, K., Brown, M. R., de Laat, R., Bothwell, M., et al. (2014). Intense and specialized dendritic localization of the fragile X mental retardation protein in binaural brainstem neurons: A comparative study in the alligator, chicken, gerbil, and human. *J. Comp. Neurol.* 522, 2107–2128. doi:10.1002/cne.23520

Whitsel, A. I., Johnson, C. B., and Forehand, C. J. (2002). An in ovo chicken model to study the systemic and localized teratogenic effects of valproic acid. *Teratology* 66, 153–163. doi:10.1002/tera.10093

Winograd, C., Clayton, D., and Ceman, S. (2008). Expression of fragile X mental retardation protein within the vocal control system of developing and adult male zebra finches. *Neuroscience* 157, 132–142. doi:10.1016/j.neuroscience.2008.09.005

Yartsev, M. M. (2017). The emperor's new wardrobe: Rebalancing diversity of animal models in neuroscience research. *Science* 358, 466–469. doi:10.1126/science.aan8865

Yazaki, Y., Matsushima, T., and Aoki, K. (1999). Testosterone modulates stimulation-induced calling behavior in Japanese quails. *J. Comp. Physiol. A* 184, 13–19. doi:10.1007/s003590050302

Zachar, G., Schrott, A., and Kabai, P. (2008). Context-dependent prey avoidance in chicks persists following complete telencephalectomy. *Brain Res. Bull.* 76 (3), 289–292. doi:10.1016/j.brainresbull.2008.02.017

Zachar, G., Tóth, A. S., Balogh, M., and Csillag, A. (2017). Effect of nucleus accumbens lesions on socially motivated behaviour of young domestic chicks. *Eur. J. Neurosci.* 45, 1606–1612. doi:10.1111/ejn.13402

Zachar, G., Tóth, A. S., Gerecsei, L. I., Zsebők, S., Ádám, Á., and Csillag, A. (2019). Valproate exposure in ovo attenuates the acquisition of social preferences of young post-hatch domestic chicks. *Front. Physiol.* 10, 881. doi:10.3389/fphys.2019.00881

Zosen, D., Austdal, L. P. E., Bjørnstad, S., Lumor, J. S., and Paulsen, R. E. (2022). Antiepileptic drugs lamotrigine and valproate differentially affect neuronal maturation in the developing chick embryo, yet with PAX6 as a potential common mediator. *Neurotoxicol. Teratol.* 90, 107057. doi:10.1016/j.ntt.2021.107057

Zsedényi, C. K., Zachar, G., Csillag, A., and Ádám, Á. (2014). Effect of synthetic cathinones: Mephedrone, butylone and 3, 4 methylene-dioxypyrovalerone (MDPV) on social separation induced distress vocalization, vigilance and postural control of young domestic chicks. *Neurosci. Lett.* 580, 88–93. doi:10.1016/j.neulet.2014.07.027

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EDITED BY
Sandra G. Velleman,
The Ohio State University, United States

REVIEWED BY
Kazuyoshi Ukena,
Hiroshima University, Japan
Miriam Friedman-Einat,
Agricultural Research Organization (ARO), Israel

*CORRESPONDENCE
Yajun Wang,
cdwyjhk@gmail.com
Juan Li,
lijuanscuhk@163.com

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Characterization of relaxin 3 and its receptors in chicken: Evidence for relaxin 3 acting as a novel pituitary hormone

Can Lv[1], Huilu Zheng[1], Biying Jiang[1], Qin Ren[1], Jiannan Zhang[1,2,3], Xin Zhang[3], Juan Li[1,2,3]* and Yajun Wang[1,2,3]*

[1]Key Laboratory of Bio-Resources and Eco-Environment of Ministry of Education, College of Life Sciences, Sichuan University, Chengdu, China, [2]Animal Disease Prevention and Food Safety Key Laboratory of Sichuan Province, College of Life Sciences, Sichuan University, Chengdu, China, [3]Joint Nutrition Center for Animal Feeding of Sichuan University-Shengliyuan Group, Chengdu, China

Mammalian relaxin (RLN) family peptides binding their receptors (RXFPs) play a variety of roles in many physiological processes, such as reproduction, stress, appetite regulation, and energy balance. In birds, although two relaxin family peptides (RLN3 and INSL5) and four receptors (RXFP1, RXFP2, RXFP2-like, and RXFP3) were predicated, their sequence features, signal properties, tissue distribution, and physiological functions remain largely unknown. In this study, using chickens as the experimental model, we cloned the cDNA of the *cRLN3* gene and two receptor (*cRXFP1* and *cRXFP3*) genes. Using cell-based luciferase reporter assays, we demonstrate that cRLN3 is able to activate both cRXFP1 and cRXFP3 for downstream signaling. cRXFP1, rather than cRXFP3, is a cognate receptor for cRLN3, which is different from the mammals. Tissue distribution analyses reveal that *cRLN3* is highly expressed in the pituitary with lower abundance in the hypothalamus and ovary of female chicken, together with the detection that cRLN3 co-localizes with pituitary hormone genes *LHB/FSHB/GRP/CART* and its expression is tightly regulated by hypothalamic factors (GnRH and CRH) and sex steroid hormone (E2). The present study supports that cRLN3 may function as a novel pituitary hormone involving female reproduction.

KEYWORDS

chickens, RLN3, RXFP1, RXFP3, pituitary

Introduction

Relaxin (RLN) was originally named in 1930 for its role in promoting the relaxation of the pubic ligament during pregnancy in mammals (e.g., guinea pigs and rabbits) (Fevold et al., 1930). Later studies report that RLN belongs to the family of peptide hormones that also include insulin and insulin-like growth factors (IGFs). The crystal structure of RLN is similar to that of insulin including the alpha helices of the A- and B-chain which are mutually supported by the two interchain cysteine bridges and one intrachain bridge in the A-chain (Haley et al., 1982; Hudson et al.,

1983; Patil et al., 2017). Increasing evidence shows that RLNs play important roles in many physiological functions, such as promoting pregnancy response, metabolism, stress, and energy balance (van der Westhuizen et al., 2008; Bathgate et al., 2013; Patil et al., 2017). In vertebrates, the relaxin family peptides vary significantly among species due to multiple (2 or 3) rounds of whole genome duplication and gene gain/loss. Till present, a total of seven structurally related peptides, relaxin 1 (RLN1), RLN2, RLN3, insulin-like peptide 3 (INSL3), INSL4, INSL5, and INSL6, have been identified in humans (Patil et al., 2017). Three RLNs (RLN1, RLN2, and RLN3) exist in higher primates (e.g., great apes), while only RLN1 and RLN3 exist in some mammals such as mice and pigs (Wilkinson et al., 2005; Patil et al., 2017). As the main form in the blood circulation and corpus luteum, RLN1 and RLN2 (in higher primates) are collectively referred to as RLN among these species (Bathgate et al., 2013).

As the typical member of the insulin superfamily, the RLNs bind their receptors to play their physiological roles. In mammals, RLN1 and RLN2 bind the relaxin family peptide 1 receptor (RXFP1) for downstream signaling (Bathgate et al., 2013). As their cognate receptor, RXFP1, which is also called the leucine-rich repeat-containing G-protein-coupled receptor 7 (LGR7), is capable of coupling to multiple G-proteins, thus triggering the pathways including cAMP accumulation (Halls et al., 2006; Halls et al., 2007; Halls et al., 2009), ERK1/2 phosphorylation, NO production, and other signaling pathways (Bathgate et al., 2013). In humans, RLN3 mainly binds the relaxin family peptide 3 receptor (RXFP3) for downstream signaling, which is coupled to the $G\alpha_{i/o}$ protein with cAMP level decrease upon ligand binding (Liu et al., 2003b; Liu et al., 2005; Bathgate et al., 2006; van der Westhuizen et al., 2007). In HEK293T cells, RLN3 was also able to activate RXFP1 inducing a dose-dependent increase in cAMP production (Sudo et al., 2003). Till present, INSL3 is reported to bind the relaxin family peptide 2 receptor (RXFP2) for downstream signaling including cAMP level increase and thymidine incorporation (Kumagai et al., 2002; Hsu et al., 2003). However, the cognate receptors for INSL4 and INSL6 remain to be identified (Bathgate et al., 2013).

In the 1980s, two RLN genes, *RLN1* and *RLN2*, were isolated from the human genome (Crawford et al., 1984). Later, the *RLN3* gene was further isolated (Bathgate et al., 2002). Sequence analyses reveal that the *RLN3* gene is the ancestral gene which continually duplicates, thus bringing the enrichment of RLN family peptides in mammals (Wilkinson et al., 2005). In humans, RLN3 is mainly localized within the brain and is involved in physiological roles including stress, motivated behavior, and appetite regulation (Smith et al., 2011; Ryan et al., 2013; McGowan et al., 2014; Smith et al., 2014; Calvez et al., 2016). As the ancestral gene copy, the *RLN3* gene also duplicates in many lower vertebrates including fish (Wilkinson et al., 2005). For example, in teleost fish, two peptides closely related to relaxin 3, RLN3a and RLN3b, have been identified (Yang et al., 2020).

In chickens, a relaxin-like peptide has been partially purified from the ovaries of actively laying hens by size-exclusion chromatography (Brackett et al., 1997). In addition, RLN3 is reported to be related to the transformation of brooding and laying eggs (Shen et al., 2016; Ye et al., 2019). In contrast to the detailed and extensive investigation of the RLN3 peptide in mammals, the sequence features, signaling properties, and expression profiles of *RLN3* within the avian species remain poorly understood. Therefore, using chicken as an experimental model, our present study aims to 1) identify the complete ORF sequences of *RLN3* and its putative receptors: *RXFP1* and *RXFP3*; 2) reveal the signaling properties of RLN3; and 3) explore the tissue distribution and regulation mechanism of *RLN3* expression. The results from our study are the first to establish a clear concept that avian RLN3 is a novel pituitary hormone which may be involved in female reproduction.

Materials and methods

Ethics statement

All the animal experiments were conducted in accordance with the Guidelines for Experimental Animals issued by the Ministry of Science and Technology of the People's Republic of China. The experimental protocol was approved by the Animal Ethics Committee of Sichuan University (Chengdu, China).

Chemicals, peptides, and primers

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), and restriction enzymes were purchased from Takara Biotechnology Co., Ltd., (Dalian, China). The recombinant human relaxin 3 (rhRLN3) was purchased from Roche R&D Center (China) Ltd. Chicken gonadotropin-releasing hormone (GnRH, GnRH1 was used in this study) and corticotropin-releasing hormone (CRH) were synthesized using solid-phase Fmoc chemistry (GL Biochem, Shanghai, China). 17β-estradiol (E2) and progesterone (P4) were purchased from Sigma-Aldrich. dihydrotestosterone (DHT) was purchased from Cayman Chemical Company (Ann Arbor, Michigan, United States). In this study, all chickens of the Lohmann Layer strain were purchased from a local commercial company (Chengdu, China). All primers used in this study were synthesized by Youkang Biological Technology Co., Ltd., (Hangzhou, China) and are listed in Table 1.

TABLE 1 Primers used in this study.

Gene name	Sense/antisense	Primer sequence (5′-3′)	Size (bp)
Primers for construction of the expression plasmids			
cRLN3	Sense	CCGGAATTCAACGGAACGGCGACGCGCCAT	621
	Antisense	CCGGAATTCCATGCACAAGTGCAGAGAGG	
cRXFP1	Sense	CCGGAATTCGCTGAAAGACAGATATGACATC	2,317
	Antisense	CCGGAATTCCCCAAACATATTTACGTGTACG	
cRXFP3	Sense	CCGGAATTCAGCATGGGATGGATGAGCTC	1,341
	Antisense	CCGGAATTCGCCATCTCAGTAGTGTTGCT	
Primers for quantitative real-time RT-PCR assay			
cRLN3	Sense	ATTCTTCTCAAGCAGCAAGT	169
	Antisense	TCTTTGAAGTCATCTGCCAT	
cRXFP1	Sense	GCTCCACGCCATCTCAATAA	160
	Antisense	CAGCGATCCCACCAATTGAC	
cRXFP3	Sense	GTTGGCAATCGTGGCTTCTC	135
	Antisense	GTTTGGTACAGACCCAGCCA	
cβ-actin	Sense	CCCAGACATCAGGGTGTGATG	123
	Antisense	GTTGGTGACAATACCGTGTTCAAT	

Total RNA extraction

In order to explore the expression of *cRLN3* and its receptors in various tissues of chickens, three adult male and three adult female chickens were purchased from local companies. All chickens were sacrificed and the tissues were isolated, including the various brain regions (telencephalon, midbrain, cerebellum, hindbrain, and hypothalamus), pituitary, heart, liver, spleen, lung, kidney, skin, muscle, adrenal gland, pancreas, duodenum, abdominal fat, spinal cord, testis, ovary, infundibulum, isthmus, uterus, and vagina. All tissue samples were stored at −80°C before use. The total RNA was extracted with RNAzol reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions and diluted in H_2O treated with diethylpyrocarbonate (DEPC). Briefly, after precipitating with DEPC-treated ultra-pure water, the RNAzol-lysed tissues were centrifuged (12,000 rpm, 10 min). BAN solution (4-bromoanisole) was added to purify the RNA and eliminate genomic DNA. Then, an equal volume of cold 100% isopropanol was added. The precipitated pellet was washed three times with 600 μl 75% ethanol and dissolved in 30 μl RNase-free water. The approximate concentration and purity of samples were determined using a OneDrop 1000 spectrophotometer.

Reverse transcription and quantitative real-time PCR

For reverse transcription, total RNA (2 μg) and 0.5 μg of oligo-deoxythymidine were mixed in a volume of 5 μl, incubated at 70°C for 10 min, and cooled at 4°C for 2 min. Then, the first strand buffer, 0.5 mM each deoxynucleotide triphosphate (dNTP), and 100 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Takara) were added to the reaction mix in a total volume of 10 μl. Reverse transcription (RT) was performed at 42°C for 90 min. After the reaction, the cDNA templates were diluted with MilliQ-H_2O and stored at −20°C.

According to our previously established method (Liu et al., 2019), quantitative real-time PCR was performed to examine the expression of *RLN3, RXFP1*, and *RXFP3* mRNA among chicken tissues. The qPCR primers of chicken *RLN3*, *RXFP1*, and *RXFP3* were designed based on sequences in the GenBank and are listed in Table 1. The primers (10 μM), dNTP (10 mM), easy Taq buffer, easy Taq DNA polymerase (TransGen Biotech), Eva Green (Biotium), MilliQ-H_2O, and templates were mixed in a total volume of 20 μl. Then the reaction mix was conducted on the CFX96 Real-Time PCR Detection System (Bio-Rad). The amplification conditions included an initial denaturation for 10 min at 94°C followed by 20 s of denaturation at 94°C, 15 s annealing at 60°C, and 30 s extension at 72°C for 40 cycles.

Cloning the cDNA of chicken *RLN3* and its receptors (*RXFP1* and *RXFP3*)

According to the GenBank database, a relaxin peptide (RLN3: NM_001113200.1) and its two putative receptors (RXFP1: XM_420385.5; RXFP3: XM_004937174.3) exist in chickens. Based on the predicted cDNAs of these genes, gene-specific primers were designed to amplify the CDS of *cRLN3*, *cRXFP1*, and *cRXFP3* with high-fidelity DNA Taq polymerase (TOYOBO, Japan). Then, these amplified PCR products were inserted into the

pcDNA3.1 (+) expression vector (for functional assays) and sequenced by Youkang Biological Technology Co., Ltd. Finally, the CDS region of each gene was determined by sequencing at least three independent clones.

Preparation of chicken RLN3 conditioned medium

To verify whether chicken RLN3 is functional, the conditioned medium of cells expressing cRLN3 ($cRLN3_{cm}$) was prepared. In brief, the expression plasmid of cRLN3 (1,000 ng) was transfected into HEK293 cells cultured in a 6-well plate. After 6 h transfection, the culture medium with 15% fetal bovine serum was replaced and continued for 18 h at 37°C with 5% CO_2. Then, the serum-rich medium was replaced with 600 μl of serum-free medium. After 24 h culture, the conditioned medium containing cRLN3 was collected and concentrated by centrifugation in an Amicon Ultra 3K (Millipore, United States) ultrafiltration tube at 4°C, 5,000 g for 30 min, with a final volume of approximately 1/4 of the original. The concentrated medium was used for subsequent functional tests. Using the same approach, the conditioned medium of cells transfected with empty pcDNA3.1 (+) expression plasmid ($pcDNA_{cm}$) was used as a negative control.

Functional characterization of chicken RLN3 and its receptors (RXFP1 and RXFP3)

According to our previously established methods (Liu et al., 2019; Jiang et al., 2022), two cell-based luciferase reporter systems (pGL3-CRE-luciferase and pGL3-NFAT-RE-luciferase) were used to verify whether $cRLN3_{cm}$ is biologically active and capable of activating cRXFP1 and cRXFP3. In brief, HEK293 cells transiently expressing each receptor (cRXFP1 and cRXFP3) were treated with 30 μl serum-free medium containing $cRLN3_{cm}$ or $pcDNA_{cm}$ (0.001–10 μl) for 6 h, and the receptor-activated cAMP/PKA pathways and calcium mobilization were then monitored by pGL3-CRE-luciferase and pGL3-NFAT-RE-luciferase reporter systems, respectively. HEK293 cells expressing empty pcDNA3.1 (+) were used as a negative control.

To compare the functional difference between chicken RLN3 and human RLN3, recombinant human RLN3 (rhRLN3, 10^{-12} to 10^{-7} M, 6 h) was used to treat HEK293 cells expressing cRXFP1 or cRXFP3, respectively, and its potency in activating cRXFP1 and cRXFP3 was also evaluated.

Preparation of the extracellular medium from chicken pituitary cells

To explore whether pituitary cells can secrete RLN3, we collected the extracellular medium of adult female chicken pituitary cells to detect its activation potential. According to the method provided in the previous article (Sun et al., 2021; Liu et al., 2022), the anterior pituitaries from a laying hen were isolated and further cut into small pieces with clean scissors. After washing with PBS, the cut pieces were then digested with 0.25% trypsin at 37°C for 30 min. The dispersed pituitary cells were cultured in Medium 199 supplemented with 10% fetal bovine serum in a Corning Cell BIND 48-well plate (Corning, Tewksbury, MA) at 37°C with 5% CO_2. After 18-h culture, the medium was replaced with a serum-free M199 medium. Four hours later, this extracellular medium from chicken pituitary cells was collected and loaded into Amicon Ultra 3K (Millipore, United States) ultrafiltration tubes for concentration by centrifugation at 4°C, 5,000 g, for 15 min, to a final volume of approximately 1/2 of the original. The HEK293 cells expressing cRXFP1 were treated with a 60 μl serum-free medium containing chicken pituitary cell extracellular medium (2×10^{-3}μl to 2×10^{1} μl) for 6 h, and then receptor-activated calcium mobilization was monitored by pGL3-NFAT-RE-luciferase reporter systems. HEK293 cells expressing empty pcDNA3.1 (+) were used as a negative control.

Effects of hypothalamic factors (gonadotropin-releasing hormone/corticotropin-releasing hormone) and sex hormone (17β-estradiol/progesterone/dihydrotestosterone) on *cRLN3* expression in cultured chicken pituitary cells

To explore the regulatory effects of GnRH/CRH/E2/P4 on cRLN3, the anterior pituitaries isolated from 4-week-old female chicks were sliced and digested by 0.25% trypsin at 37°C for 20 min. The dispersed pituitary cells were cultured at a density of 5×10^5 cells/well in Medium 199 supplemented with 15% fetal bovine serum. Then, the medium was replaced with a serum-free M199 medium and the cells were incubated with GnRH (1–100 nM), CRH (0.1–10 nM), E2 (100 nM), and P4 (100 nM) for different duration times (4 h/24 h). In the present study, the pituitary cells from 4-week-old male chicks were also treated with DHT (100 nM) for 4 h/24 h. The total RNA was then extracted from cultured pituitary cells and the expression of *RLN3* and β-actin mRNA was assayed by qPCR.

Data analysis

The luciferase activities of HEK293 cells expressing cRXFP1/cRXFP3/pcDNA3.1 (+) in the treated group were expressed as a

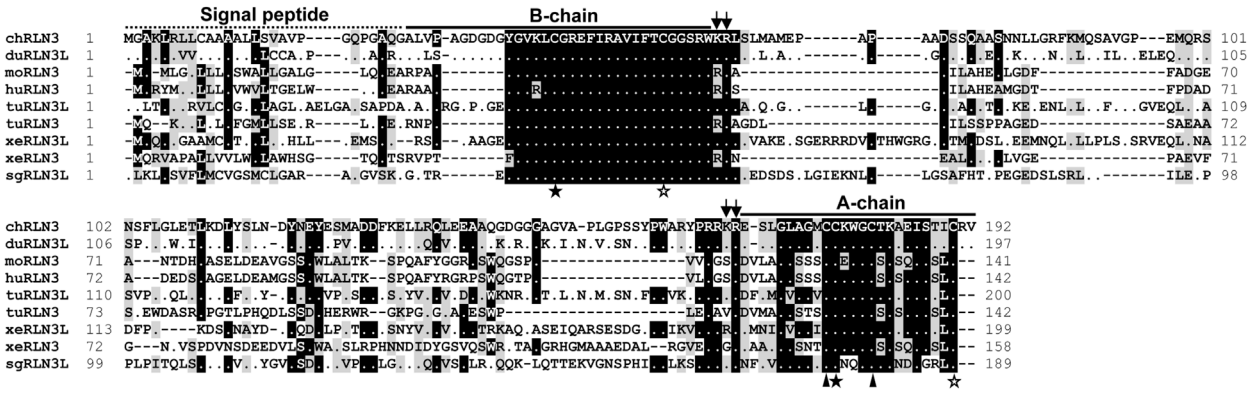

FIGURE 1
Amino acid alignment of chicken RLN3 (chRLN3) with that of ducks (duRLN3L, XP_032061192.1), mice (moRLN3, NP_775276.1), humans (huRLN3, NP_543140.1), Western painted turtles (tuRLN3L, XP_005296973.1; tuRLN3, XP_005310753.1), *Xenopus tropicalis* (xeRLN3L, NP_001072488.1; xeRLN3, NP_001116073.1), and spotted gars (sgRLN3L, XP_015192667.1). The conserved dibasic residues (KR) for proteolytic processing are marked by two arrows. The cysteine residues of two pairs of disulfide bonds for A-chain and B-chain formation are represented by solid stars and hollow stars, respectively. The two cysteine residues used to form the disulfide bond in the A-chain are represented by solid triangles. Dashes denote gaps in the alignment.

relative fold increase compared to the control group (without peptide treatment). The relative mRNA levels of target genes were first calculated as the ratios to that of β-actin and then expressed as fold change (or the percentage) compared to their respective controls (chosen tissues). The data were analyzed by Student's t-test (between two groups), or by one-way ANOVA followed by the Dunnett test in GraphPad Prism 7 (GraphPad Software, San Diego, CA). To validate our results, all experiments were repeated twice or thrice.

Results

Cloning of *cRLN3*, *cRXFP1* and *cRXFP3*

In this study, the complete ORFs of chicken (c) *RLN3*, *RXFP1*, and *RXFP3* were cloned for the first time from the chicken brain. The cloned *cRLN3* cDNA is 579 bp in length, which encodes a precursor of 192 amino acids (a.a.), with a signal peptide of 22 a.a. located at the N-terminus, followed by the B-chain, C-chain, and A-chain at the C-terminus (Figure 1). Sequence analyses demonstrated that the chicken RLN3 precursor showed a very low sequence identity (28% ~41%) with that of humans, mice, *Xenopus tropicalis*, and spotted gars. However, as shown in Figure 1, the a.a. sequence identity of the mature peptides among the species is very high. For example, the A- and B-chain of chicken RLN3 share 54% and 67% a.a. sequence identity with those of human RLN3, respectively (Table 2). Like human relaxin family peptides, the cRLN3 precursor showed the conserved two pairs of dibasic residues (K61R62 and K166R167) that were essential for proteolytic processing (Bathgate et al., 2002). In addition, similar to the insulin superfamily members, the six cysteine residues in B- and A-chains critical for the formation of the disulfide bonds (Cys43–Cys177; Cys176–Cys181; and Cys55–Cys190) were detected to be conserved among the species. Moreover, a conserved RxxxRxxI/V motif was detected in the B-chain of cRLN3.

In the present study, the coding region of *cRXFP1* is 2,292 bp in length encoding the receptor of 763 a.a.. Sequence analyses revealed that chicken RXFP1 shared high sequence identity with that of ducks (94%), mice (73%), humans (77%), turtles (82%), *X. tropicalis* (65%), and spotted gars (56%) (Figure 2A). As a typical G-protein-coupled receptor, cRXFP1 also showed the seven transmembrane domains (TM) and a highly conserved NSxLNP(L/I)Y motif for G-protein coupling. Like human RXFP1, chicken RXFP1 showed a very large N-terminus, including a unique low-density lipoprotein receptor type A (LDLa) module and the next 10 leucine-rich repeat (LRR) regions connected by a hinge region (Bathgate et al., 2013).

In the present study, the complete ORF of *cRXFP3* is 1,347 bp in length encoding the receptor of 448 a.a.. Sequence analyses revealed that cRXFP3 also showed high a.a. sequence identity with RXFP3 of ducks (92%), mice (59%), humans (58%), turtles (60%), *X. tropicalis* (63%), and spotted gars (66%) (Figure 2B). In the present study, as shown in Figure 2B, the conserved seven transmembrane domains (TM) and a highly conserved NSxLNP(L/I)Y motif for G-protein coupling were also detected to be conserved in cRXFP3. Unlike cRXFP1, chicken RXFP3 showed a relatively short NH_2-terminal domain.

TABLE 2 Amino acid sequence identity of chicken (c) RLN3 A-chain and B-chain with that of human (h) RLN1/2/3.

	hRLN1 A/B (%)	hRLN2 A/B (%)	hRLN3 A/B (%)
cRLN3 A-chain	41.18	38.24	66.70
cRLN3 B-chain	34.62	34.62	53.85

Synteny analyses of *cRLN3*, *cRXFP1* and *cRXFP3*

To examine whether chicken *RLN3*, *RXFP1,* and *RXFP3* are orthologous to the genes identified in other vertebrates, synteny analyses were performed by searching their conserved neighboring genes in the genomes of human and other vertebrates, including ducks (*Anas platyrhynchos*), mice (*Mus musculus*), turtles (*Pelodiscus sinensis or Chrysemys picta*), frogs (*X. tropicalis*), and spotted gars (*Lepisosteus oculatus*) (Figure 3). Interestingly, chicken/duck *RLN3* is orthologous to *RLN3* identified in turtles, frogs, and spotted gars, but not orthologous to *RLN3* identified in humans and mice (Figure 3A). In Figures 3B,C, chicken *RXFP1* and *RXFP3* are orthologous to the genes identified in ducks, humans, mice, turtles, and frogs and spotted gars, respectively.

Functional characterization of cRLN3, cRXFP1 and cRXFP3

To verify whether chicken RLN3 can activate RXFP1 or RXFP3, a cRLN3-conditioned medium ($cRLN3_{cm}$, 0.001–10 μl) was used to treat HEK293 cells expressing cRXFP1/cRXFP3. Receptor activation was examined by pGL3-CRE-luciferase and pGL3-NFAT-RE-luciferase reporter systems established in our previous studies (Liu et al., 2019).

As shown in Figure 4A, using a pGL3-CRE-luciferase reporter system, we demonstrated that $cRLN3_{cm}$ could stimulate luciferase activity of HEK293 cells expressing cRXFP1 in a dose-dependent manner, while it could inhibit forskolin (2 μM, an adenlyate cyclase activator)-induced luciferase activity of HEK293 cells expressing cRXFP3 dose-dependently. The present finding indicated that cRXFP1 was coupled to the Gs-cAMP signaling pathway, while cRXFP3 was coupled to the Gi-cAMP signaling pathway. Moreover, we found that $cRLN3_{cm}$ was at least 10-fold more potent in activating cRXFP1 than cRXFP3.

As shown in Figure 4B, using a pGL3-NFAT-RE-luciferase reporter system, $cRLN3_{cm}$ could also stimulate the luciferase activity of HEK293 cells expressing cRXFP1 (not cRXFP3) dose-dependently, indicating that cRXFP1 activation may trigger calcium mobilization.

Considering the close structural and evolutionary relationship between chicken RLN3 and human RLN3, we further tested whether recombinant human RLN3 (rhRLN3) could activate cRXFP1 and cRXFP3. As shown in Figure 5A, using a pGL3-CRE-luciferase reporter system, we demonstrated that rhRLN3 could potently stimulate the luciferase activity of HEK293 expressing cRXFP1 with an EC_{50} value of 2.68 nM. In addition, rhRLN3 also could inhibit forskolin-stimulated luciferase activities of HEK293 cells expressing cRXFP3 with an EC_{50} value of 41.55 nM (Table 3). Using a pGL3-NFAT-RE-luciferase reporter system, we demonstrated that rhRLN3 could stimulate luciferase activities of HEK293 cells expressing cRXFP1 (not cRXFP3) with an EC_{50} value of 21.15 nM (Figure 5B; Table 3). These findings supported that cRLN3 was functionally similar to human RLN3.

Expression of *cRLN3*, *cRXFP1* and *cRXFP3* in chicken tissues

Using qPCR, we examined the mRNA expression of *cRLN3* in adult chicken tissues. As shown in Figure 6A, chicken *RLN3* was abundantly expressed in the pituitary and had a lower abundance in the tissue hypothalamus and ovary. The expression abundance of *cRLN3* mRNA in chicken pituitary can reach a high level with a TPM value (>2000), similar to the expression of the pituitary hormone genes *LHB/FHB/GRP* (gastrin-releasing peptide)/*CART* (cocaine- and amphetamine-regulated transcript) (data not shown). Moreover, *cRLN3* shows a dimorphic expression pattern with high abundance in female chickens. As shown in Figure 6B, the expression abundance of *cRLN3* in the female pituitary was about 60 times higher than that in the male pituitary. In the female hypothalamus, its *cRLN3* expression abundance was about 10 times higher than that in male chickens.

In the present study, the high abundance of *cRLN3* in the chicken pituitary led us to further investigate its distribution in pituitary cell clusters from a single-cell dataset reported by our research group (Zhang et al., 2021). As shown in Figure 6D, *cRLN3* has been found mainly to localize in the gonadotroph cell clusters of female chickens. In addition to the gonadotroph cell clusters, *cRLN3* also was found to localize within the lactotroph cell clusters and the folliculo-stellate cells.

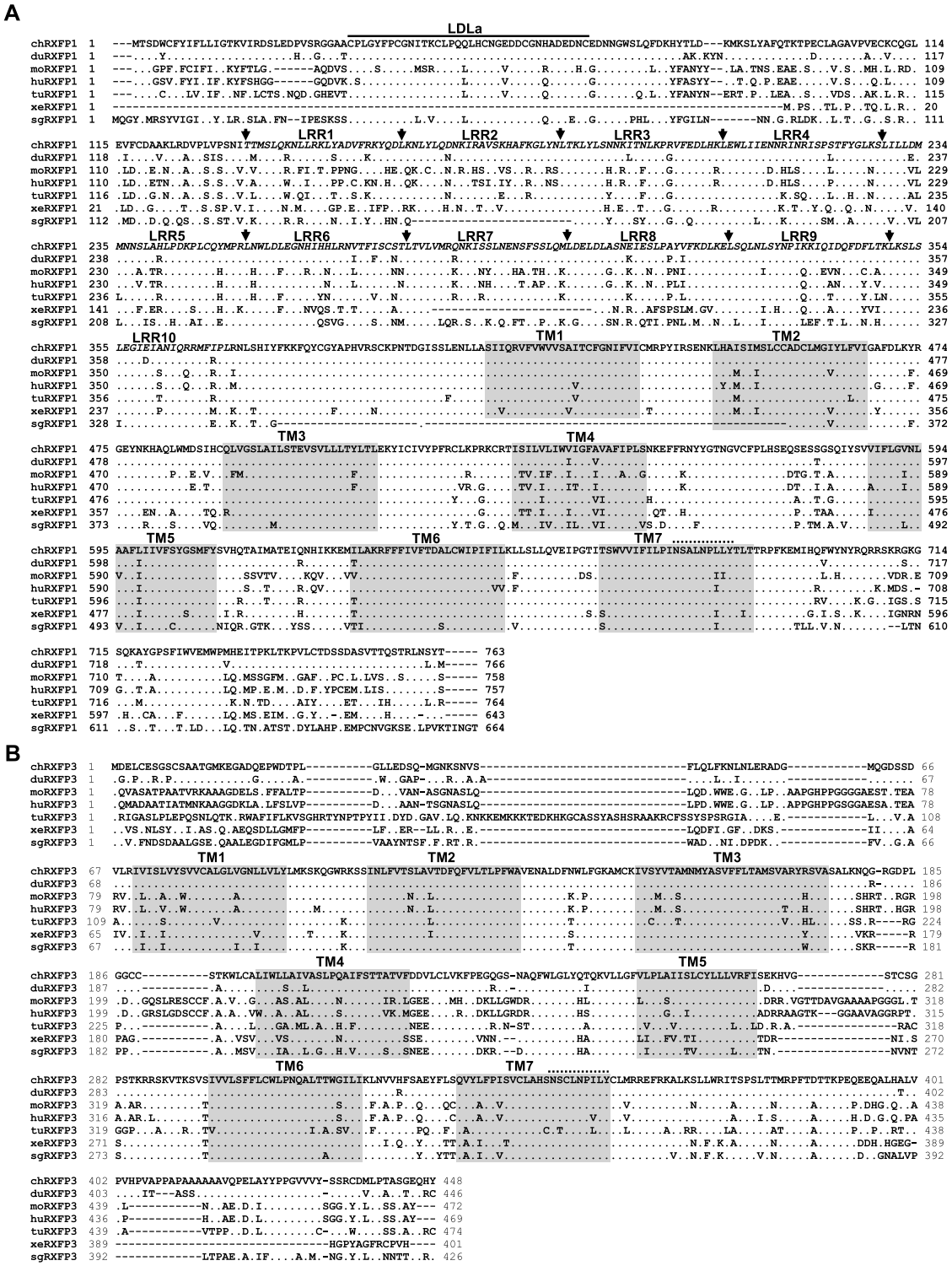

FIGURE 2
Amino acid sequence alignment of chicken RXFP1/3 with that from other species. **(A)** Amino acid alignment of chicken RXFP1 (chRXFP1) with that of ducks (duRXFP1, XP_012952098.1), mice (moRXFP1, NP_997617.1), humans (huRXFP1, NP_067647.2), Chinese soft-shell turtles (tuRXFP1, XP_006121928.1), *X. tropicalis* (xeRXFP1, XP_017951372.1), and spotted gars (sgRXFP1, XP_015200025.1). **(B)** Amino acid alignment of chicken RXFP3 (chRXFP3) with that of ducks (duRXFP3, XP_027302601.1), mice (moRXFP3, NP_848832.1), humans (huRXFP3, NP_057652.1), Chinese soft-shell turtles (tuRXFP1, XP_006116663.1), *X. tropicalis* (xeRXFP3, XP_002941625.1), and spotted gars (sgRXFP3, XP_006627308.1). The seven transmembrane domains (TM1-7) are shaded; the highly conserved NSxLNP(L/I)Y motif present in the TM7 is represented by a dashed overline; dots indicate the amino acid; dashes denote gaps in the alignment. For cRXFP1, the unique low-density lipoprotein receptor type A (LDLa) module is represented by a solid overline; the next 10 leucine-rich repeat (LRR) regions are shown in italics and separated by arrows.

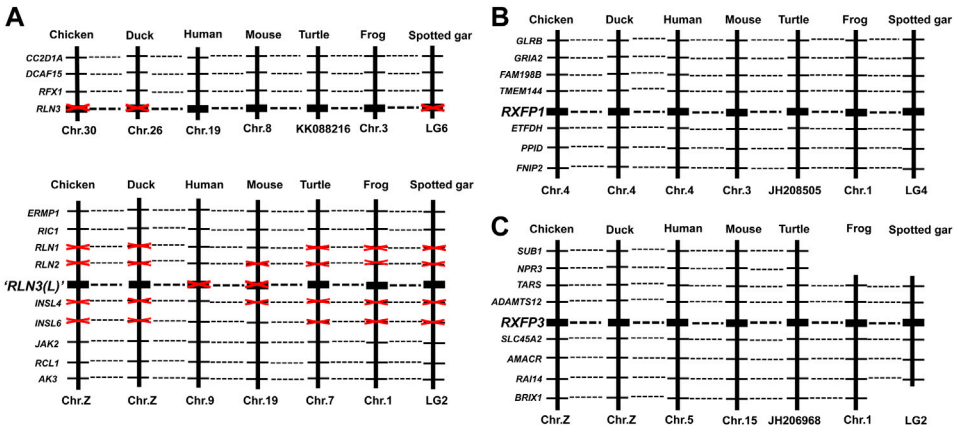

FIGURE 3
Synteny analyses of *RLN3* **(A)**, *RXFP1* **(B),** and *RXFP3* **(C)** among chicken (*Gallus gallus*), duck (*Anas platyrhynchos*), human (*Homo sapiens*), mouse (*Mus musculus*), turtle [RLN3: painted turtle (*Chrysemys picta*); RXFP1/3: Chinese soft-shell turtle (*Pelodiscus sinensis*)], frog (*Xenopus tropicalis*), and spotted gar (*Lepisosteus oculatus*).

In the present study, the expression of *cRXFP1* and *cRXFP3* genes was also investigated in chicken tissues based on qPCR. *cRXFP1* and *cRXFP3* were detected to be widely but differentially expressed among chicken tissues. As shown in Figure 6E, *cRXFP1* was highly expressed in the infundibulum, uterus, hypothalamus, and the varied functional brain areas (except the cerebellum). In contrast, *cRXFP3* was detected to be predominantly expressed in the hypothalamus and kidney with moderate/weak expression among tissues including the spinal cord, hindbrain, and ovary (Figure 6F).

The signaling of the secreted cRLN3 from chicken pituitary cells

In the present study, in order to explore whether the chicken pituitary can synthesize and secret the biologically active cRLN3 protein, the extracellular medium of adult female chicken pituitary cells was collected to explore its activation potential for cRXFP1. Using the pGL3-NFAT-RE-luciferase reporter system, we found that cRXFP1 could be activated by the culture medium of pituitary cells with luciferase activity increase in HEK293 cells (Figure 6C).

The regulation of *cRLN3* mRNA expression in cultured pituitary cells

In the present study, the high abundance of *cRLN3* mRNA in the pituitary gland of female chickens led us to further investigate its expression regulation profile. Since *cRLN3* was detected to be localized mainly in the gonadotroph cells, where the GnRHRs and CRHRs were found to be abundantly expressed (Shimizu and Bédécarrats, 2006; Joseph et al., 2009; Zhang et al., 2021), the hypothalamic factors GnRH and CART were employed to investigate their roles in the regulation of *cRLN3* mRNA expression by qPCR. As shown in Figure 7A, the GnRH (10 nM) was able to slightly increase the expression of *cRLN3* mRNA after 4 h of treatment. However, the GnRH (10 nM) was not able to further regulate the expression of *cRLN3* mRNA after 24 h of treatment. In contrast, the CRH was able to significantly increase the expression of *cRLN3* mRNA in a time-dependent manner. In the present study, the different concentrations of the GnRH (1–100 nM, 4 h) and CRH (0.1–10 nM, 24 h) were further employed to examine their effect on the *cRLN3* mRNA expression. As shown in Figures 7B,C, both GnRH and CRH treatments could increase the expression level of *cRLN3* mRNA in a dose-dependent manner, while the stimulation effect of CRH was higher than that of GnRH.

In the present study, the regulatory effects of sex hormones E2 (100 nM), P4 (100 nM), and DHT (100 nM) on the expression of *cRLN3* mRNA in the cultured pituitary cells were also investigated. As shown in Figure 7D, E2 treatment for 4 h could increase the expression level of *cRLN3* mRNA in the cultured pituitary cells. However, the longer E2 treatment (24 h) could not regulate the mRNA expression of *cRLN3*. In the present study, both P4 and DHT treatments could not affect the expression of *cRLN3* mRNA in the cultured pituitary cells.

Discussion

In the present study, the genes encoding the chicken relaxin 3 peptide (cRLN3) and its two receptors cRXFP1 and cRXFP3 have been cloned for the first time. Functional studies reveal that cRLN3 can activate cRXFP1 and cRXFP3 for downstream signaling. Between the two receptors, cRXFP1 showed a higher

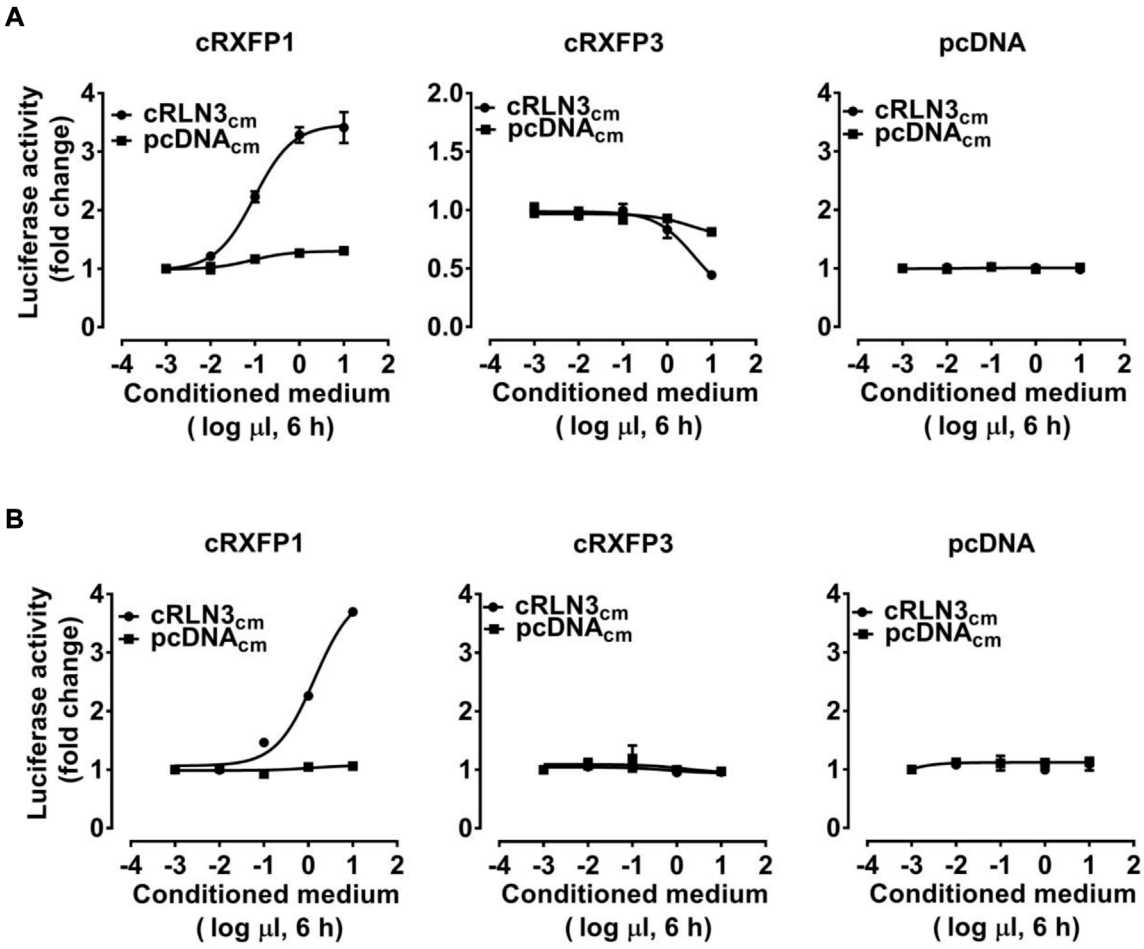

FIGURE 4
Effects of the chicken RLN3-conditioned medium (1×10^{-3}μl to 1×10^{1} μl $cRLN3_{cm}$ in 30 μl medium, 6 h) on the activation of cRXFP1, cRXFP3 expressed in HEK293 cells, monitored by a pGL3-CRE-luciferase reporter system **(A)** or a pGL3-NFAT-RE-luciferase reporter system **(B)**. HEK293 cells transfected with an empty pcDNA3.1 (+) vector were used as a control and $cRLN3_{cm}$ treatment did not change the luciferase activity, indicating the specific action on receptor activation. Each data point represents the mean ± SEM of three replicates ($N = 3$).

binding potency with cRLN3 than cRXFP3, supporting their differential roles among the tissues. In the cultured pituitary cells, the expression of *cRLN3* mRNA is tightly regulated by the hypothalamic factors GnRH and CRH and sex steroid hormone E2. In combination with the detection that the high abundance of *RLN3* mRNA in the pituitary and its co-localization with pituitary hormones genes *LHB/FSHB/GRP/CART* in gonadotroph cells in a dimorphic pattern, the present study supports that RLN3 is a novel pituitary hormone involving female reproduction in avian species.

Chicken *RLN3* gene is the duplicated copy of the ancestral *RLN3* gene

In this study, the chicken *RLN3* gene has been cloned for the first time. Sequence analyses reveal that the precursor protein deduced from cRLN3 shares a low sequence identity with that of other species. However, its A-chain and B-chain from mature proteins still show a higher sequence identity than that of humans, supporting their functional conservation across species (Patil et al., 2017). In addition, the motif RxxxRxxI/V sequences were detected to be conserved in the B-chain, also supporting its function for receptor binding (Bathgate et al., 2013). In the present study, synteny analyses reveal that chicken/duck *RLN3* is orthologous to *RLN3* identified in turtles, frogs, and spotted gars, but not orthologous to *RLN3* identified in humans and mice (Figure 3A). The present study supports that the isolated relaxin 3 gene is likely to be the duplicated copy (*RLN3-L*) from the ancestral relaxin 3 gene, which emerged prior to the divergence of fish (Wilkinson et al., 2005; Yegorov and Good, 2012; Yegorov et al., 2014). Thus, among the lower vertebrates, the gene duplication of *RLN3-L* brings

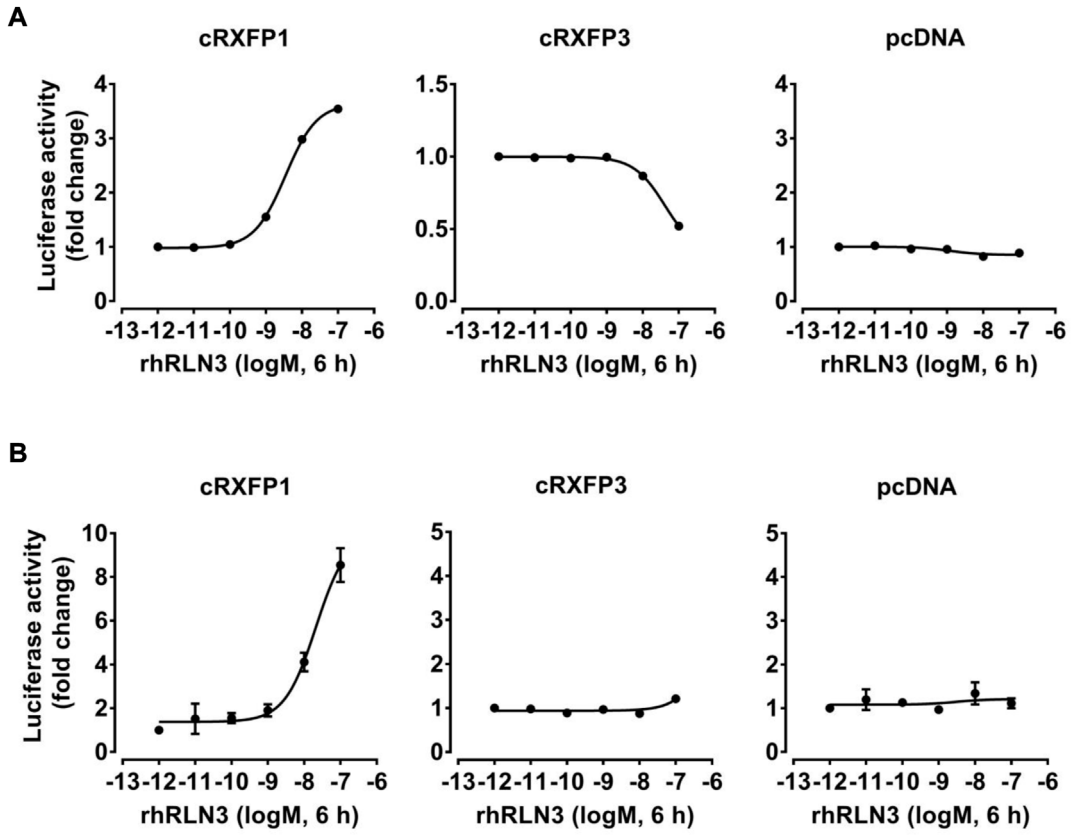

FIGURE 5
Effects of rhRLN3 (10^{-12}–10^{-7} M, 6 h) on the activation of cRXFP1, cRXFP3 expressed in HEK293 cells, monitored by a pGL3-CRE-luciferase reporter system **(A)** or a pGL3-NFAT-RE-luciferase reporter system **(B)**. HEK293 cells transfected with an empty pcDNA3.1 (+) vector were used as a control and rhRLN3 treatment did not change the luciferase activity, indicating the specific action on receptor activation. Each data point represents the mean ± SEM of three replicates (N = 3).

TABLE 3 EC_{50} values of rhRLN3 in activating different signaling pathways in HEK293 cells expressing chicken (c) RXFP1 and RXFP3.

Signaling pathway	cAMP/PKA (nM)	Ca^{2+} (nM)
cRXFP1	2.68	21.15
cRXFP3	41.55	—

RLN3a and *RLN3b* reported in teleost fish (Wilkinson et al., 2005; Yegorov et al., 2014). In chicken, the gene duplication of *RLN3-L* brings the *cRLN3* gene reported in the present study and the predicted *cINSL5* gene (Wilkinson et al., 2005). In contrast, the ancestral *RLN3* gene, which disappeared in chicken, further duplicates in mammals, thus bringing the gene cluster where *RLN1*, *RLN2*, and *RLN3* are located among the mammalian species (Good et al., 2012; Bathgate et al., 2018). In vertebrates, genome duplication and gene gain/loss result in the abundance of relaxin family peptides. The isolation and synteny analyses of *cRLN3* in the present study provide a unique clue for the relaxin family members' evolution across species.

In the present study, two RLN3 receptors, *RXFP1* and *RXFP3* also have been cloned in chickens. As shown in Figure 2A, chicken RXFP1 shows a high a.a. sequence identity with that of other species. The cloned cRXFP1 shows a large N-terminal, including an LDLa module that is essential for downstream signaling (Halls et al., 2005; Hopkins et al., 2006; Scott et al., 2006) and an LRR region for ligand binding (Scott et al., 2006). In addition, the cloned cRXFP1 also shows the typical seven transmembrane domains with the conserved site for G-protein coupling (Bathgate et al., 2013). Synteny analyses show that the cloned *cRXFP1* is orthologous to *RXFP1* from other vertebrates including humans (Figure 3B), thus suggesting their conserved downstream signaling.

Similar to cRXFP1 to be conserved among the species, the cloned chicken RXFP3 also shares a high sequence identity with that of other species. Like mammalian RXFP3, chicken RXFP3 also contains a short N-terminus, which is crucial for

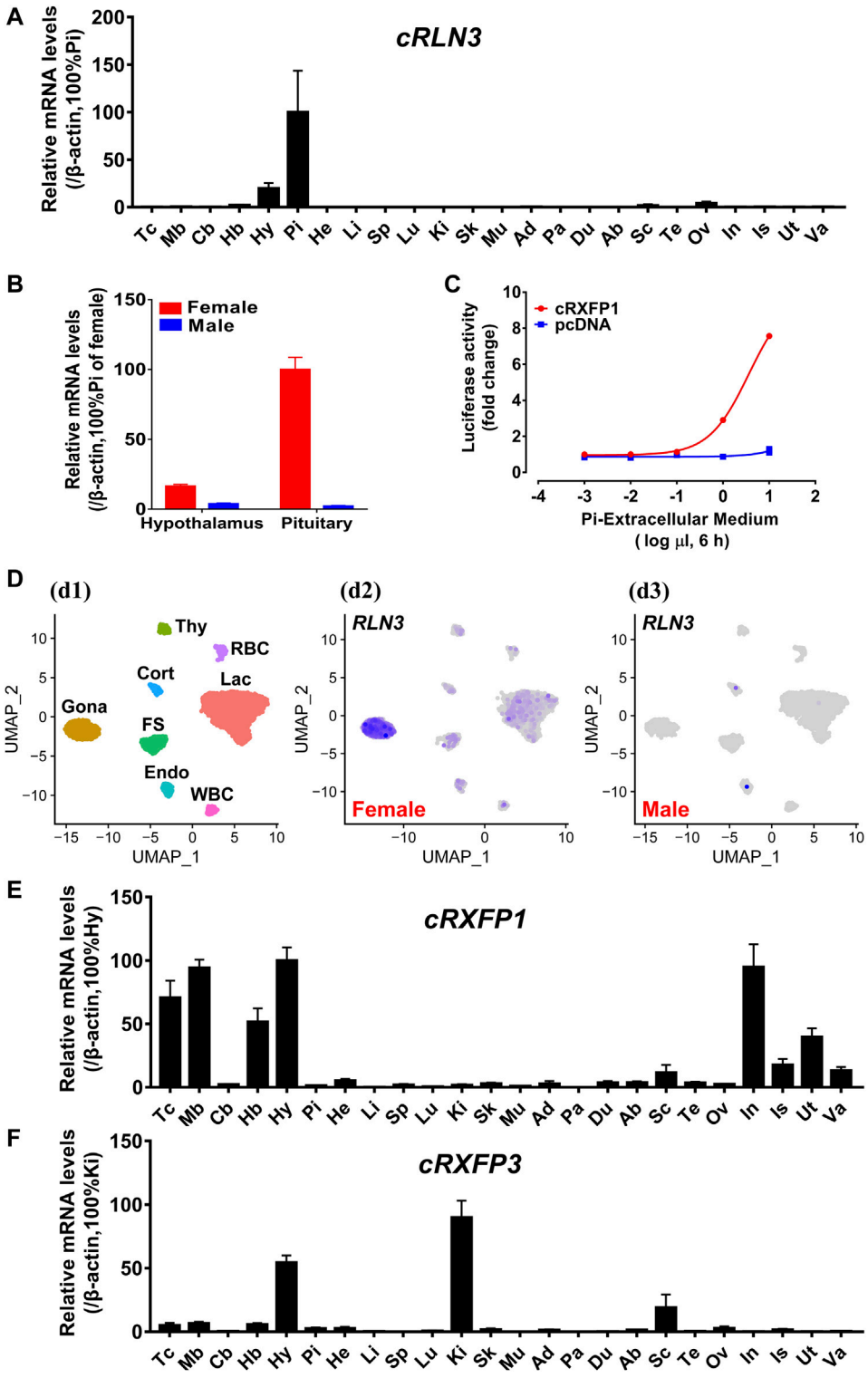

FIGURE 6
(A) Quantitative real-time RT-PCR (qPCR) analysis showing the abundant expression of *cRLN3* in various adult chicken tissues. Each data point represents the mean ± SEM of six individual adult chickens (three males and three females) ($N = 6$). Tc, Telencephalon; Mb, Midbrain; Cb, Cerebellum; Hb, Hindbrain; Hy, Hypothalamus; Pi, Pituitary; He, Heart; Li, Liver; Sp, Spleen; Lu, Lung; Ki, Kidney; Sk, Skin; Mu, Muscle; Ad, Adrenal gland; Pa, Pancreas; Du, Duodenum; Ab, Abdominal fat; Sc, Spinal cord; Te: Testis; Ov, Ovary; In, Infundibulum; Is, Isthmus; Ut, Uterus; Va, Vagina. **(B)** Quantitative real-time RT-PCR analysis showing the sex-differential expression of *cRLN3* in the pituitary and hypothalamus of chickens. Each data
(*Continued*)

FIGURE 6 (Continued)
point represents the mean ± SEM of three individual adult chickens (three males or three females) (N = 3). **(C)** Effects of the chicken pituitary cell (Pi) extracellular medium (1×10^{-3}µl to 1×10^{1} µl extracellular medium in 30 µl medium, 6 h) on the activation of cRXFP1 expressed in HEK293 cells, monitored by a pGL3-NFAT-RE-luciferase reporter system. HEK293 cells transfected with an empty pcDNA3.1 (+) vector were used as a control and Pi extracellular medium treatment did not change the luciferase activity, indicating the specific action on receptor activation. Each data point represents the mean ± SEM of three replicates (N = 3). **(D)** Chicken pituitary single-cell transcriptome data (at https://scrna.avianscu.com/pit/) showing that *cRLN3* is highly expressed in female chicken pituitary gonadotrophs. (d1) Uniform Manifold Approximation and Projection (UMAP) map showing the identified eight pituitary cell clusters. Cells are colored by Seurat clustering and annotated by cell clusters (each point represents a single cell). Gona, gonadotrophs; Lac, lactotrophs; Cort, corticotrophs; Thy, thyrotrophs; FS, folliculo-stellate cells; Endo, endothelial cells; WBC, white blood cells; RBC, red blood cells. (d2) UMAP map showing the expression of *cRLN3* in distinct cell clusters of the female chicken pituitary. The overall expression level of *cRLN3* was high in all pituitary cell clusters of female chickens, and the highest expression level was found in gonadotrophs. (d3) UMAP map showing the expression of *cRLN3* in distinct cell clusters of the male chicken pituitary. In the male chicken pituitary, *cRLN3* was only very poorly expressed in the corticotrophs, lactotrophs, and endothelial cells and was not expressed in other pituitary cell clusters. **(E,F)** Quantitative real-time RT-PCR analysis showing the abundant expression of *cRXFP1* **(E)** and *cRXFP3* **(F)** in various adult chicken tissues. Each data point represents the mean ± SEM of six individual adult chickens (three males and three females) (N = 6).

ligand binding and signal transduction (Bathgate et al., 2013). In the present study, the seven transmembrane domains and the conserved site for G-protein coupling also have been detected in chicken RXFP3, supporting it to be the typical G-protein-coupled receptor. Synteny analysis shows that the cloned *cRXFP3* is orthologous to that of other vertebrates including humans, supporting their conserved evolution profile.

Chicken RLN3 binds cRXFP1 and cRXFP3 for downstream signaling

In the present study, the chicken RLN3-conditioned medium ($cRLN3_{cm}$) could activate cRXFP1 expressed in HEK293 cells, thus stimulating the Gs-cAMP/PKA signaling pathway. Our finding is consistent with the reports in mammals that RXFP1 can be activated by RLN3 (Sudo et al., 2003; Halls et al., 2006). However, only a dose-dependent increase in luciferase activity in HEK293 cells expressing chicken cRXFP1 is detected in the present study which is different from the report in mammalian species that RXFP1 is capable of coupling to Gs and other G-proteins (Gi/o), thus showing a biphasic pattern of cAMP accumulation (Halls et al., 2006; Halls et al., 2007).

In the present study, the chicken RLN3-conditioned medium ($cRLN3_{cm}$) can also activate cRXFP3 through the inhibition of forskolin-stimulated luciferase activity, indicating that cRXFP3 is functionally coupled to the Gi protein (Figure 4A). The present finding is consistent with the findings in mammals (Kocan et al., 2014). In humans, the activation of RXFP3 inhibits forskolin-stimulated cAMP accumulation in a variety of cell lines (Liu et al., 2003b; Liu et al., 2005; van der Westhuizen et al., 2010). The present study supports conserved downstream signaling of RXFP3 between mammalian and avian species.

Although cRLN3 is able to activate both cRXFP1 and cRXFP3, the potency of cRLN3 for cRXFP1 activation is at least 10-fold higher than that for cRXFP3. Similarly, rhRLN3 shows a higher potency for cRXFP1 activation (EC_{50}: 2.68 nM) than for cRXFP3 (EC_{50}: 41.55 nM). The present study supports that chicken RLN3 is a functional analog of rhRLN3. However, our finding contrasts with that of mammalian species. In mammals, as an endogenous ligand for RXFP3, RLN3 shows a higher potency for RXFP3 activation than for RXFP1 (Liu et al., 2005). The difference may result from ligand–receptor interaction model change during evolution (Bathgate et al., 2003; Bathgate et al., 2018).

In addition to the Gs-cAMP signaling pathway, we also found that cRXFP1 activation can increase intracellular Ca^{2+} levels (Figure 4B). This finding differs from that in mammals that RXFP1 activation cannot increase Ca^{2+} levels (Bani et al., 2002). Like mammalian RXFP3, cRXFP3 cannot activate the Ca^{2+} signaling pathway in the present study, thus supporting its conserved signaling properties between birds and mammals (Liu et al., 2003a; Liu et al., 2003b).

Chicken RLN3 functions as a novel pituitary hormone involving female reproduction

In the present study, *cRLN3* mRNA expression has been detected to be expressed abundantly in the chicken pituitary (Figure 6). Its abundance in our single-cell transcriptome dataset from the pituitary is very high, reaching a level similar to that of the pituitary genes *LHB/FSHB/GRP/CART*. As the key pituitary hormone in reproduction, LH and FSH are involved in follicular development and ovulation in chickens (Palmer and Bahr, 1992; Oguike et al., 2005). GRP, as a novel "gonadotrophic factor" in chickens (Mo et al., 2017), is reported to be involved in the regulation of reproductive and gastrointestinal activities. CART, expressed abundantly in pituitary cells and regulated by hypothalamic factors CRH and GnRH, is reported to be involved in the regulation of stress and reproductive

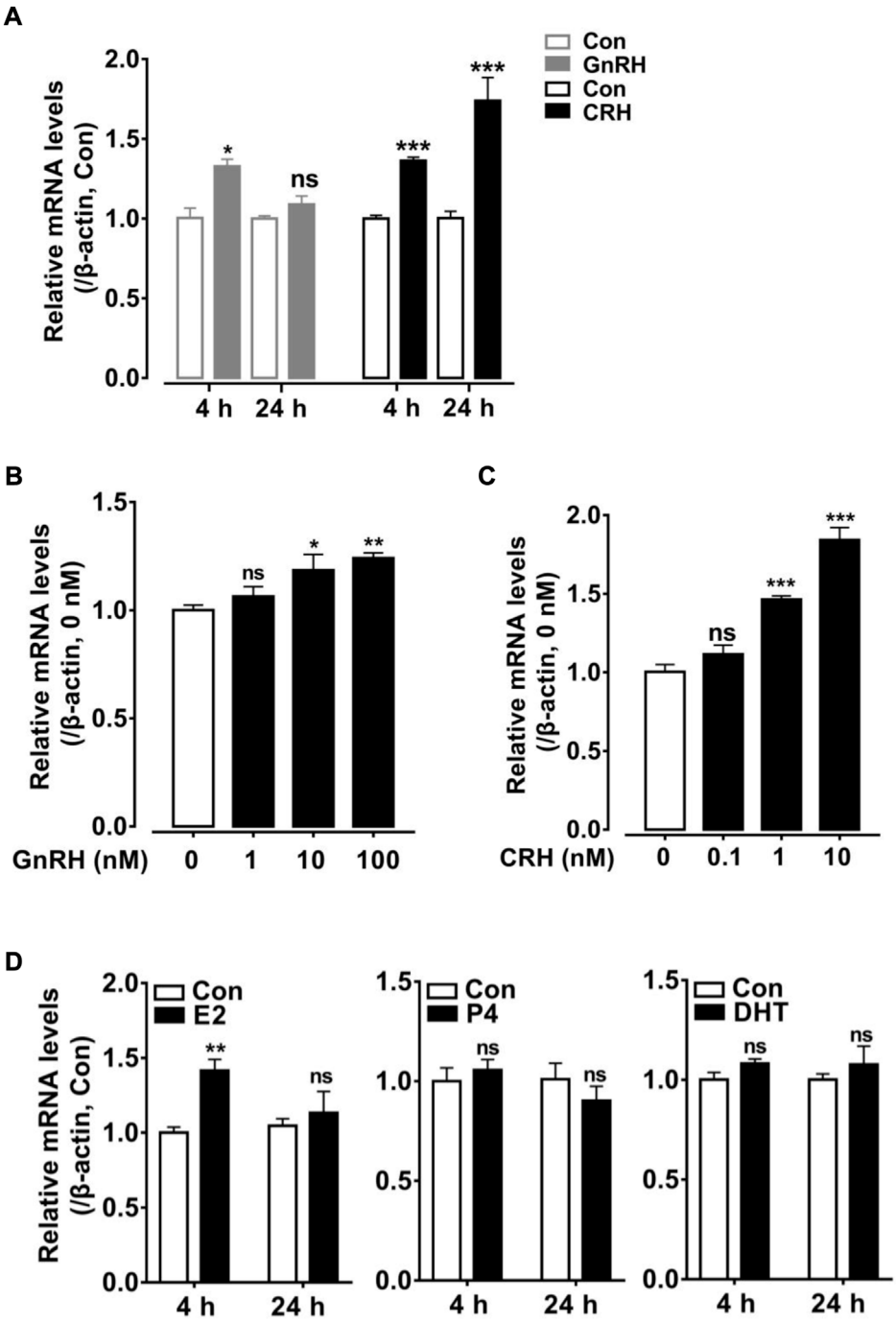

FIGURE 7
Effects of hypothalamic factors (GnRH or CRH) and sex steroid (E2/P4/DHT) treatment on *cRLN3* expression in cultured chicken pituitary cells detected by qPCR. **(A)** Effects of GnRH (10 nM) or CRH (10 nM) treatment for 4 h, 24 h on *cRLN3* expression in cultured pituitary cells. **(B)** Effects of GnRH (1, 10, and 100 nM) treatment for 4 h on *cRLN3* expression in cultured pituitary cells. **(C)** Effects of CRH (0.1, 1, and 10 nM) treatment for 24 h on *cRLN3* expression in cultured pituitary cells. Each data point represents the mean ± SEM of four replicates ($N = 4$). **(D)** Effects of E2 (100 nM), P4 (100 nM) or DHT (100 nM) treatment for 4 h, 24 h on *cRLN3* expression in cultured pituitary cells. *, $p < 0.05$ vs. control, **, $p < 0.005$ vs. control, and ***, $p < 0.001$ vs. control.

behavior in domestic chickens (Mo et al., 2015; Mo et al., 2019). Together with the cell signaling experiments which prove that cRLN3 can be secreted from the chicken pituitary cells (Figure 6C), the present study supports that cRLN3 plays its function as a pituitary hormone. Following the detection that its receptors (*cRXFP1* and *cRXFP3*) are differentially expressed in a wide range of chicken tissues, cRLN3 may, on the one hand, be functional in the brain as a neuropeptide (Smith et al., 2011) in mammals (Bathgate et al., 2002; Burazin et al., 2002; Liu et al., 2003b; Smith et al., 2010) and other species including fish (Donizetti et al., 2008). On the other hand, as a pituitary hormone, cRLN3 may also play a role in the endocrine system, targeting wide tissues and, thus, be involved in a wide range of physiological processes.

In the present study, pituitary *cRLN3* mRNA expression was shown to be regulated by the hypothalamic factors GnRH and CRH which further supports cRLN3 to be functional as a pituitary hormone (Figure 7). The present study reveals that CRH potently stimulates the expression of cRLN3 in a dose- and time-dependent manner (Figure 7C). In contrast, GnRH could only weakly stimulate the mRNA expression of *cRLN3* (Figure 7B). Since GnRH is pulsed secreted and negatively regulated by CRH (Ciechanowska et al., 2010), the expression of *RLN3* mRNA may be regulated by CRH. As the key player in the hypothalamus–pituitary–adrenal (HPA) axis of vertebrates, CRH controls multiple physiological processes associated with the HPA axis in birds and mammals (Papadimitriou and Priftis, 2009; Cornett et al., 2013). The RLN3 expression/secretion induced by CRH supports its conserved physiological functions in vertebrates. For example, RLN3 can stimulate rat hypothalamic explants to release GnRH and CRH, and its intraventricular (icv) injection can also increase plasma LH, ACTH, and corticosterone levels (McGowan et al., 2008; McGowan et al., 2014). In chickens, in the transcriptional analyses of newly hatched chicks during fasting and delayed feeding, *RLN3* has been identified in a gene network supporting its important role in nutrition (Higgins et al., 2010).

In the present study, the *cRLN3* mRNA expression was shown to be sexually dimorphic with a much higher abundance in the female pituitary and hypothalamus, strongly suggesting its involvement in female reproduction. Supporting evidence also comes from the high expression of its cognate receptor (*cRXFP1*) in chicken tissues. Similar to rats, *RXFP1* is highly expressed in the uterus involving uterine contractions (Hsu et al., 2002; Vodstrcil et al., 2010). In addition, *cRXFP1* is also detected to be expressed highly in a wide range of female tissues (infundibulum, isthmus, uterus, and vagina), thus supporting its role in reproduction. Supporting evidence also comes from steroid hormone feedback regulation. In the present, E2 has been found to increase the expression of cRLN3 (Figure 7D). As a sex steroid hormone, E2 is mainly secreted by the growing follicles and the ovary, which play an important role in female reproduction, including acting as the growth factor for reproductive organs' growth (Cooke et al., 1998; Cooke et al., 2021), triggering hypothalamic events *via* a positive feedback system (Micevych et al., 2008; Micevych and Sinchak, 2011), and maintaining follicle growth and maturation (Britt et al., 2004; Drummond, 2006; Padmanabhan et al., 2018). The findings that cRLN3 is involved in female reproductive biology are in line with the report that female teleost fish that RLN3 promotes the production of E2 during follicular development (Wilson et al., 2009; Yang et al., 2020). In another transcriptome analysis between the two divergent chicken breeds, which are in the laying phase and the brooding phase, the expression of *RLN3* mRNA shows downregulation in brooding chickens, thus pointing out its role actively involved in reproductive biology (Shen et al., 2016). The report that RLN3 is actively involved in female reproductive biology also comes from the study by Ghanem and Johnson (2021) in a shotgun proteomics study using isobaric tags to characterize the proteins in chicken ovarian follicles immediately before and after cyclic recruitment. RLN3 is revealed to be distinctively expressed in a total of 1,535 proteins. Based on quantitative PCR, the expression of *RLN3* and its receptors were further revealed to be expressed abundantly in granulosa cells and the theca cells, thus implicating the important role of RLN3 in chicken female reproductive biology.

Till present, a total of five genes (*LHB*, *FSHB*, *GRP*, *CART*, and *RLN3*) have been detected to be abundantly expressed in chicken pituitary gonadotrope cells (Mo et al., 2017; Zhang et al., 2021). Although these genes concentrate on the same cell cluster, these genes vary in sexual distribution patterns, cell co-localization profiles, and the hypothalamus regulation networks. For example, *RLN3* and *GRP* show an abundance in female gonadotrope cells while *LHB*, *FSHB*, and *CART* show a higher expression in male pituitary cells. In addition, all cells expressing *RLN3* overlap with the cells expressing *LHB* and *GRP*. In contrast, the cells expressing *RLN3* only partially co-localize with the cells expressing *FSHB* and *CART* (Zhang et al., 2021). At the hypothalamus regulation level, RLN3 is mainly regulated by CRH while CART is regulated by the GnRH and CRH with equal potency (Cai et al., 2015; Mo et al., 2015; Mo et al., 2019). The present study supports that these genes play differential roles in chicken pituitary function. With their especially complicated regulation networks reported in chickens, the present study enriches our understanding of avian pituitary biology, thus shedding light on their function across species.

In summary, we identified a relaxin peptide (cRLN3) and its two receptors (cRXFP1 and cRXFP3) in chickens. Functional studies have shown that cRLN3 can activate cRXFP1 and cRXFP3, and cRXFP1 is a cognate receptor for cRLN3. Tissue distribution analyses show that *cRLN3* is highly expressed in the female chicken pituitary. Moreover, *cRLN3* in pituitary cells is regulated by hypothalamic factors GnRH and CRH and sex steroid hormone E2. These results suggest that cRLN3 is a novel hormone from the

pituitary, which is associated with female reproduction in birds. Undoubtedly, these findings will help us to better understand the structure, ligand–receptor binding, and physiological functions of the relaxin system in birds and other vertebrates.

Data availability statement

Publicly available datasets were analyzed in this study. The scRNA-seq data used in this study have been deposited in the Genome Sequence Archive in the National Genomics Data Center, China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences, under accession number CRA003604 and are publicly accessible at https://ngdc.cncb.ac.cn/gsa/. A supplementary online web server (https://scrna.avianscu.com/pit/) was developed to facilitate the use of this dataset.

Ethics statement

The animal study was reviewed and approved by the Animal Ethics Committee of Sichuan University (Chengdu, China).

Author contributions

Conceptualization: CL, JL, and YW; formal analysis: HZ and BJ; funding acquisition: JZ, JL, and YW; investigation: CL and HZ; methodology: CL, BJ, and XZ; supervision: YW; and writing—review and editing: JZ, YW, and JL.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Bani, D., Baronti, R., Vannacci, A., Bigazzi, M., Sacchi, T. B., Mannaioni, P. F., et al. (2002). Inhibitory effects of relaxin on human basophils activated by stimulation of the Fcε receptor. The role of nitric oxide. *Int. Immunopharmacol.* 2 (8), 1195–1204. doi:10.1016/S1567-5769(02)00079-6

Bathgate, R. A. D., Kocan, M., Scott, D. J., Hossain, M. A., Good, S. V., Yegorov, S., et al. (2018). The relaxin receptor as a therapeutic target - perspectives from evolution and drug targeting. *Pharmacol. Ther.* 187, 114–132. doi:10.1016/j.pharmthera.2018.02.008

Bathgate, R. A. D., Samuel, C. S., Burazin, T. C. D., Gundlach, A. L., and Tregear, G. W. (2003). Relaxin: new peptides, receptors and novel actions. *Trends Endocrinol. Metab.* 14 (5), 207–213. doi:10.1016/s1043-2760(03)00081-x

Bathgate, R. A., Halls, M. L., van der Westhuizen, E. T., Callander, G. E., Kocan, M., and Summers, R. J. (2013). Relaxin family peptides and their receptors. *Physiol. Rev.* 93 (1), 405–480. doi:10.1152/physrev.00001.2012

Bathgate, R. A., Ivell, R., Sanborn, B. M., Sherwood, O. D., and Summers, R. J. (2006). International union of pharmacology LVII: recommendations for the nomenclature of receptors for relaxin family peptides. *Pharmacol. Rev.* 58 (1), 7–31. doi:10.1124/pr.58.1.9

Bathgate, R. A., Samuel, C. S., Burazin, T. C., Layfield, S., Claasz, A. A., Reytomas, I. G., et al. (2002). Human relaxin gene 3 (H3) and the equivalent mouse relaxin (M3) gene. Novel members of the relaxin peptide family. *J. Biol. Chem.* 277 (2), 1148–1157. doi:10.1074/jbc.M107882200

Brackett, K. H., Fields, P. A., Dubois, W., Chang, S. M., Mather, F. B., and Fields, M. J. (1997). Relaxin: an ovarian hormone in an avian species (*Gallus domesticus*). *Gen. Comp. Endocrinol.* 105 (2), 155–163. doi:10.1006/gcen.1996.6819

Britt, K. L., Saunders, P. K., McPherson, S. J., Misso, M. L., Simpson, E. R., and Findlay, J. K. (2004). Estrogen actions on follicle formation and early follicle development. *Biol. Reprod.* 71 (5), 1712–1723. doi:10.1095/biolreprod.104.028175

Burazin, T. C., Bathgate, R. A., Macris, M., Layfield, S., Gundlach, A. L., and Tregear, G. W. (2002). Restricted, but abundant, expression of the novel rat gene-3 (R3) relaxin in the dorsal tegmental region of brain. *J. Neurochem.* 82 (6), 1553–1557. doi:10.1046/j.1471-4159.2002.01114.x

Cai, G., Mo, C., Huang, L., Li, J., and Wang, Y. (2015). Characterization of the two CART genes (CART1 and CART2) in chickens (Gallus gallus). *PLoS One* 10 (5), e0127107. doi:10.1371/journal.pone.0127107

Calvez, J., de Avila, C., Matte, L. O., Guevremont, G., Gundlach, A. L., and Timofeeva, E. (2016). Role of relaxin-3/RXFP3 system in stress-induced binge-like eating in female rats. *Neuropharmacology* 102, 207–215. doi:10.1016/j.neuropharm.2015.11.014

Ciechanowska, M., Łapot, M., Mateusiak, K., and Przekop, F. (2010). Neuroendocrine regulation of GnRH release and expression of GnRH and GnRH receptor genes in the hypothalamus-pituitary unit in different physiological states. *Reprod. Biol.* 10 (2), 85–124. doi:10.1016/s1642-431x(12)60054-0

Cooke, P. S., Buchanan, D. L., Lubahn, D. B., and Cunha, G. R. (1998). Mechanism of estrogen action: lessons from the estrogen receptor-α knockout mouse. *Biol. Reprod.* 59 (3), 470–475. doi:10.1095/biolreprod59.3.470

Cooke, P. S., Mesa, A. M., Sirohi, V. K., and Levin, E. R. (2021). Role of nuclear and membrane estrogen signaling pathways in the male and female reproductive tract. *Differentiation.* 118, 24–33. doi:10.1016/j.diff.2020.11.002

Cornett, L. E., Kang, S. W., and Kuenzel, W. J. (2013). A possible mechanism contributing to the synergistic action of vasotocin (VT) and corticotropin-releasing

hormone (CRH) receptors on corticosterone release in birds. *Gen. Comp. Endocrinol.* 188, 46–53. doi:10.1016/j.ygcen.2013.02.032

Crawford, R., Hudson, P., Shine, J., Niall, H., Eddy, R., and Shows, T. (1984). Two human relaxin genes are on chromosome 9. *EMBO J* 3 (10), 2341–2345. doi:10.1002/j.1460-2075.1984.tb02136.x

Donizetti, A., Grossi, M., Pariante, P., D'Aniello, E., Izzo, G., Minucci, S., et al. (2008). Two neuron clusters in the stem of postembryonic zebrafish brain specifically express relaxin-3 gene: first evidence of nucleus incertus in fish. *Dev. Dyn.* 237 (12), 3864–3869. doi:10.1002/dvdy.21786

Drummond, A. E. (2006). The role of steroids in follicular growth. *Reprod. Biol. Endocrinol.* 4 (1), 16–11. doi:10.1186/1477-7827-4-16

Fevold, H. L., Hisaw, F. L., and Meyer, R. K. (1930). The relaxative hormone of the corpus luteum. Its purification and Concentration1. *J. Am. Chem. Soc.* 52 (8), 3340–3348. doi:10.1021/ja01371a051

Ghanem, K., and Johnson, A. L. (2021). Proteome profiling of chicken ovarian follicles immediately before and after cyclic recruitment. *Mol. Reprod. Dev.* 88 (8), 571–583. doi:10.1002/mrd.23522

Good, S., Yegorov, S., Martijn, J., Franck, J., and Bogerd, J. (2012). New insights into ligand-receptor pairing and coevolution of relaxin family peptides and their receptors in teleosts. *Int. J. Evol. Biol.* 2012, 310278. doi:10.1155/2012/310278

Haley, J., Hudson, P., Scanlon, D., John, M., Cronk, M., Shine, J., et al. (1982). Porcine relaxin: Molecular cloning and cDNA structure. *DNA* 1 (2), 155–162. doi:10.1089/dna.1.1982.1.155

Halls, M. L., Bathgate, R. A., and Summers, R. J. (2007). Comparison of signaling pathways activated by the relaxin family peptide receptors, RXFP1 and RXFP2, using reporter genes. *J. Pharmacol. Exp. Ther.* 320 (1), 281–290. doi:10.1124/jpet.106.113225

Halls, M. L., Bathgate, R. A., and Summers, R. J. (2006). Relaxin family peptide receptors RXFP1 and RXFP2 modulate cAMP signaling by distinct mechanisms. *Mol. Pharmacol.* 70 (1), 214–226. doi:10.1124/mol.105.021691

Halls, M. L., Bond, C. P., Sudo, S., Jin, K., Ferraro, T., Layfield, S., et al. (2005). Multiple binding sites revealed by interaction of relaxin family peptides with native and chimeric relaxin family peptide receptors 1 and 2 (LGR7 and LGR8). *J. Pharmacol. Exp. Ther.* 313 (2), 677–687. doi:10.1124/jpet.104.080655

Halls, M. L., Hewitson, T. D., Moore, X. L., Du, X. J., Bathgate, R. A., and Summers, R. J. (2009). Relaxin activates multiple cAMP signaling pathway profiles in different target cells. *Ann. N. Y. Acad. Sci.* 1160, 108–111. doi:10.1111/j.1749-6632.2008.03814.x

Higgins, S. E., Ellestad, L. E., Trakooljul, N., McCarthy, F., Saliba, J., Cogburn, L. A., et al. (2010). Transcriptional and pathway analysis in the hypothalamus of newly hatched chicks during fasting and delayed feeding. *BMC Genomics* 11, 162. doi:10.1186/1471-2164-11-162

Hopkins, E. J., Layfield, S., Ferraro, T., Bathgate, R. A. D., and Gooley, P. R. (2006). The NMR solution structure of the relaxin (RXFP1) receptor lipoprotein receptor class A module and identification of key residues in the N-terminal region of the module that mediate receptor activation. *J. Biol. Chem.* 282 (6), 4172–4184. doi:10.1074/jbc.M609526200

Hsu, S. Y., Nakabayashi, K., Nishi, S., Kumagai, J., Kudo, M., Sherwood, O. D., et al. (2002). Activation of orphan receptors by the hormone relaxin. *Science* 295 (5555), 671–674. doi:10.1126/science.1065654

Hsu, S. Y. T., Nakabayashi, K., Nishi, S., Kumagai, J., Kudo, M., Bathgate, R. A. D., et al. (2003). Relaxin signaling in reproductive tissues. *Mol. Cell. Endocrinol.* 202 (1-2), 165–170. doi:10.1016/s0303-7207(03)00078-9

Hudson, P., Haley, J., John, M., Cronk, M., Crawford, R., Haralambidis, J., et al. (1983). Structure of a genomic clone encoding biologically active human relaxin. *Nature* 301 (5901), 628–631. doi:10.1038/301628a0

Jiang, B., Cao, B., Zhou, Z., Li, Z., Lv, C., Zhang, J., et al. (2022). Characterization of chicken α2a-adrenoceptor: Molecular cloning, functional analysis, and its involvement in ovarian follicular development. *Genes* 13 (7), 1113. doi:10.3390/genes13071113

Joseph, N. T., Morgan, K., Sellar, R., McBride, D., Millar, R. P., and Dunn, I. C. (2009). The chicken type III GnRH receptor homologue is predominantly expressed in the pituitary, and exhibits similar ligand selectivity to the type I receptor. *J. Endocrinol.* 202 (1), 179–190. doi:10.1677/JOE-08-0544

Kocan, M., Sarwar, M., Hossain, M. A., Wade, J. D., and Summers, R. J. (2014). Signalling profiles of H3 relaxin, H2 relaxin and R3(BΔ23-27)R/I5 acting at the relaxin family peptide receptor 3 (RXFP3). *Br. J. Pharmacol.* 171 (11), 2827–2841. doi:10.1111/bph.12623

Kumagai, J., Hsu, S. Y., Matsumi, H., Roh, J. S., Fu, P., Wade, J. D., et al. (2002). INSL3/Leydig insulin-like peptide activates the LGR8 receptor important in testis descent. *J. Biol. Chem.* 277 (35), 31283–31286. doi:10.1074/jbc.C200398200

Liu, C., Chen, J., Kuei, C., Sutton, S., Nepomuceno, D., Bonaventure, P., et al. (2005). Relaxin-3/insulin-like peptide 5 chimeric peptide, a selective ligand for G protein-coupled receptor (GPCR)135 and GPCR142 over leucine-rich repeat-containing G protein-coupled receptor 7. *Mol. Pharmacol.* 67 (1), 231–240. doi:10.1124/mol.104.006700

Liu, C., Chen, J., Sutton, S., Roland, B., Kuei, C., Farmer, N., et al. (2003a). Identification of relaxin-3/INSL7 as a ligand for GPCR142. *J. Biol. Chem.* 278 (50), 50765–50770. doi:10.1074/jbc.M308996200

Liu, C., Eriste, E., Sutton, S., Chen, J., Roland, B., Kuei, C., et al. (2003b). Identification of relaxin-3/INSL7 as an endogenous ligand for the orphan G-protein-coupled receptor GPCR135. *J. Biol. Chem.* 278 (50), 50754–50764. doi:10.1074/jbc.M308995200

Liu, H., Luo, Q., Zhang, J., Mo, C., Wang, Y., and Li, J. (2019). Endothelins (EDN1, EDN2, EDN3) and their receptors (EDNRA, EDNRB, EDNRB2) in chickens: Functional analysis and tissue distribution. *Gen. Comp. Endocrinol.* 283, 113231. doi:10.1016/j.ygcen.2019.113231

Liu, M., Bu, G., Wan, Y., Zhang, J., Mo, C., Li, J., et al. (2022). Evidence for neuropeptide W acting as a physiological corticotropin-releasing inhibitory factor in male chickens. *Endocrinology* 163 (7), bqac073. doi:10.1210/endocr/bqac073

McGowan, B. M., Minnion, J. S., Murphy, K. G., Roy, D., Stanley, S. A., Dhillo, W. S., et al. (2014). Relaxin-3 stimulates the neuro-endocrine stress axis via corticotrophin-releasing hormone. *J. Endocrinol.* 221 (2), 337–346. doi:10.1530/JOE-13-0603

McGowan, B. M., Stanley, S. A., Donovan, J., Thompson, E. L., Patterson, M., Semjonous, N. M., et al. (2008). Relaxin-3 stimulates the hypothalamic-pituitary-gonadal axis. *Am. J. Physiol. Endocrinol. Metab.* 295 (2), E278–E286. doi:10.1152/ajpendo.00028.2008

Micevych, P., and Sinchak, K. (2011). The neurosteroid progesterone underlies estrogen positive feedback of the LH surge. *Front. Endocrinol.* 2, 90. doi:10.3389/fendo.2011.00090

Micevych, P., Soma, K. K., and Sinchak, K. (2008). Neuroprogesterone: Key to estrogen positive feedback? *Brain Res. Rev.* 57 (2), 470–480. doi:10.1016/j.brainresrev.2007.06.009

Mo, C., Cai, G., Huang, L., Deng, Q., Lin, D., Cui, L., et al. (2015). Corticotropin-releasing hormone (CRH) stimulates cocaine- and amphetamine-regulated transcript gene (CART1) expression through CRH type 1 receptor (CRHR1) in chicken anterior pituitary. *Mol. Cell. Endocrinol.* 417, 166–177. doi:10.1016/j.mce.2015.09.007

Mo, C., Huang, L., Cui, L., Lv, C., Lin, D., Song, L., et al. (2017). Characterization of NMB, GRP and their receptors (BRS3, NMBR and GRPR) in chickens. *J. Mol. Endocrinol.* 59 (1), 61–79. doi:10.1530/JME-17-0020

Mo, C., Lv, C., Huang, L., Li, Z., Zhang, J., Li, J., et al. (2019). Regulation of pituitary cocaine- and amphetamine-regulated transcript expression and secretion by hypothalamic gonadotropin-releasing hormone in chickens. *Front. Physiol.* 10, 882. doi:10.3389/fphys.2019.00882

Oguike, M. A., Igboeli, G., Ibe, S. N., and Ironkwe, M. O. (2005). Physiological and endocrinological mechanisms associated with ovulatory cycle and induced-moulting in the domestic chicken—a review. *Worlds Poult. Sci. J.* 61 (4), 625–632. doi:10.1079/wps200574

Padmanabhan, V., Puttabyatappa, M., and Cardoso, R. C. (2018). "Hypothalamus–pituitary–ovary Axis," in *Encyclopedia of reproduction*, Netherlands: Elsevier. 121–129. doi:10.1016/B978-0-12-801238-3.64632-9

Palmer, S. S., and Bahr, J. M. J. B. P. (1992). Follicle stimulating hormone increases serum oestradiol-17 beta concentrations, number of growing follicles and yolk deposition in aging hens (*Gallus gallus* domesticus) with decreased egg production. *Br. Poult. Sci.* 33 (2), 403–414. doi:10.1080/00071669208417478

Papadimitriou, A., and Priftis, K. N. (2009). Regulation of the hypothalamic-pituitary-adrenal axis. *Neuroimmunomodulation* 16 (5), 265–271. doi:10.1159/000216184

Patil, N. A., Rosengren, K. J., Separovic, F., Wade, J. D., Bathgate, R. A. D., and Hossain, M. A. (2017). Relaxin family peptides: structure-activity relationship studies. *Br. J. Pharmacol.* 174 (10), 950–961. doi:10.1111/bph.13684

Ryan, P. J., Kastman, H. E., Krstew, E. V., Rosengren, K. J., Hossain, M. A., Churilov, L., et al. (2013). Relaxin-3/RXFP3 system regulates alcohol-seeking. *Proc. Natl. Acad. Sci. U. S. A.* 110 (51), 20789–20794. doi:10.1073/pnas.1317807110

Scott, D. J., Layfield, S., Yan, Y., Sudo, S., Hsueh, A. J., Tregear, G. W., et al. (2006). Characterization of novel splice variants of LGR7 and LGR8 reveals that receptor signaling is mediated by their unique low density lipoprotein class A modules. *J. Biol. Chem.* 281 (46), 34942–34954. doi:10.1074/jbc.M602728200

Shen, X., Bai, X., Xu, J., Zhou, M., Xu, H., Nie, Q., et al. (2016). Transcriptome sequencing reveals genetic mechanisms underlying the transition between the laying and brooding phases and gene expression changes associated with

divergent reproductive phenotypes in chickens. *Mol. Biol. Rep.* 43 (9), 977–989. doi:10.1007/s11033-016-4033-8

Shimizu, M., and Bédécarrats, G. Y. (2006). Identification of a novel pituitary-specific chicken gonadotropin-releasing hormone receptor and its splice variants. *Biol. Reprod.* 75 (5), 800–808. doi:10.1095/biolreprod.105.050252

Smith, C. M., Chua, B. E., Zhang, C., Walker, A. W., Haidar, M., Hawkes, D., et al. (2014). Central injection of relaxin-3 receptor (RXFP3) antagonist peptides reduces motivated food seeking and consumption in C57BL/6J mice. *Behav. Brain Res.* 268, 117–126. doi:10.1016/j.bbr.2014.03.037

Smith, C. M., Ryan, P. J., Hosken, I. T., Ma, S., and Gundlach, A. L. (2011). Relaxin-3 systems in the brain-the first 10 years. *J. Chem. Neuroanat.* 42 (4), 262–275. doi:10.1016/j.jchemneu.2011.05.013

Smith, C. M., Shen, P. J., Banerjee, A., Bonaventure, P., Ma, S., Bathgate, R. A., et al. (2010). Distribution of relaxin-3 and RXFP3 within arousal, stress, affective, and cognitive circuits of mouse brain. *J. Comp. Neurol.* 518 (19), 4016–4045. doi:10.1002/cne.22442

Sudo, S., Kumagai, J., Nishi, S., Layfield, S., Ferraro, T., Bathgate, R. A., et al. (2003). H3 relaxin is a specific ligand for LGR7 and activates the receptor by interacting with both the ectodomain and the exoloop 2. *J. Biol. Chem.* 278 (10), 7855–7862. doi:10.1074/jbc.M212457200

Sun, C., Qiu, Y., Ren, Q., Zhang, X., Cao, B., Zou, Y., et al. (2021). Molecular cloning and functional characterization of three 5-HT receptor genes (HTR1B, HTR1E, and HTR1F) in chickens. *Genes* 12 (6), 891. doi:10.3390/genes12060891

van der Westhuizen, E. T., Christopoulos, A., Sexton, P. M., Wade, J. D., and Summers, R. J. (2010). H2 relaxin is a biased ligand relative to H3 relaxin at the relaxin family peptide receptor 3 (RXFP3). *Mol. Pharmacol.* 77 (5), 759–772. doi:10.1124/mol.109.061432

van der Westhuizen, E. T., Halls, M. L., Samuel, C. S., Bathgate, R. A., Unemori, E. N., Sutton, S. W., et al. (2008). Relaxin family peptide receptors-from orphans to therapeutic targets. *Drug Discov. Today* 13 (15-16), 640–651. doi:10.1016/j.drudis.2008.04.002

van der Westhuizen, E. T., Werry, T. D., Sexton, P. M., and Summers, R. J. (2007). The relaxin family peptide receptor 3 activates extracellular signal-regulated kinase 1/2 through a protein kinase C-dependent mechanism. *Mol. Pharmacol.* 71 (6), 1618–1629. doi:10.1124/mol.106.032763

Vodstrcil, L. A., Shynlova, O., Verlander, J. W., Wlodek, M. E., and Parry, L. J. (2010). Decreased expression of the rat myometrial relaxin receptor (RXFP1) in late pregnancy is partially mediated by the presence of the conceptus. *Biol. Reprod.* 83 (5), 818–824. doi:10.1095/biolreprod.110.083931

Wilkinson, T. N., Speed, T. P., Tregear, G. W., and Bathgate, R. A. (2005). Evolution of the relaxin-like peptide family. *BMC Evol. Biol.* 5, 14. doi:10.1186/1471-2148-5-14

Wilson, B. C., Burnett, D., Rappaport, R., Parry, L. J., and Fletcher, E. K. (2009). Relaxin-3 and RXFP3 expression, and steroidogenic actions in the ovary of teleost fish. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 153 (1), 69–74. doi:10.1016/j.cbpa.2008.09.020

Yang, L., Li, Y., Wu, Y., Sun, S., Song, Q., Wei, J., et al. (2020). Rln3a is a prerequisite for spermatogenesis and fertility in male fish. *J. Steroid Biochem. Mol. Biol.* 197, 105517. doi:10.1016/j.jsbmb.2019.105517

Ye, P., Ge, K., Li, M., Yang, L., Jin, S., Zhang, C., et al. (2019). Egg-laying and brooding stage-specific hormonal response and transcriptional regulation in pituitary of Muscovy duck (*Cairina moschata*). *Poult. Sci.* 98 (11), 5287–5296. doi:10.3382/ps/pez433

Yegorov, S., Bogerd, J., and Good, S. V. (2014). The relaxin family peptide receptors and their ligands: new developments and paradigms in the evolution from jawless fish to mammals. *Gen. Comp. Endocrinol.* 209, 93–105. doi:10.1016/j.ygcen.2014.07.014

Yegorov, S., and Good, S. (2012). Using paleogenomics to study the evolution of gene families: origin and duplication history of the relaxin family hormones and their receptors. *PLoS One* 7 (3), e32923. doi:10.1371/journal.pone.0032923

Zhang, J., Lv, C., Mo, C., Liu, M., Wan, Y., Li, J., et al. (2021). Single-cell RNA sequencing analysis of chicken anterior pituitary: A bird's-eye view on vertebrate pituitary. *Front. Physiol.* 12, 562817. doi:10.3389/fphys.2021.562817

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EDITED BY
Sandra G. Velleman,
The Ohio State University, United States

REVIEWED BY
Colin Guy Scanes,
University of Arkansas, United States
Servet Yalcin,
Ege University, Turkey

*CORRESPONDENCE
I. Rozenboim,
rully.r@mail.huji.ac.il

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Targeted differential photostimulation alters reproductive activities of domestic birds

I. Rozenboim[1]*, J. Bartman[1], N. Avital Cohen[1], N. Mobarkey[1], S. Zaguri[1], M. E. El Halawani[2], Y. Chaiseha[3] and A. Marco[1]

[1]Department of Animal Sciences, Robert H. Smith Faculty of Agriculture, Food and Environment, Hebrew University of Jerusalem, Rehovot, Israel, [2]Department of Animal Science, University of Minnesota, Minneapolis, MN, United States, [3]School of Biology, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, Thailand

Modern poultry production systems use environmentally controlled houses providing only artificial illumination. The role of light in reproduction of poultry depends on light quality (photoperiod, intensity/brightness, and spectrum), which enables us to provide custom-made illumination, targeted for the elevation of reproductive activities. Artificial targeted illumination significantly affects poultry reproduction. This phenomenon is based on the mechanism of light absorption in birds, which consists of two main components: the eye (retinal photoreceptors) and brain extraretinal photoreceptors. Several experiments on turkey hens and broiler breeder males and females have shown that photostimulation of brain extraretinal photoreceptors, while maintaining retinal photoreceptors under non-photostimulatory conditions, elevates reproductive activity by increasing egg production of hens and semen quality of roosters. In addition, we found acceleration in all gonadal axis parameters, leading to the acceleration in the production rate. Furthermore, we studied the role of retinal activation in gonadal axis suppuration and identified the role of serotonin in this phenomenon. As for today, several broiler breeder farms use targeted illumination based on our studies with excellent results.

KEYWORDS

broilers, photostimulation, extraretinal photoreceptors, reproduction, targeted illumination

Introduction

In modern environmentally controlled poultry houses, artificial illumination is provided (Rozenboim et al., 2013). Light quality is defined and manifested by photoperiod, intensity/brightness, and spectrum (Figure 1) and plays a pivotal role as an environmental factor activating reproduction. Its effect on bird reproduction has been studied for many years in order to accelerate productivity. Photoperiod as a use for the diphenism of day length is a common tool for the activation or deactivation of

reproductive activities (Lewis and Morris, 2006). The terms light intensity and light brightness are mistakenly mixed due to misunderstanding of light perception in avian species. The intensity of any electromagnetic radiation (visual light is a small part of electromagnetic radiation) is measured by watts/m^2, while brightness (a unit that reflects the effect of light on retinal photoreceptors) is measured by lux (lx), foot candle, and lumen. The third component of light quality is the spectral output of the light source measured in nanometers (nm).

Light perception in most of the avian species occurs at two major sites: the eye through the retina (retinal photoreceptors) and several places in the brain by extra-retinal photoreceptors (ERPRs) (Menaker and Underwood, 1976). Several studies presented extraretinal locations that included the pineal gland, olfactory bulb, and hypothalamus (Scanes and Dridi, 2021) and were defined as deep brain photoreceptors. In mammalian species, where no ERPR can be found, the only place governing the circadian rhythm is retinal photoreceptors (Tosini et al., 2016). The avian retinal system is not required for controlling the circadian rhythm and circannual cycle, as shown in enucleated ducks, which responded to photostimulation, by activating reproduction (Benoit and Assenmacher, 1954). In addition, a follow-up study (Benoit, 1964) found that covering the head to eliminate light penetration to the skull resulted in photorefractoriness. The biochemistry of photoreceptors presents opsin–protein complexes that bind to vitamin-A—which isomerizes in response to light (Bownds, 1967; Hart, 2001). This phenomenon of isomerization allows the opsin molecule to bind to a protein involved in signal transfer to the brain—which by activating the biochemical cascade alters neurotransmitter release from the photoreceptor (Applebury and Hargrave, 1986).

Photostimulation initiates activity in several parts of the brain by activating neuroendocrine response in several axes and causes a broad cascade of hormonal changes. By activating the release of hypothalamic gonadotropin-releasing hormone (GnRH), followed by the secretion of gonadotropins (LH and FSH) from the pituitary gland into the blood, there is an initiation of gonadal recrudescence (Chaiseha and El Halawani, 2005). Moreover, photostimulation reduces the production of gonadotropin-inhibitory hormone (GnIH), which acts as an inhibitory neuropeptide regulating the production of GnRH (Zaguri et al., 2020). While acting as a catalyst for the activation of the gonadal axis, photostimulation can cause a decline in the activity of various neuroendocrine axes, such as lactotropic and serotonergic axes (El Halawani et al., 1983; Dawson et al., 2002). Photostimulation is associated with a decrease in serotonin, which creates a chain reaction lowering levels of VIP, prolactin, and melatonin (Mobarkey et al., 2010).

Several studies have indicated that the eyes play an inhibitory role in reproductive activities of birds. Siopes and Wilson (1980) demonstrated that the eye of the Japanese quail was not essential for photostimulation and sexual development. However, the eyes appear to be essential for short-day-induced testicular regression. Yokoyama and Farner (1976) demonstrated the inhibitory effect of retinal photoreceptors on reproduction of white-crowned sparrows, manifested by a reduction in LH serum. In addition, Homma et al. (1972) and Siopes and Wilson (1974) similarly demonstrated the debilitating role of eyes in the photosexual responses of quails by reduction until termination of egg production and deteriorating cloacal gland activity due to shortened photoperiods, which affected only the birds with retinal vision.

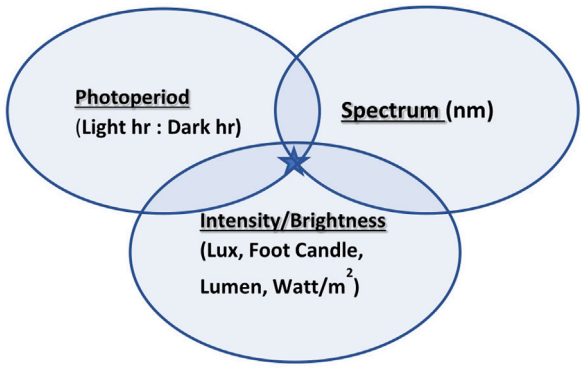

FIGURE 1
Three components of light quality: photoperiod, intensity/brightness, and spectrum join together to create targeted illumination (marked with a star).

The decline in the egg production rate during the reproductive season of domestic birds is a well-known phenomenon. Many studies were conducted in order to reveal the factors associated with this natural decline in productivity. Both incubation behavior and photorefractoriness are associated with environmental light stimulation.

By using differential targeted photostimulation, that is, activating the ERPR while maintaining the retina under non-photostimulatory conditions, we were able, among others, to determine the role of the eye and the brain in the decline of reproductive activities. This was the main objective of several studies conducted on turkey hens and broiler breeder hens and roosters in our laboratory.

Early observations on turkey hens

Our first study was conducted in collaboration with Prof. Mohamed El Halawani from the University of Minnesota. In this first trial, we tested our theory on turkey hens. In brief, 384 large white turkey hens aged 20 weeks were housed in three environmental- and light-controlled rooms (n = 128). In each room, birds were housed in 16 pens (n = 8). In this experiment, we used filtered light (Lee filters). Two parallel light systems were installed in two experimental rooms. The first system (red) had peak emission in the 650–725 nm range (0.565 W/cm^2; 4.45 lx), and the second system (Green) had peak emission between

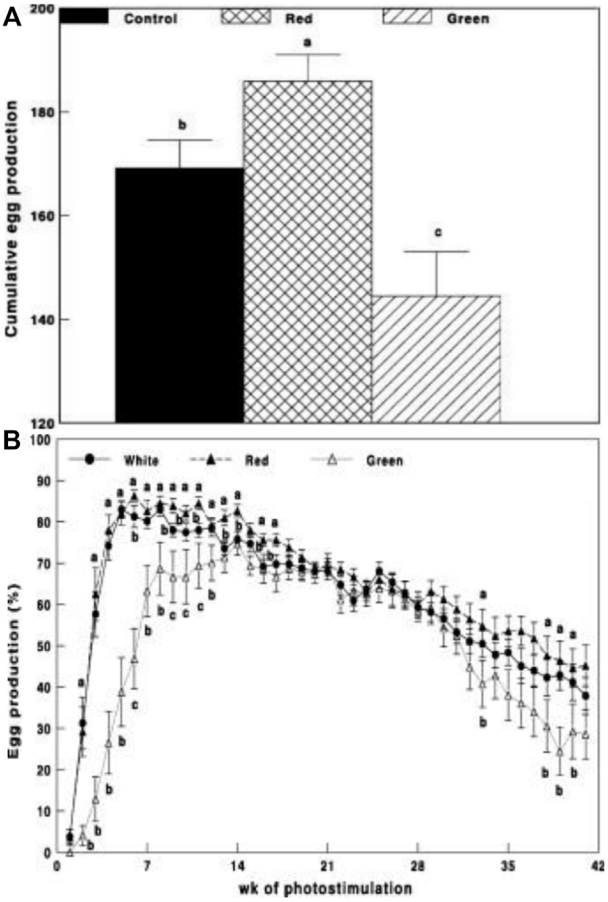

FIGURE 2
Cumulative egg production **(A)** and egg production through weeks of photostimulation (%) **(B)** of Cobb broiler breeder hens in the control group (29 lx, 0.1 W/m^2); red group, photostimulated with the red light (0.1 W/m^2) combined with the non-photostimulatory green light (29 lx); and green group, photostimulated with the green light combined with the non-photostimulatory red light. Data are presented as mean ± standard error of the mean (N = 45). Values with different letters are significantly different ($p \leq 0.05$) (Mobarkey et al., 2010).

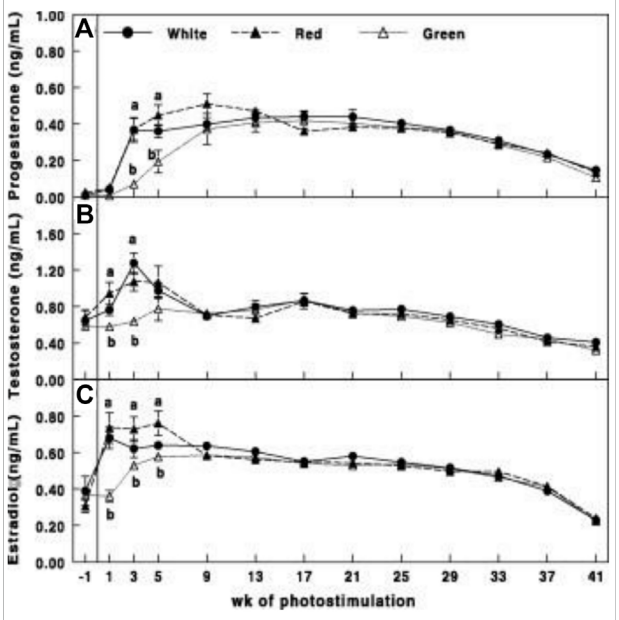

FIGURE 3
Plasma progesterone **(A)**, testosterone **(B)**, and estradiol **(C)** concentrations of Cobb broiler breeder hens in the control group (29 lux 0.1 W/m^2); red group, photostimulated with the red light (0.1 W/m^2) combined with the non-photostimulatory green light (29 lx); and green group, photostimulated with the green light combined with the non-photostimulatory red light. Plasma steroid concentrations were determined by enzyme-linked immunosorbent assay. Data are presented as mean ± standard error of the mean (N = 45). Values with different letters are significantly different ($p \leq 0.05$) (Mobarkey et al., 2010).

500 and 575 nm (0.248 W/cm^2; 23.1 lx). Before photostimulation birds were kept under 6 h of light using both red and green light systems, photostimulation was conducted by increasing the day length to 16 h of light either using the red-light system (red group) or the green-light system (green group) and providing the light for another 6 hr at the middle of the day. The third group was photostimulated by white light (full spectrum provided using the 60-W incandescent lamp, 13.4 W/cm^2; 33.7 lx).

We observed that hens photostimulated with the green light that was exposed also to a short day of the red wavelength showed very low egg production. Hens receiving the full spectrum of light showed slightly higher egg production, while birds photostimulated with the red light that were exposed to short green photostimulation showed the highest egg production. Total egg production for 27 weeks was higher in the red group, followed by the white group and the green-treated birds.

At the end of this preliminary trial, we found that rearing turkey hens under a long day of red light combined with a short day of green light caused significant acceleration in egg production. By using dual lighting systems, we were able to create two parallel photostimulation conditions. First, by using the red light, the extraretinal photoreceptors were photostimulated with little photostimulation of the retinal system, and second, by using the green light, the retinal photoreceptors were photostimulated with little photostimulation of the extraretinal photoreceptors. It is clear from our observations that photostimulation of the retina causes an inhibitory effect on reproductive activities of turkey hens, while photostimulation of the extraretinal photoreceptors accelerates reproductive activities.

The effect of targeted photostimulation on broiler breeder hens

In this trial, we tested our hypothesis on broiler breeder hens with a few technological upgrades by switching to LED devices

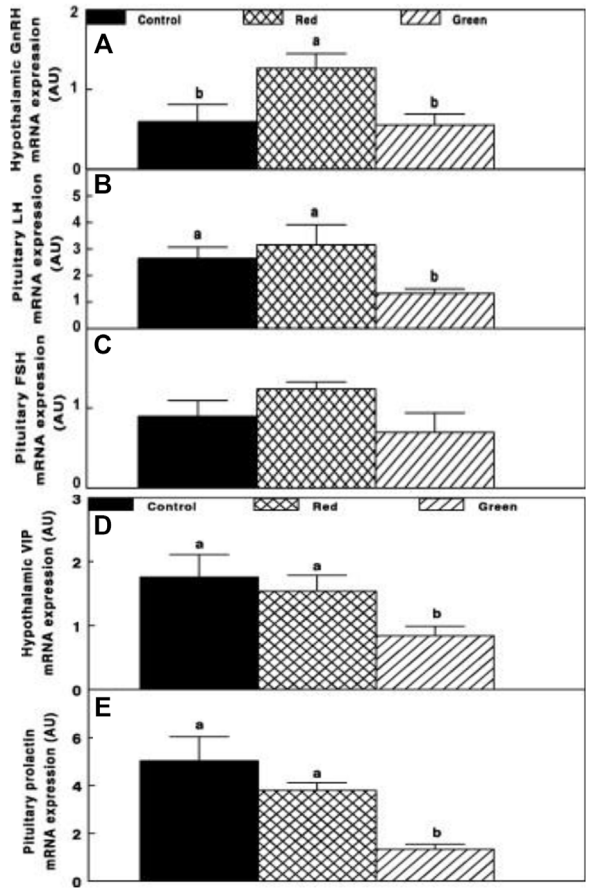

FIGURE 4
Chicken GnRH **(A)**, LH **(B)**, FSH **(C)**, hypothalamic vasoactive intestinal peptide (VIP) **(D)**, and pituitary prolactin **(E)** mRNA expression of the control group (29 lx, 0.1 W/m^2); red group, photostimulated with the red light (0.1 W/m^2) combined with the non-photostimulatory green light (29 lx); and green group, photostimulated with the green light combined with the non-photostimulatory red light. Expression of gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH) was determined by semiquantitative PCR. Abbreviation: A.U., arbitrary units. Data are presented as mean ± standard error of the mean (n = 4). Values with different letters are significantly different ($p \leq 0.05$) (Mobarkey et al., 2010).

that provided a similar illumination environment to that described in the first initial trial. Before the study, white light intensity and brightness were measured using a LI-COR light meter (LI-COR, Lincoln, NE, United States) under the standard conditions of the chicken house. Intensity levels (0.1 W/m^2) and brightness (29 x) were used for green and red illumination, respectively. Annual egg production was significantly elevated in the group exposed to long-day red light together with short-day exposure to green light (Figure 2).

Gonadal axis activity is elevated in the red group compared to all other illuminated groups. This was manifested by the elevation of gonadal steroids at the initiation of egg production (Figure 3) and hypothalamic GnRH-I, pituitary LH, and FSH mRNA gene expression (Figures 4A–C).

Relative photostimulation of retinal and extraretinal photoreceptors also affected the lactotropic axis. Hypothalamic vasoactive intestinal peptide (VIP) mRNA expression was reduced in retinal photostimulated hens (green group, Figure 4D) and was correlated with decreased prolactin mRNA expression ($p \leq 0.05$; Figure 4E).

In addition, activating the brain (ERPR) (red group) significantly elevated ($p \leq 0.05$) hypothalamic red opsin mRNA expression (Figure 5A). In addition, the expression of red opsin was also observed in the retina (Figure 5B). Selective retinal photoreceptor photostimulation in the green group significantly elevated retinal green opsin (Figure 5C), whereas the expression of green opsin in the hypothalamus was very low in all groups (Figure 5D).

Similar to our previous study conducted on turkeys, the activation of ERPR combined with non-photostimulatory conditions to the retinal photoreceptors of broiler breeder hens significantly elevated gonadal axis activity. A unique finding was observed in the hypothalamus. Photostimulation of the ERPR significantly elevated mRNA gene expression of red opsin, suggesting that it might be related to the elevation of GnRH-I mRNA gene expression of increased cumulative egg production (9.87%, compared to the control group). Furthermore, we suggest a possible direct connection between the hypothalamic ERPR and GnRH synapses (Mobarkey et al., 2010).

The debilitating effect of green photostimulation on reproduction was studied in a separate experiment. Understanding the mechanism(s) of the adverse effect of retinal photostimulation on reproduction was the main objective of the next study presented in this paper. Two target candidates were studied: the lactotropic axis and the serotonergic axis.

Serotonin, which is synthesized in the retina (Dawson and Goldsmith, 1997; Cho et al., 1998) and in the hypothalamus (Dunn and Sharp, 1999) during the day (Dawson and Goldsmith, 1997; Cho et al., 1998; Péczely and Kovács, 2000), has been reported to inhibit avian reproduction (Proudman and Opel, 1981; Bacon and Long, 1996; El Halawani et al., 1996). Elevation of serotonin levels directly inhibits GnRH synthesis (Kikuchi et al., 1998) and LH secretion (Proudman and Opel, 1981; El Halawani et al., 1996). In addition, deactivation of the serotonergic axis generally elevates gonadotropin secretion, followed by gonadal development (El Halawani et al., 1996). VIP's synthesis and release are controlled by serotonin (Wong et al., 1991; You et al., 1995; Kang et al., 2006), and levels are changed every 24 h (increase during the day and reduce at night) (Menaker and Keatts, 1968). Furthermore, VIP is considered to be a major prolactin-releasing factor (Menaker et al., 1970), and high plasma concentrations of prolactin inhibit reproduction (Benoit and Ott, 1944). Photostimulation increases hypothalamic

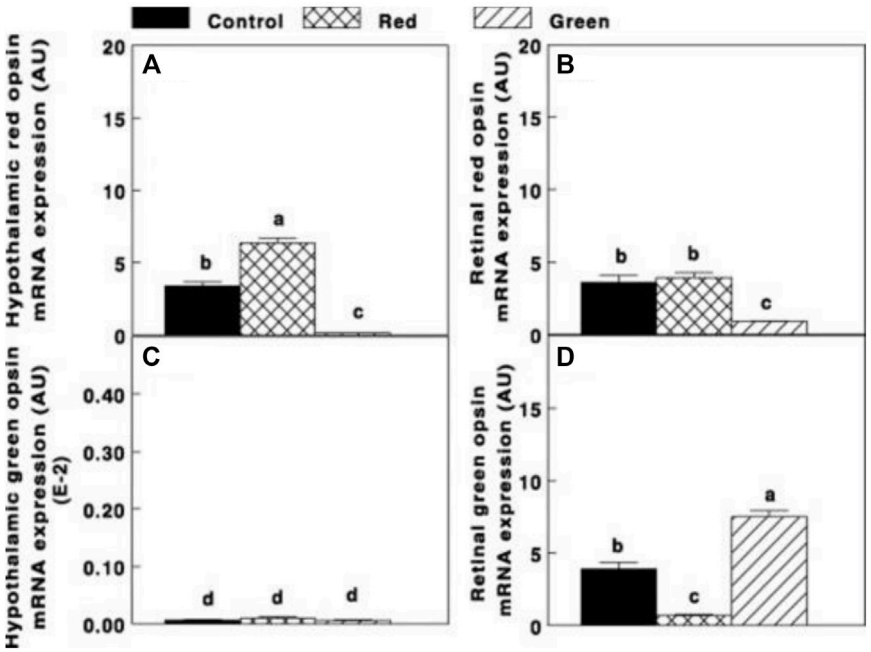

FIGURE 5
Hypothalamic and retinal red opsin mRNA expression [**(A,B)**, respectively] and hypothalamic and retinal green opsin [**(C,D)**, respectively] of the control group (29 lx, 0.1 W/m^2); red group, photostimulated with the red light (0.1 W/m^2) combined with the non-photostimulatory green light (29 lx); and green group, photostimulated with the green light combined with the non-photostimulatory red light. Data are presented as mean ± standard error of the mean (n = 5). Values with different letters are significantly different ($p \leq 0.05$) (Mobarkey et al., 2010).

VIP mRNA content (Homma et al., 1977) and secretion (Foster and Hankins, 2002), which increases prolactin synthesis and secretion (Berson, 2003).

In an experiment published by Mobarkey et al. (2013), broiler breeder hens were photostimulated with the green light while maintaining red light under non-photostimulatory conditions. Parallel to photostimulation, birds were either vaccinated against VIP or orally treated with parachlorophenylalanine (PCPA), which blocks serotonin synthesis by inhibiting tryptophan hydroxylase (an enzyme involved in the pathway of serotonin synthesis) (Silver et al., 1988).

Retinal photostimulation of ERPR by the green light under the non-photostimulatory condition by exposing the birds to a short day of red light caused a significant delay in the onset of egg production (Figure 6). Hens that were photostimulated with the green light and treated with PCPA showed improved ($p < 0.05$) egg production compared with that of the green-control and green-VIP groups. Egg production of the green-PCPA group did not differ from that of the white-control, white-PCPA, and white-VIP groups that were photostimulated for 8 weeks. Active immunization against VIP had no effect on egg production under either the white light or green light.

Retinal activation of ERPR with the green light under a non-photostimulatory condition by a short day of red light significantly reduced gonadal axis activity (reduction in

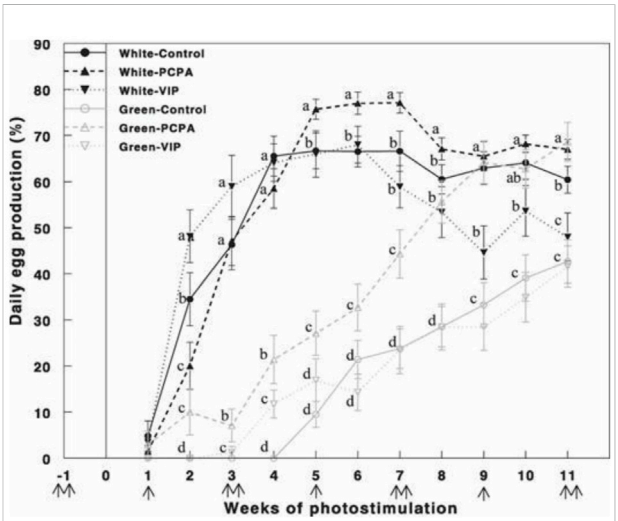

FIGURE 6
Egg production (%) of Cobb broiler breeder hens reared under photostimulatory white light (29 lx, 0.1 W/m^2 White) or green light (29 lx) combined with the non-photostimulatory red light (0.1 W/m^2) (Green). Hens were treated with PCPA, actively immunized against VIP, or left untreated (control). A single arrow indicates PCPA treatment, and double arrows indicate the timing of VIP immunization or PCPA treatment. Data are means ± SEM (n = 15). Values with different letters are significantly different (Mobarkey et al., 2013).

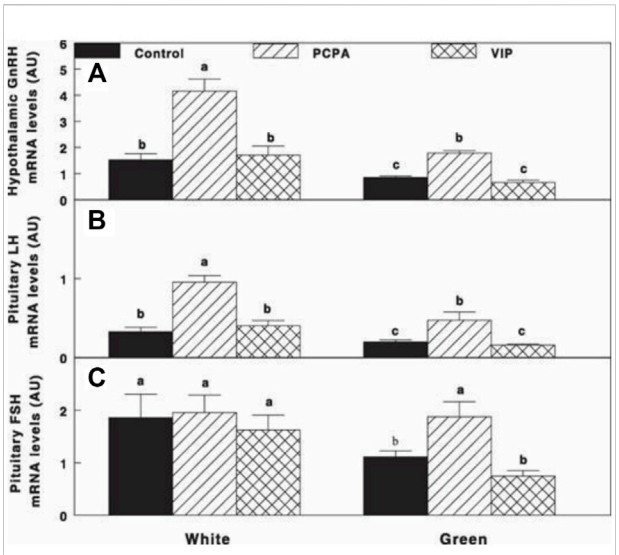

FIGURE 7
Expression of GnRH-I **(A)**, LH-β **(B)**, and FSH-β **(C)** mRNA of Cobb broiler breeder hens reared under the photostimulatory white light (29 lx, 0.1 W/m^2 White) or green light (29 lx) combined with the non-photostimulatory red light (Green). Hens were treated with PCPA, actively immunized against VIP, or left untreated (Control). Values with different letters are significantly different ($p < 0.05$) (Mobarkey et al., 2013).

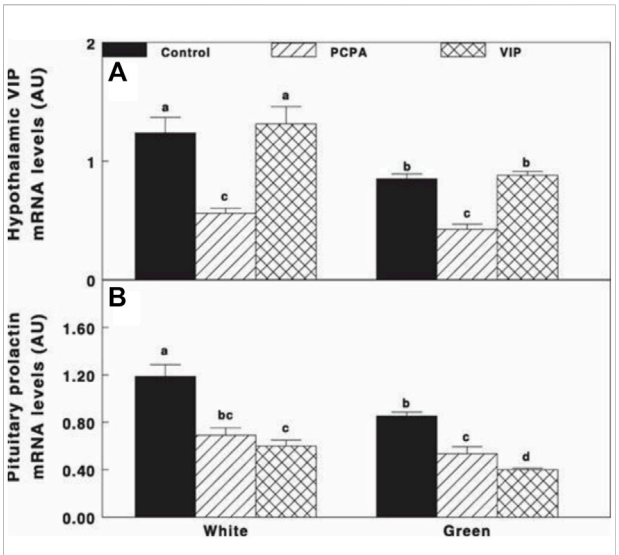

FIGURE 8
VIP **(A)** and prolactin **(B)** mRNA gene expression of Cobb broiler breeder hens reared under the photostimulatory white light (29 lx, 0.1 W/m2 white) or green light (29 lx) combined with the non-photostimulatory red light (green). Hens were treated with PCPA, actively immunized against VIP, or left untreated (control). Data are means ± SEM ($n = 4$). Values with different letters are significantly different ($p < 0.05$) (Mobarkey et al., 2013).

GnRH-I, LH-β, and FSH-β mRNA expression) (Figures 7A–C, respectively); PCPA treatment significantly elevated mRNA gene expression of GnRH-I and LH-β to similar levels of the white-control group. Active immunization against VIP had no effect on mRNA gene expression of the aforementioned components of the gonadal axis also in the white-control or green-photostimulated groups.

Oral administration of PCPA significantly reduced VIP and prolactin mRNA gene expression under both white light and green light (Figures 8A,B), followed by significant decrease in prolactin mRNA gene expression without any effect on VIP mRNA gene expression that was similar to that of the control groups of each treatment light.

PCPA treatment of green light-photostimulated birds significantly elevated plasma LH compared to green-control hens and green-VIP groups (Figure 9E). Plasma LH levels in the green light PCPA-treated group were elevated at 5 weeks of photostimulation, with no significant difference in the white-control groups.

PCPA and VIP treatments of all light groups (white and green light) significantly reduced plasma prolactin levels (Figure 9D). Similarly, the green light-photostimulated group showed lower gonadal steroid levels. Oral administration of PCPA elevated plasma gonadal steroids, thus overcoming the debilitating effect of photostimulation with the green light. Furthermore, active immunization against VIP also significantly elevated plasma progesterone and testosterone levels; however, this elevation was smaller than that in the PCPA treatment group. Active immunization against VIP had no effect on plasma estrogen concentrations (Figures 9A-C).

The mechanism(s) by which retinal photostimulation inhibits reproduction is far from clear. Several speculations can be made: gonadotropin-inhibitory hormone (GnIH) was found to inhibit gonadotropin release from the anterior pituitary of chickens (Ohta and Homma, 1987; Meddle and Follett, 1997). Further studies are needed in order to verify whether retinal photostimulation debilitates reproductive activities by elevating GnIH. We suggest that serotonin plays a pivotal role and should be investigated in relation to retinal inhibition of reproduction. This suggestion is based on several studies indicating that serotonin is synthesized in the retina (Millam et al., 2003; Yasuo et al., 2003) and in the hypothalamus (Harrison, 1972) that inhibits avian reproduction (El Halawani et al., 1983; Hall et al., 1986). Levels of inhibition can be shown at the hypothalamic level by GnRH inhibition (El Halawani et al., 1995) and LH secretion (Hall et al., 1986). Conversely, a blockade of the serotonergic system generally stimulates gonadotropin secretion and enhances gonadal development (Ubuka et al., 2005).

The effects of targeted wavelength illumination on reproductive activities have been much less studied in roosters than in hens. Early studies presented the effect of wavelength stimulation on sexual maturation of several male birds, in which

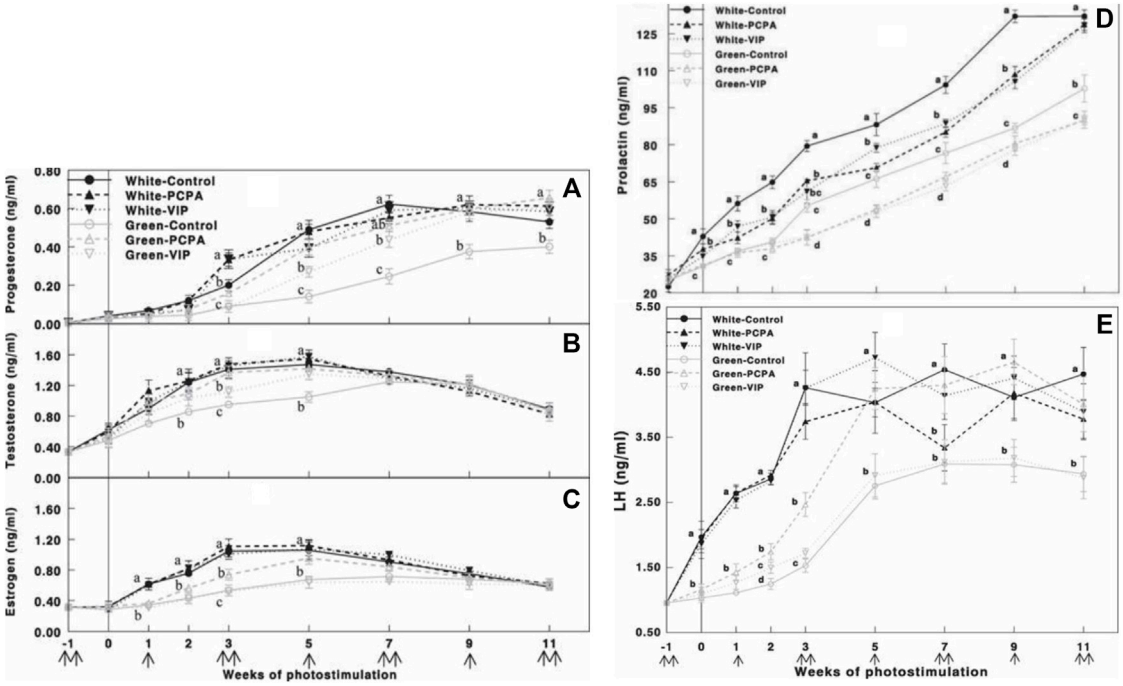

FIGURE 9
Plasma progesterone **(A)**, testosterone **(B)**, estrogen **(C)**, prolactin **(D)**, and LH **(E)** concentrations determined by ELISA of Cobb broiler breeder hens reared under the photostimulatory white light (29 lx, 0.1 W/m^2 white) or green light (29 lx) combined with the non-photostimulatory red light (green). Hens were treated with PCPA, actively immunized against VIP, or left untreated (control). Data are means ± SEM (n = 15). Values with different letters are significantly different ($p < 0.05$) (Mobarkey et al., 2013).

TABLE 1 Lighting regime and treatments.

Treatment	White light	Blue light	Red light	Green light	Total hours
Control group	0,700–2,100 h	—	—	—	14
Blue–red group	—	0,700–2,100 h	0,700–1,300 h	—	14
Red–blue group	—	0,700–1,300 h	0,700–2,100 h	—	14
Green–red group	—	—	0,700–1,300 h	0,700–2,100 h	14
Red–green group	—	—	0,700–2,100 h	0,700–1,300 h	14

long wavelength stimulation has been shown to accelerate maturation in roosters (Johnson et al., 1982), ducks (Benoit et al., 1950), and quails (Woodard et al., 1969). In addition, roosters that were photostimulated with the white or red light for 6 h had lower spermatogenesis and GnRH levels and lower testis weight than roosters that were photostimulated for 14 h. A similar study using green or blue wavelengths did not have the same effect (Casey et al., 1969). Male semen quality (concentration, viability, and motility) is highly correlated with fertility. Lower levels of semen quality can result in subfertile rooster and great economic losses (McDaniel et al., 1998). As in females, we hypothesized that targeted illumination in roosters might elevate fertility and reproductive performances.

The effect of targeted photostimulation on broiler breeder males

In a newly published study, differential targeted photostimulation was tested on broiler breeder roosters. Similar to the illumination protocol that was used on broiler breeder hens, ERPR photostimulation was applied by red-light illumination (14 h) while maintaining non-photostimulatory conditions for the retinal photoreceptors (illumination for 6 h with either blue or green light), as shown in Table 1.

After roosters were photostimulated (as previously described for broiler breeder hens), individual semen quality analysis (semen

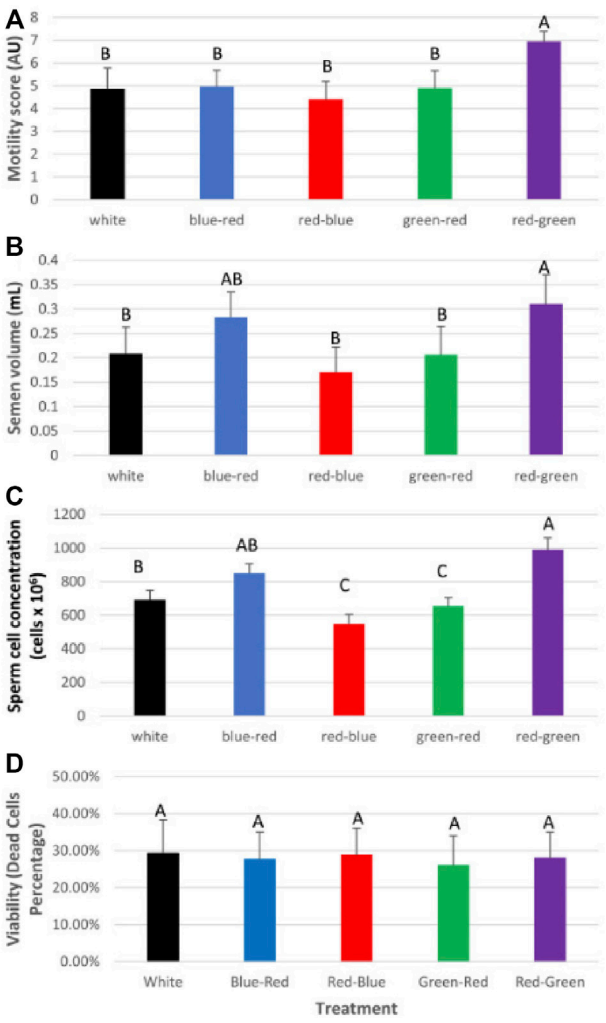

FIGURE 10
Semen analysis (average) for the experimental duration of broiler breeder roosters (Ross) exposed to: 14 h white light, control (white); blue light for 14 h combined with 6 h red light (blue–red); red light for 14 h with 6 h blue light (red–blue); green light for 14 h with 6 h red light (green–red); and red light for 14 h with 6 h green light (red–green). **(A)** Motility rate in all groups. **(B)** Semen volume. **(C)** Concentration of sperm cells per ejaculate. Data are presented as average ±standard error. Levels with different letters are significantly different ($p \leq 0.05$). **(D)** Viability of sperm cells. Data are presented as average ± standard error. Levels with different letters are significantly different ($p \leq 0.05$) (Bartman et al., 2021).

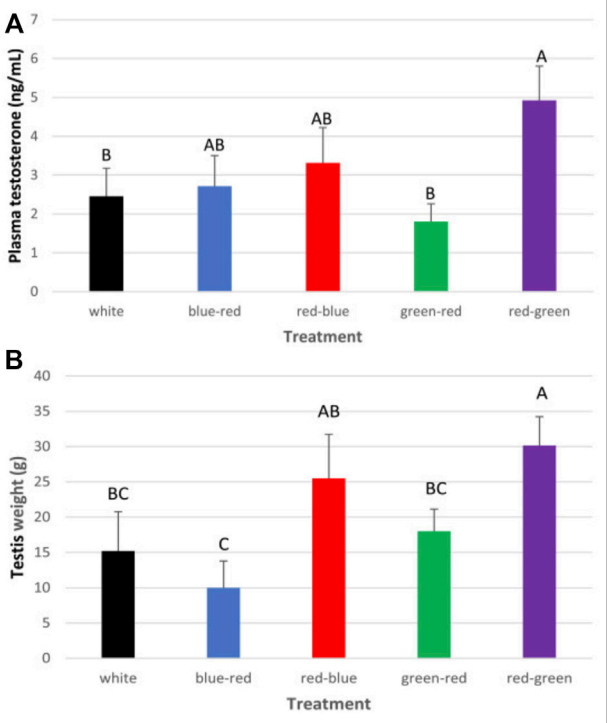

FIGURE 11
Cumulative plasma testosterone levels **(A)** and weight of testes **(B)** of all roosters after exposure to: 14 h white light, control (white); blue light for 14 h combined with 6 h red light (blue–red); red light for 14 h with 6 h blue light (red–blue); green light for 14 h with 6 h red light (green–red); and red light for 14 h with 6 h green light (red–green). Data are presented as average ± standard error. Levels with different letters are significantly different ($p \leq 0.05$) (Bartman et al., 2021).

volume, motility, sperm cell concentration/ml, concentration/ejaculate, and viability) was conducted weekly until the end of the experiment at 65 weeks of age. Plasma samples were tested monthly for prolactin, estradiol, progesterone, and testosterone levels. At 65 weeks of age, all roosters were euthanized, and selected tissues were collected for mRNA gene expression analysis. The results of this study show that semen quality parameters are significantly elevated in red–green group (long day red light combined with short day green light) compared to the control white light-photostimulated group (Figure 10). Furthermore, both long-day photostimulated groups (red–green and red–blue) had higher testis weight at 65 weeks of age (Figure 11B). Moreover, plasma testosterone levels were significantly elevated in the red–green group (average 4.99 ng/ml), compared to those of all other treatment and control groups that averaged 3.04 ng/ml (Figure 11A). The difference was significant compared to the green–red treatment and control group.

Similarly to broiler breeder hens, acceleration of gonadal axis activity was detected in the red–green treatment group manifested by higher hypothalamic GnRH mRNA gene expression levels and pituitary LH and FSH mRNA levels (Figures 12A–C, respectively). In addition, LH and FSH receptor mRNA gene expression in the testes was lower in the red–green group than that in all other treatment groups, including the control group (Figures 12D,E). Aromatase mRNA levels in the testes were the lowest in the red–green treatment group (Figure 12F). Although the difference was not significant, the long-day red treatments resulted in lower expression of this gene than all other treatments and most of

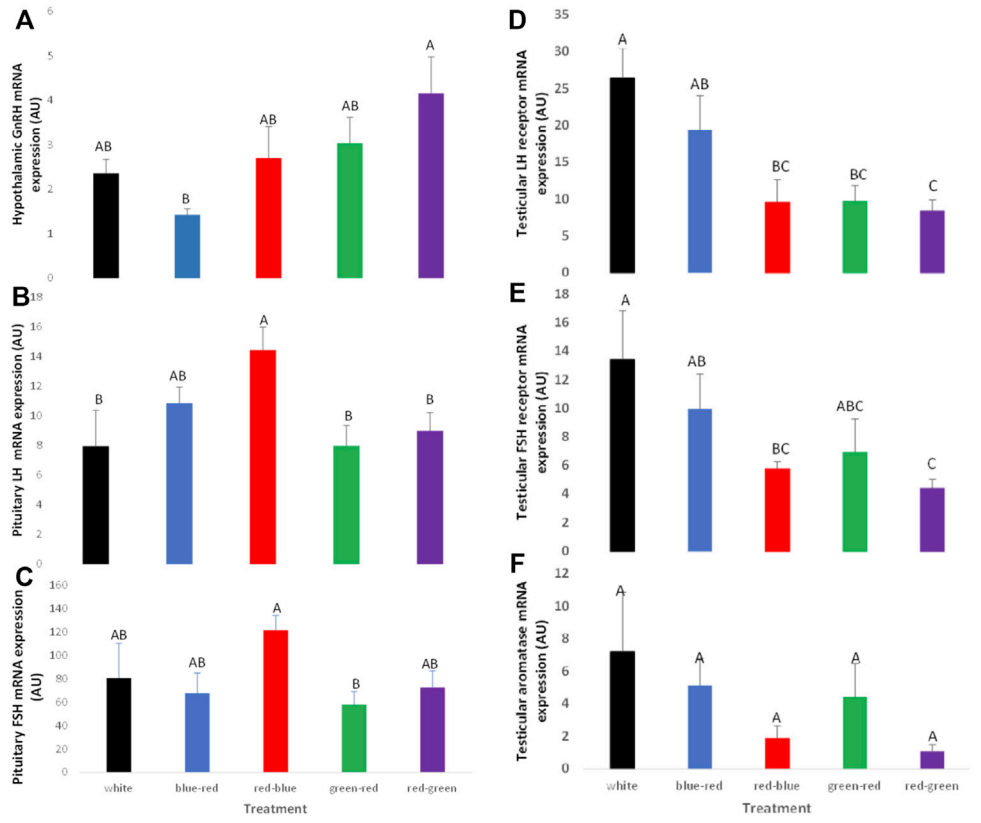

FIGURE 12
Expression of GnRH-I **(A)**, LH-β **(B)**, FSH-β **(C)**, testicular LH receptors **(D)**, FSH receptors **(E)**, and aromatase **(F)** mRNA of broiler breeder roosters (Ross) exposed to: 14 h white light, control (white); blue light for 14 h combined with 6 h red light (blue–red); red light for 14 h with 6 h blue light (red–blue); green light for 14 h with 6 h red light (green–red); red light for 14 h with 6 h green light (red–green). Data are presented as average ± standard error. Levels with different letters are significantly different ($p \leq 0.05$) (Bartman et al., 2021).

all to the control, which showed the highest level of expression (Bartman et al., 2021).

Discussion

Temperate zone birds, including broilers and turkeys, are photoperiodic, with increasing day length stimulating sexual activity, which is caused by long wavelengths of the spectrum (Benoit, 1964; Woodard et al., 1969). Photoperiodic activation of the reproduction state is mediated by ERPR (Benoit and Ott, 1944; Menaker et al., 1970). The location of the ERPR associated to photoperiodic stimulation of reproduction is the medio-basal region of the hypothalamus (Homma et al., 1977; Foster and Hankins, 2002). Unfortunately, little is known about extraretinal photoreceptors, and consequently, we know very little about their sensory physiology or molecular biology. Several opsins have been characterized both in cells and tissues beyond the traditionally accepted retinal photoreceptors (the rods and cones) in several vertebrate species (Berson, 2003; Van Gelder, 2003; Silver et al., 1988; Zaguri et al., 2020; Mobarkey et al., 2010). Immunoreactivity of opsin neurons were found in the quail and duck medio-basal hypothalamus (MBH) (Ohta et al., 1984). Furthermore, electrical stimulation of this region resulted LH secretion and gonadal growth (Sharp and Follett, 1969; Konishi et al., 1987). In addition, MBH lesions block the photo-induced release of LH and testicular growth (Davies and Follett, 1975; Ohta and Homma, 1987; Meddle and Follett, 1997). Quail and turkey neuronal activation, manifested by fos-like protein expression, occurs in the MBH and is associated with photoperiodically driven LH rise (Millam et al., 2003; Yasuo et al., 2003). All evidence points to the MBH as a pivotal site for circadian measurement of day length (Harrison, 1972).

The retina is the most obvious photoreceptive tissue that is activated by the green spectrum (Prescott and Wathes, 1999; Lewis and Morris, 2000). There are indications that the activation of retinal photoreceptors by visible radiation is inhibitory to reproduction (Homma et al., 1972; Siopes and Wo, 1980). Orbital enucleation increased egg production in chickens (Ubuka et al., 2005), and orbital enucleation combined with pinealectomy

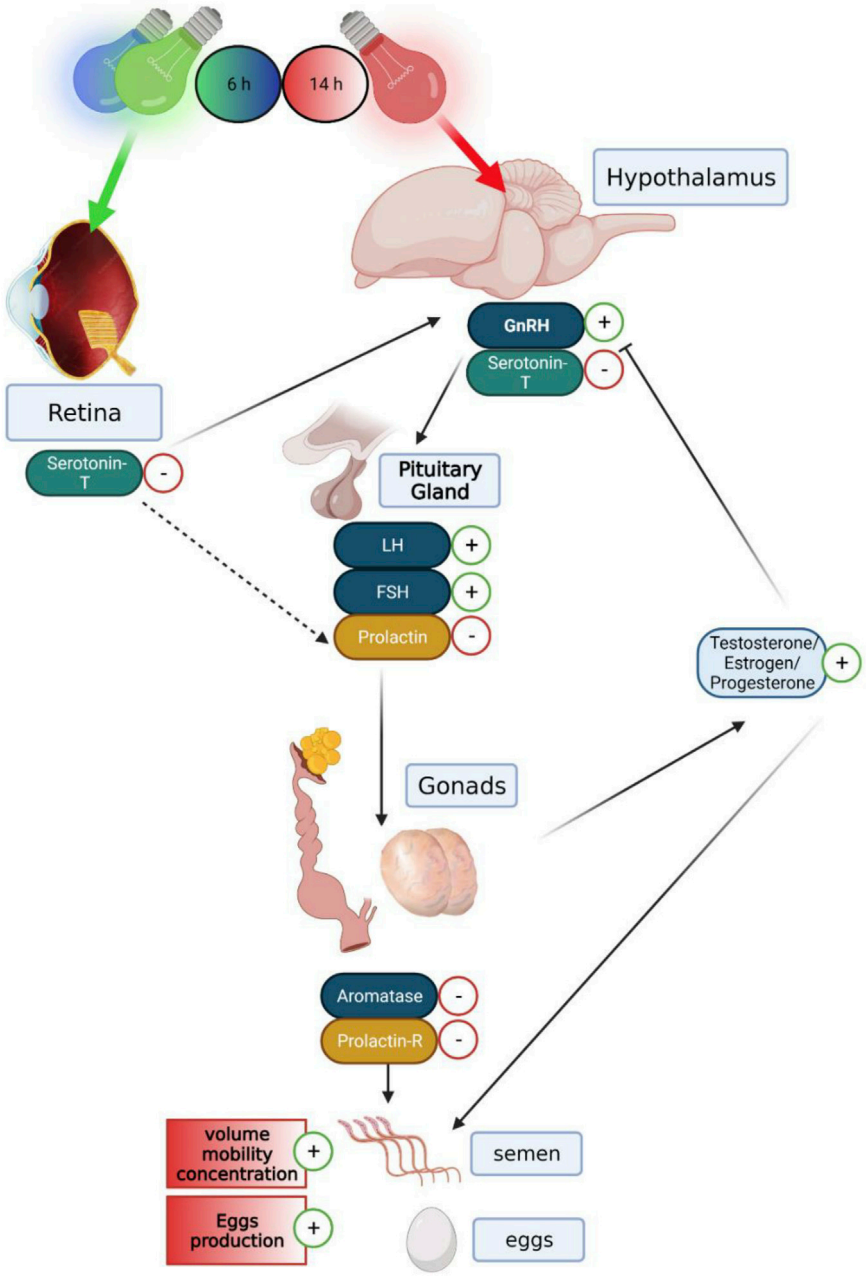

FIGURE 13
Graphical illustration demonstrating the hormonal and genomic changes of the gonadal, serotonergic, and lactotropic axes in the hypothalamus–pituitary–gonadal pathway, in both breeding broiler males and females: a long day of red illumination combined with a short day of green or blue illumination resulting in elevated expression levels of GnRH mRNA and reduced levels of serotonin transporter in the hypothalamus. Furthermore, a lower level of serotonin transporter expression levels in the retina can be observed. This causes raised levels of LH and FSH gene expression and lower gene expression of prolactin in the pituitary gland. This follows a reduction in aromatase and prolactin receptor mRNA expression in the reproductive systems, thus causing elevation in testosterone levels in the plasma and higher semen quality in males and elevation in estrogen and progesterone in the plasma and larger egg production in females. Created with BioRender.com.

decreased the hypothalamic concentration of gonadotropin-inhibitory factor (GnIH) mRNA and its peptide (Underwood et al., 1990). These findings, taken together with our results, suggest of two light pathways that regulate reproduction in birds, as shown in Figure 13: a stimulatory pathway mediated by hypothalamic photoreceptors, which are activated by the red spectrum/630 nm wavelength and an inhibitory retinal-hypothalamic pathway activated by the green spectrum/525 nm

wavelength. The functional significance of the interaction between the two hypothesized pathways in the regulation of the avian reproductive cycle is currently unclear. The possibility remains that the retinal pathway may be of importance at the termination of egg-laying activity, as in the case of the onset of photorefractoriness. The mechanism underlying photorefractoriness is unknown, but there are indications that the retinal-hypothalamic pathway, which involves the melatonin system (Underwood et al., 1984; Guyomarc'h et al., 2001), may be involved in the inhibition of the avian reproductive neuroendocrine system and the termination of sexual and egg-laying activities (Rozenboim et al., 2002). More recently, melatonin injection has been shown to increase hypothalamic levels of GnIH mRNA and its peptide (Underwood et al., 1990).

Long wavelengths (red light) penetrate the skull and tissues and stimulate ERPR—activating the gonadal axis (Oishi and Lauber, 1973; Benoit, 1978; Mobarkey et al., 2010). Shorter wavelengths, (green–yellow lights) mainly activating the retina, stimulate the secretion of gonadotropin-inhibitory hormone (GnIH), followed by reproduction inhibition (Benoit and Assenmacher, 1966; Mobarkey et al., 2013; Bédécarrats, 2015). Selective photostimulation of different photoreceptor sites can be used as an environmental tool for acceleration of reproductive activities in domestic birds.

Several endocrine axes are involved in reproductive activities of domestic birds, and the most pronounced ones are the gonadotropic axis and the lactotropic/serotonergic axis, both known to be activated by photostimulation. The gonadotropic axis activation by photostimulation has been well characterized (Sharp et al., 1998; Saldanha et al., 2001), whereas the lactotropic and serotonergic axes are known to deactivate reproduction (Avital- Cohen et al., 2012). The mechanisms through which photic cues are transduced to neuroendocrine effector neurons remain unknown. There is still much to discover about the connection between the brain ERPR and the reproductive axis. Previous studies have shown that brain photoreceptors communicate directly with the GnRH neurons that stimulate the activation of reproduction (Saldanha et al., 2001; Scanes and Dridi, 2021). Another possible connection is with vasoactive intestinal peptide (VIP) cells, which colocalize with all opsin-expressing cells in birds. Within the opsin system, VIP could potentially regulate reproduction through synaptic interactions all along the trajectory of its axons through the lateral septum and hypothalamus (Hof et al., 1991; Saldanha et al., 2001). More importantly, we previously demonstrated that complementary treatment with PRL in old breeder roosters vaccinated against VIP reactivated the gonadal axis activity and improved sperm quality (Avital- Cohen et al., 2012). Thus, the decline in VIP and PRL gene expression in the green–red group might explain the damage caused to LH gene expression and reproductive performance.

Elevation in GnIH mRNA gene expression was shown in birds subjected to long-day green light combined with short-day red light (green–red group) (Zaguri et al., 2020). Since GnIH inhibits the synthesis and secretion of gonadotropins LH and FSH in domestic fowl (Ciccone et al., 2004) and disrupts gonadal development and activity in quail (Ubuka et al., 2006), we suggest the involvement of this hormone as a deactivator of reproduction.

Author contributions

All author listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Applebury, M. L., and Hargrave, P. A. (1986). Molecular biology of the visual pigments. *Vis. Res.* 26 (12), 1881–1895. doi:10.1016/0042-6989(86)90115-x

Avital-Cohen, N., Heiblum, R., Argov, N., Rosenstrauch, A., Chaiseha, Y., Mobarkey, N., et al. (2012). The effect of active immunization against vasoactive intestinal peptide (VIP) and inhibin on reproductive performance of aging White Leghorn roosters. *Poult. Sci.* 91 (1), 161–174. doi:10.3382/ps.2011-01439

Bacon, W. L., and Long, D. W. (1996). Secretion of luteinizing hormone during a forced molt in Turkey hens. *Poult. Sci.* 75 (12), 1579–1586. doi:10.3382/ps.0751579

Bartman, J., Zaguri, S., Avital-Cohen, N., Dishon, L., Druyan, S., Gumułka, M., et al. (2021). Targeted differential illumination improves reproductive traits of broiler breeder males. *Poult. Sci.* 100 (6), 101109. doi:10.1016/j.psj.2021.101109

Bédécarrats, G. Y. (2015). Control of the reproductive axis: Balancing act between stimulatory and inhibitory inputs. *Poult. Sci.* 94 (4), 810–815. doi:10.3382/ps/peu042

Benoit, J., and Assenmacher, I. (1954). Comparative sensitivity of superficial and deep receptors in photosexual reflex in duck. *C. R. Hebd. Seances Acad. Sci.* 239 (1), 105–107.

Benoit, J., and Assenmacher, I. (1966). Research on the photosensitivity of superficial and deep neural receptors in gonad stimulation by visible radiation in the immature Pekin duck. *C. R. Acad. Hebd. Seances Acad. Sci. D.* 262 (26), 2750–2752.

Benoit, J. M. (1978). Chronobiologic study in the domestic duck. II. Physiological mechanism of the chronobiologic action of visible light on the gonads of the male duck. *Chronobiologia* 5 (2), 158–168.

Benoit, J., and Ott, L. (1944). External and internal factors in sexual activity: Effect of irradiation with different wave-lengths on the mechanisms of photostimulation of the hypophysis and on testicular growth in the immature duck. *Yale J. Biol. Med.* 17 (1), 27–46.2.

Benoit, J. (1964). *The role of the eye and of the hypothalamus in the photostimulation of gonads in the duck*. New York, United States: Annals of the New York Academy of Sciences.

Benoit, J., Walter, F. X., and Assenmacher, I. (1950). [Further research on the effect of light of different wave lengths on gonad stimulation of the impuberal male duck]. *C. R. Seances Soc. Biol. Fil.* 144, 1206–1211.

Berson, D. M. (2003). Strange vision: Ganglion cells as circadian photoreceptors. *Trends Neurosci.* 26 (6), 314–320. doi:10.1016/S0166-2236(03)00130-9

Bownds, D. (1967). Site of attachment of retinal in rhodopsin. *Nature* 216 (5121), 1178–1181. doi:10.1038/2161178a0

Casey, J. M., Harrison, P. C., Latshaw, J. D., and McGinnis, J. (1969). January. Effects of photoperiod and colored lights on sexual maturaty of domestic FowlNORTH DUNLAP AVE, SAVOY, IL 61874: Poultry science assoc INC. *Poult. Sci.* 48, 1111.

Chaiseha, Y., and El Halawani, M. E. (2005). Neuroendocrinology of the female Turkey reproductive cycle. *J. Poult. Sci.* 42 (2), 87–100. doi:10.2141/jpsa.42.87

Cho, N., Harada, M., Ogi, K., Endo, S., Onda, H., Fujino, M., et al. (1998). Discovery of a novel, potent, and orally active nonpeptide antagonist of the human luteinizing hormone-releasing hormone (LHRH) receptor. *J. Med. Chem.* 41 (22), 4190–4195. doi:10.1021/jm9803673

Ciccone, N. A., Dunn, I. C., Boswell, T., Tsutsui, K., Ubuka, T., Ukena, K., et al. (2004). Gonadotrophin inhibitory hormone depresses gonadotrophin α and follicle-stimulating hormone β subunit expression in the pituitary of the domestic chicken. *J. Neuroendocrinol.* 16 (12), 999–1006. doi:10.1111/j.1365-2826.2005.01260.x

Davies, D. T., and Follett, B. K. (1975). The neuroendocrine control of gonadotrophin release in the Japanese quail. I. The role of the tuberal hypothalamus. *Proc. R. Soc. Lond. B Biol. Sci.* 191 (1103), 285–301. doi:10.1098/rspb.1975.0129

Dawson, A., and Goldsmith, A. R. (1997). Changes in gonadotrophin-releasing hormone (GnRH-I) in the pre-optic area and median eminence of starlings (*Sturnus vulgaris*) during the recovery of photosensitivity and during photostimulation. *J. Reprod. Fertil.* 111 (1), 1–6. doi:10.1530/jrf.0.1110001

Dawson, A., Talbot, R. T., Dunn, I. C., and Sharp, P. J. (2002). Changes in basal hypothalamic chicken gonadotropin-releasing hormone-I and vasoactive intestinal polypeptide associated with a photo-induced cycle in gonadal maturation and prolactin secretion in intact and thyroidectomized starlings (sturnus vulgaris). *J. Neuroendocrinol.* 14 (7), 533–539. doi:10.1046/j.1365-2826.2002.00807.x

Dunn, I. C., and Sharp, P. J. (1999). Photo-induction of hypothalamic gonadotrophin releasing hormone-I mRNA in the domestic chicken: A role for oestrogen? *J. Neuroendocrinol.* 11 (5), 371–375. doi:10.1046/j.1365-2826.1999.00335.x

El Halawani, M. E., Pitts, G. R., Sun, S., Silsby, J. L., and Sivanandan, V. (1996). Active immunization against vasoactive intestinal peptide prevents photo-induced prolactin secretion in turkeys. *Gen. Comp. Endocrinol.* 104 (1), 76–83. doi:10.1006/gcen.1996.0143

El Halawani, M. E., Silsby, J. L., Fehrer, S. C., and Behnke, E. J. (1983). Reinitiation of ovulatory cycles in incubating female turkeys by an inhibitor of serotonin synthesis, p-chlorophenylalanine. *Biol. Reprod.* 28 (1), 221–228. doi:10.1095/biolreprod28.1.221

El Halawani, M. E., Silsby, J. L., Rozenboim, I., and Pitts, G. R. (1995). Increased egg production by active immunization against vasoactive intestinal peptide in the Turkey (*Meleagris gallopavo*). *Biol. Reprod.* 52 (1), 179–183. doi:10.1095/biolreprod52.1.179

Foster, R. G., and Hankins, M. W. (2002). Non-rod, non-cone photoreception in the vertebrates. *Prog. Retin. Eye Res.* 21 (6), 507–527. doi:10.1016/s1350-9462(02)00036-8

Guyomarc'h, C., Lumineau, S., Vivien-Roels, B., Richard, J. P., and Derégnaucourt, S. (2001). Effect of melatonin supplementation on the sexual development in European quail (*Coturnix coturnix*). *Behav. Process.* 53 (1-2), 121–130. doi:10.1016/s0376-6357(01)00133-4

Hall, T. R., Cheung, A., and Harvey, S. (1986). Serotoninergic inhibition of LH secretion in the domestic fowl. *J. Endocrinol.* 110 (2), 239–244. doi:10.1677/joe.0.1100239

Harrison, P. C. (1972). Extraretinal photocontrol of reproductive responses of Leghorn hens to photoperiods of different length and spectrum. *Poult. Sci.* 51 (6), 2060–2064. doi:10.3382/ps.0512060

Hart, N. S. (2001). The visual ecology of avian photoreceptors. *Prog. Retin. Eye Res.* 20 (5), 675–703. doi:10.1016/s1350-9462(01)00009-x

Hof, P. R., Dietl, M. M., Charnay, Y., Martin, J. L., Bouras, C., Palacios, J. M., et al. (1991). Vasoactive intestinal peptide binding sites and fibers in the brain of the pigeon *Columba livia*: An autoradiographic and immunohistochemical study. *J. Comp. Neurol.* 305 (3), 393–411. doi:10.1002/cne.903050304

Homma, K., Sakakibara, Y., and Ohta, M. (1977). "Potential sites and action spectra for encephalic photoreception in the Japanese quail," in *First international symposium on avian endocrinology* (Bangor, United Kingdom: University College of North Wales), 25–26.

Homma, K., Wilson, W. O., and Siopes, T. D. (1972). Eyes have a role in photoperiodic control of sexual activity of Coturnix. *Science* 178 (4059), 421–423. doi:10.1126/science.178.4059.421

Johnson, A. L., Foss, D. C., and Carew, L. B., Jr (1982). Effect of selected light treatments on pineal weight and lipid content in the cockerel (*Gallus domesticus*). *Poult. Sci.* 61 (1), 128–134. doi:10.3382/ps.0610128

Kang, S. W., Thayananuphat, A., Rozenboim, I., Millam, J. R., Proudman, J. A., and El Halawani, M. E. (2006). Expression of hypothalamic GnRH-I mRNA in the female Turkey at different reproductive states and following photostimulation. *Gen. Comp. Endocrinol.* 146 (2), 91–99. doi:10.1016/j.ygcen.2005.10.017

Kikuchi, M., Kobayashi, M., Ito, T., Kato, Y., and Ishii, S. (1998). Cloning of complementary deoxyribonucleic acid for the follicle-stimulating hormone-β subunit in the Japanese quail. *Gen. Comp. Endocrinol.* 111 (3), 376–385. doi:10.1006/gcen.1998.7123

Konishi, H., Foster, R. G., and Follett, B. K. (1987). Evidence for a daily rhythmicity in the acute release of luteinizing hormone in response to electrical stimulation in the Japanese quail. *J. Comp. Physiol. A* 161 (2), 315–319. doi:10.1007/BF00615251

Lewis, P. D., and Morris, T. R. (2000). Poultry and coloured light. *World's. Poult. Sci. J.* 56 (3), 189–207. doi:10.1079/wps20000015

Lewis, P. D., and Morris, T. R. (2006). *Poultry lighting: The theory and practice*. North Parramatta, Australia: Northcot.

McDaniel, C. D., Hannah, J. L., Parker, H. M., Smith, T. W., Schultz, C. D., and Zumwalt, C. D. (1998). Use of a sperm analyzer for evaluating broiler breeder males. 1. Effects of altering sperm quality and quantity on the sperm motility index. *Poult. Sci.* 77 (6), 888–893. doi:10.1093/ps/77.6.888

Meddle, S. L., and Follett, B. K. (1997). Photoperiodically driven changes in Fos expression within the basal tuberal hypothalamus and median eminence of Japanese quail. *J. Neurosci.* 17 (22), 8909–8918. doi:10.1523/jneurosci.17-22-08909.1997

Menaker, M., and Keatts, H. (1968). Extraretinal light perception in the sparrow. II. Photoperiodic stimulation of testis growth. *Proc. Natl. Acad. Sci. U. S. A.* 60 (1), 146–151. doi:10.1073/pnas.60.1.146

Menaker, M., Roberts, R., Elliott, J., and Underwood, H. (1970). Extraretinal light perception in the sparrow, III: The eyes do not participate in photoperiodic photoreception. *Proc. Natl. Acad. Sci. U. S. A.* 67 (1), 320–325. doi:10.1073/pnas.67.1.320

Menaker, M., and Underwood, H. (1976). Extraretinal photoreception in birds. *Photophysiology* 23, 299–306. doi:10.1111/j.1751-1097.1976.tb07251.x

Millam, J. R., Craig-Veit, C. B., and Siopes, T. D. (2003). Photostimulated fos-like immunoreactivity in tuberal hypothalamus of photosensitive vs. photorefractory Turkey hens. *Gen. Comp. Endocrinol.* 134 (2), 175–181. doi:10.1016/s0016-6480(03)00249-1

Mobarkey, N., Avital, N., Heiblum, R., and Rozenboim, I. (2013). The effect of parachlorophenylalanine and active immunization against vasoactive intestinal peptide on reproductive activities of broiler breeder hens photostimulated with green light. *Biol. Reprod.* 88 (4), 83, 1–7. doi:10.1095/biolreprod.112.103697

Mobarkey, N., Avital, N., Heiblum, R., and Rozenboim, I. (2010). The role of retinal and extra-retinal photostimulation in reproductive activity in broiler breeder hens. *Domest. Anim. Endocrinol.* 38 (4), 235–243. doi:10.1016/j.domaniend.2009.11.002

Ohta, M., and Homma, K. (1987). Detection of neural connection to the infundibular complex by partial or complete hypothalamic deafferentation in male quail. *Gen. Comp. Endocrinol.* 68 (2), 286–292. doi:10.1016/0016-6480(87)90040-2

Ohta, M., Wada, M., and Homma, K. (1984). Induction of rapid testicular growth in Japanese quail by phasic electrical stimulation of the hypothalamic photosensitive area. *J. Comp. Physiol.* 154 (4), 583–589. doi:10.1007/bf00610171

Oishi, T. A. D. A. S. H. I., and Lauber, J. K. (1973). Photoreception in the photosexual response of quail. II. Effects of intensity and wavelength. *Am. J. Physiol.* 225 (4), 880–886. doi:10.1152/ajplegacy.1973.225.4.880

Péczely, P., and Kovács, K. J. (2000). Photostimulation affects gonadotropin-releasing hormone immunoreactivity and activates a distinct neuron population in

the hypothalamus of the mallard. *Neurosci. Lett.* 290 (3), 205–208. doi:10.1016/s0304-3940(00)01360-4

Prescott, N. B., and Wathes, C. M. (1999). Reflective properties of domestic fowl (Gallus g. domesticus), the fabric of their housing and the characteristics of the light environment in environmentally controlled poultry houses. *Br. Poult. Sci.* 40 (2), 185–193. doi:10.1080/00071669987584

Proudman, J. A., and Opel, H. (1981). Turkey prolactin: Validation of a radioimmunoassay and measurement of changes associated with broodiness. *Biol. Reprod.* 25 (3), 573–580. doi:10.1095/biolreprod25.3.573

Rozenboim, I., Aharony, T., and Yahav, S. (2002). The effect of melatonin administration on circulating plasma luteinizing hormone concentration in castrated White Leghorn roosters. *Poult. Sci.* 81 (9), 1354–1359. doi:10.1093/ps/81.9.1354

Rozenboim, I., El Halawani, M. E., Kashash, Y., Piestun, Y., and Halevy, O. (2013). The effect of monochromatic photostimulation on growth and development of broiler birds. *Gen. Comp. Endocrinol.* 190, 214–219. doi:10.1016/j.ygcen.2013.06.027

Saldanha, C. J., Silverman, A. J., and Silver, R. (2001). Direct innervation of GnRH neurons by encephalic photoreceptors in birds. *J. Biol. Rhythms* 16 (1), 39–49. doi:10.1177/074873040101600105

Scanes, C. G., and Dridi, S. (Editors) (2021). *Sturkie's avian physiology* (Massachusetts, United States: Academic Press).

Sharp, P. J., Dawson, A., and Lea, R. W. (1998). Control of luteinizing hormone and prolactin secretion in birds. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.* 119 (3), 275–282. doi:10.1016/s0742-8413(98)00016-4

Sharp, P. J., and Follett, B. K. (1969). The effect of hypothalamic lesions on gonadotrophin release in Japanese quail (*Coturnix coturnix* japonica). *Neuroendocrinology* 5 (3-4), 205–218. doi:10.1159/000121861

Silver, R., Witkovsky, P., Horvath, P., Alones, V., Barnstable, C. J., and Lehman, M. N. (1988). Coexpression of opsin-and VIP-like-immunoreactivity in CSF-contacting neurons of the avian brain. *Cell. Tissue Res.* 253 (1), 189–198. doi:10.1007/BF00221754

Siopes, T. D., and Wilson, W. O. (1974). Extraocular modification of photoreception in intact and pinealectomized coturnix. *Poult. Sci.* 53 (6), 2035–2041. doi:10.3382/ps.0532035

Siopes, T. D., and Wilson, W. O. (1980). Participation of the eyes in the photostimulation of chickens. *Poult. Sci.* 59 (5), 1122–1125. doi:10.3382/ps.0591122

Siopes, T. D., and Wo, W. (1980). Participation of the eyes in the photosexual response of Japanese quail (*Coturnix coturnix* japonica). *Biol. Reprod.* 23, 352–357. doi:10.1095/biolreprod23.2.352

Tosini, G., Ferguson, I., and Tsubota, K. (2016). Effects of blue light on the circadian system and eye physiology. *Mol. Vis.* 22, 61–72.

Ubuka, T., Bentley, G. E., Ukena, K., Wingfield, J. C., and Tsutsui, K. (2005). Melatonin induces the expression of gonadotropin-inhibitory hormone in the avian brain. *Proc. Natl. Acad. Sci. U. S. A.* 102 (8), 3052–3057. doi:10.1073/pnas.0403840102

Ubuka, T., Ukena, K., Sharp, P. J., Bentley, G. E., and Tsutsui, K. (2006). Gonadotropin-inhibitory hormone inhibits gonadal development and maintenance by decreasing gonadotropin synthesis and release in male quail. *Endocrinology* 147 (3), 1187–1194. doi:10.1210/en.2005-1178

Underwood, H., Barrett, R. K., and Siopes, T. (1990). Melatonin does not link the eyes to the rest of the circadian system in quail: A neural pathway is involved. *J. Biol. Rhythms* 5 (4), 349–361. doi:10.1177/074873049000500406

Underwood, H., Binkley, S., Siopes, T., and Mosher, K. (1984). Melatonin rhythms in the eyes, pineal bodies, and blood of Japanese quail (*Coturnix coturnix* japonica). *Gen. Comp. Endocrinol.* 56 (1), 70–81. doi:10.1016/0016-6480(84)90063-7

Wong, E. A., Ferrin, N. H., Silsby, J. L., and El Halawani, M. E. (1991). Cloning of a Turkey prolactin cDNA: Expression of prolactin mRNA throughout the reproductive cycle of the domestic Turkey (*Meleagris gallopavo*). *Gen. Comp. Endocrinol.* 83 (1), 18–26. doi:10.1016/0016-6480(91)90101-b

Woodard, A. E., Moore, J. A., and Wilson, W. O. (1969). Effect of wave length of light on growth and reproduction in Japanese quail (*Coturnix coturnix* japonica). *Poult. Sci.* 48 (1), 118–123. doi:10.3382/ps.0480118

Yasuo, S., Watanabe, M., Okabayashi, N., Ebihara, S., and Yoshimura, T. (2003). Circadian clock genes and photoperiodism: Comprehensive analysis of clock gene expression in the mediobasal hypothalamus, the suprachiasmatic nucleus, and the pineal gland of Japanese quail under various light schedules. *Endocrinology* 144 (9), 3742–3748. doi:10.1210/en.2003-0435

Yokoyama, K., and Farner, D. S. (1976). Photoperiodic responses in bilaterally enucleated female white-crowned sparrows, Zonotrichia leucophrys gambelii. *Gen. Comp. Endocrinol.* 30 (4), 528–533. doi:10.1016/0016-6480(76)90124-6

You, S., Foster, L. K., Silsby, J. L., El Halawani, M. E., and Foster, D. N. (1995). Sequence analysis of the Turkey LH β subunit and its regulation by gonadotrophin-releasing hormone and prolactin in cultured pituitary cells. *J. Mol. Endocrinol.* 14 (1), 117–129. doi:10.1677/jme.0.0140117

Zaguri, S., Bartman, J., Avital-Cohen, N., Dishon, L., Gumułka, M., Chaiseha, Y., et al. (2020). Targeted differential monochromatic lighting improves broiler breeder reproductive performance. *Poult. Sci.* 99 (7), 3697–3708. doi:10.1016/j.psj.2020.03.007

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EDITED BY
Sandra G. Velleman,
the Ohio State University, United States

REVIEWED BY
Walter Gay Bottje,
the University of Arkansas, United States
Colin Guy Scanes,
University of Arkansas, United States

*CORRESPONDENCE
Neelu Jain Gupta,
drneelujgupta@hotmail.com,

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Altered dynamics of mitochondria and reactive oxygen species in the erythrocytes of migrating red-headed buntings

Nitin Bhardwaj[1], Anit Kumar[2] and Neelu Jain Gupta [2]*

[1]Department of Zoology and Environmental Science, Gurukula Kangri (Deemed to be University), Haridwar, Uttarakhand, India, [2]Department of Zoology, Chaudhary Charan Singh University, Meerut, Uttar Pradesh, India

Background: Blood antioxidants provide propensity to mitigate reactive oxygen species (ROS) apart from other oxidative challenges during a high-energy state of migration in night migratory songbirds. The study investigated the modulation of erythrocytes, mitochondrial abundance, hematocrit changes, and relative expression of fat transport-related genes during migration in red-headed buntings (*Emberiza bruniceps*). We hypothesized an increase in antioxidants along with the mitigation of mitochondria-related reactive oxygen species elevation and consequential apoptosis occurring during migration.

Methods: Male red-headed buntings (n = 6) were placed under short days (8 h of light and 16 h of dark, 8L:16D)/long days (14L:10D) and photo induced to simulated non-migratory, nMig; pre-migratory, pMig; and migratory, Mig, states. Erythrocyte shape, reactive oxygen species production, mitochondrial membrane potential (MMP), reticulocyte proportion, and apoptosis were analyzed using flow cytometry and relative expression of fat metabolizing and antioxidant genes was measured by using qPCR.

Results: There was a significant increase in hematocrit, erythrocyte area, and mitochondrial membrane potential. Reactive oxygen species and apoptotic erythrocyte proportion declined in the Mig state. The changes in antioxidant genes (SOD1 and NOS2), fatty acid translocase (CD36), and metabolic (FABP3, DGAT2, GOT2, and ATGL) genes showed a significant increment during the Mig state.

Conclusion: These results suggested that adaptive changes occur in mitochondrial behavior and apoptosis of erythrocytes. The transition in erythrocytes, antioxidant genes, and fatty acid metabolism gene expressions suggested differences in regulatory strategies at the cellular/transcriptional level during different states of simulated migration in birds.

KEYWORDS

erythrocytes, apoptosis, reactive oxygen species, mitochondrial potential, migration, photoperiod

Introduction

Physiological revamping in night migratory songbirds is associated with migratory success. These animals fly long distances every autumn and spring, from breeding to wintering grounds and contrariwise. To sustain intense migration exercise, flight muscles must receive unhampered oxygen supply, putting the blood vessels into migratory hyperemia. Birds'

body physiology redirects to the series of tissue-dependent adaptive changes (Handby et al., 2022). Heart enlargement (Trivedi et al., 2014), hematocrit increase (Butler, 2016), the liver hypofunction aiding fat mobilization from adipose tissue (Guglielmo, 2010), altered muscle size dynamics with insulin-like growth factor, and IGF1 rise (Price et al., 2011) are prominent physiological changes. Blood mediates the multitude of supplies such as oxygen and energy metabolites amid heightened hypoxia-reoxygenation physiology (McWilliams et al., 2021).

In blood, erythrocytes are hemoglobin (Hb)-containing cells that exhibit dynamic morphology to aid seasonal physiological changes in birds (Bańbura et al., 2007). The total Hb concentration in erythrocytes is proportional to the oxygen binding capacity. Many avian species exhibit changes in Hb concentration with seasonal and other physiological changes, such as moult, and might relate to birds' ability to fulfill their respective oxygen requirements (Kostelecka-Myrcha, 1997; Minias, 2015). The hematocrit (packed cell volume, PCV) indicates erythrocytes' proportion in blood volume, which may (Butler, 2010) or may not change during avian migration (Fair et al., 2007). Smaller and more elongated erythrocytes during migration facilitated oxygenation/deoxygenation and aerobic metabolism (Soulsbury et al., 2021). Refueling compensated hematocrit lowering in late-arriving bar-tailed godwits, thus correcting in-flight light anemia (Merila and Svenson, 1995; Piersma et al., 1996; Jenni et al., 2006). In zebra finch, erythrocytes get affected due to the enhanced reactive oxygen species (ROS) levels (Stier et al., 2013). In migratory birds, no reports on erythrocyte dynamics and their putative role on alleviating exercise accrued ROS are available.

ROS are short-lived by-products (Halliwell, 2011; Winterbourn, 2015) of high mitochondrial activity, a prerequisite of elevated metabolism (Dmitry et al., 2014). Inside cells, ROS levels above homeostatic balance aggravate apoptosis (Kamata et al., 2005), a well-executed suicidal plan of the cell, induced by high mitochondrial activity. In addition to cellular ROS, nitro-oxidative damage is also indicated by NOS2 (nitric oxide synthase) levels (Bredt and Snyder, 1994). Antioxidants such as superoxide dismutase (SOD1) show the first line of cellular defense to counteract ROS and other oxidants. In mitochondria, ubiquitin-mediated degradation *via* proteolysis regulates energy metabolism (Lavie et al., 2018).

Regular flight during migration stimulates antioxidant protection in addition to fat catabolic pathways. For example, mitochondrial fatty acid oxidation co-occurs with a rise in palmitoylethanolamide, PEA levels, which have anti-inflammatory and cannabinomimetic properties (Gupta et al., 2020). Increased transcriptional activity of genes (fatty acid transporter, CD36; fatty acid binding protein, FABP; glutamic-oxaloacetic transaminase; GOT2, diacylglycerol acyl transferase DGAT2 and adipose triglyceride lipase, ATGL) implicated in fat internalization, transport, and breakdown in the liver and/or muscle has been shown in buntings (Sharma et al., 2021). Furthermore, nucleated avian erythrocytes are capable of exhibiting cellular metabolism (Stier et al., 2013). Relative expression of genes of energy metabolism in avian erythrocytes has never been investigated alongside mitochondrial membrane potential (MMP), ROS, and apoptosis during metabolic stress of migration. It is also possible that birds achieve this feat through increased erythropoiesis.

Migratory buntings (*Emberiza* species) exhibit pre-migratory hyperphagia, an increase in body weight, hormones (for example, thyroxine; please see Jain and Kumar, 1995), trigger of intense night-flight behavior (Gupta and Kumar, 2013), and the metabolic ability to support high aerobic capacity (Gupta et al., 2020). In the present study, we sought to comprehensively outline seasonal changes in blood biochemistry and cytology of red-headed buntings, such as reticulocyte regeneration, hematocrit changes, MMP, ROS, apoptosis, and changes in the concentration of selected genes implicated in the ROS and energy metabolism. The flow cytometry method was used for counting and analyzing size range using optical detection of erythrocytes, enabling inherent analysis of several 10,000 cells within a shorter time in addition to reducing the statistical noise. Specific antibodies included 1) anti-transferrin that binds to surface transferrin receptors of reticulocytes, 2) annexin V for analyzing the apoptotic cells, 3) CM-H2DCFDA stain for ROS, and 4) mitotracker red stains for MMP analysis were used. We hypothesized an increment in antioxidants in response to the ROS elevation, in addition to a change in the mitochondrial functioning during migration. We also predicted an altered ROS and apoptosis, associated elevation in fat metabolizing gene levels, and antioxidant gene in blood, as consequences of migration.

Materials and methods

Birds

The experiment was conducted using male red-headed bunting (*Emberiza bruniceps*), as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEAs) guidelines and duly approved by the Chaudhary Charan Singh University, Institutional Animal Ethics Committee (IAEC Project Codes: IAEC/2020/02 and IAEC/2022/09). Red-headed buntings are night migratory songbirds which, over winter in the Indian subcontinent 28°N, exhibit a non-migratory diurnal state (*nMig*). With increasing day lengths in January to March, they overeat to go through a pre-migratory preparation state (*pMig*) before undertaking spring migratory (*Mig*) night flight to return to their breeding grounds 40°N. To begin with, acclimatized buntings (n = 18) were brought indoors and placed under a short photoperiod of 8 h of light and 16 h of dark (SD, 8L:16D) for 14 days in positive-pressure air-conditioned units (22°C ± 2°C, 50% relative humidity; monitored using Easy Log USB, Lascar electronics Inc. PA, United States) in three groups. Birds of group 1 (n = 6) were singly housed in well-ventilated activity recording cages, installed with passive infra-red sensor connected to the Chronobiology Kit hardware + software program system from Stanford Software Systems, Santa Cruz, CA, United States, that supports collection, plotting, and analyzing each bird's flight behavior. Birds of group 2 and 3 were placed in groups (n = 6, each). Food (foxtail millet, *Setaria italica*, and egg mixture) and water were provided *ad libitum*, without direct animal handling. After 2 weeks of SD, birds of groups 1 and 2 were transferred to long days (LD, 14L:10D), while group 3 was retained under SD, as controls, whose body weight and relative mRNA gene expression level was simultaneously monitored (see Figures 1A, 1A.1–A1.3). The day of LD transfer was treated as day 0. For group 1, activity was continuously monitored and plotted as described earlier (Das and Gupta, 2016; Figures 1C, D). For group 2, body weight and blood sample collection was initiated on days 0, 7, and 28 when birds exhibited simulated non-migratory (*nMig*), pre-migratory (*pMig*), and migratory (*Mig*) annual life history states. About 25–50 µL of blood was drawn from the left brachial wing vein (see Gupta et al., 2020 for more details) and

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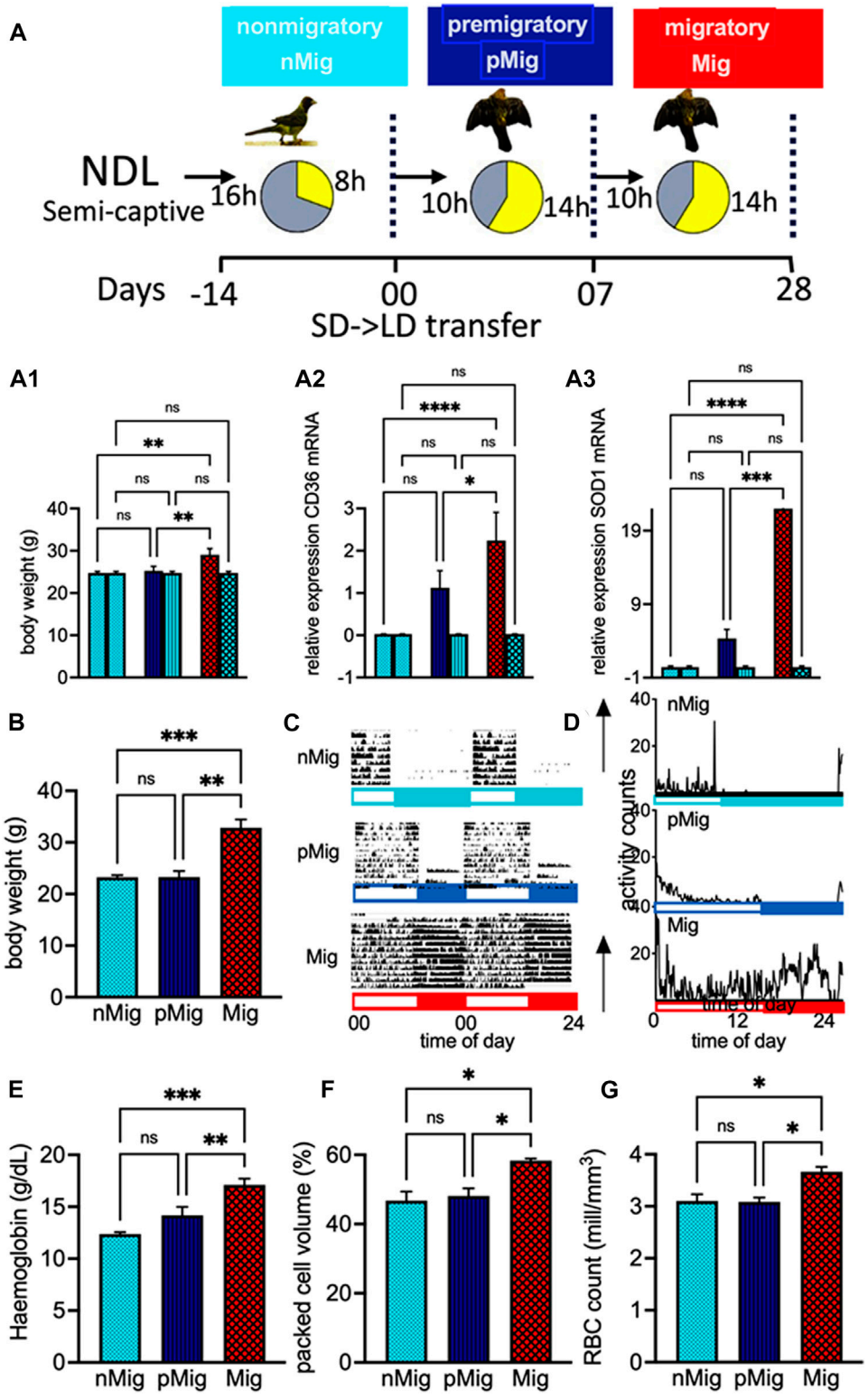

FIGURE 1
(A) Experimental design. Male red-headed buntings, *Emberiza bruniceps*, either in activity cages (n = 6) or in two groups (n = 6, each) were brought indoors from natural daylength, NDL, conditions during February and placed under a short photoperiod of 8 h of light and 16 h of dark (SD, 8L:16D) for 14 days. Birds in six activity cages and one group were transferred to long days (LD, 14L:10D) on day 0. **(A.1–A.3)** Comparison of body weight (g) and mRNA expression of CD36 and SOD1 of groups 2 and 3, the latter continuously held under SD. Buntings exhibited non-migratory (*nMig*, cyan blue), pre-migratory (*pMig*, blue), and migratory (*Mig*, red) annual life history states, on days 0, 7, and 28 of blood sampling. **(B)** Mean (±SEM) of body weight of *nMig*, *pMig*, and *Mig* buntings. **(C)** Double-plotted actogram representative of *nMig*, *pMig*, and *Mig* buntings. **(D)** Mean activity profile of buntings (n = 6), in Figure 1A. Open and closed bars on the X-axis show light and dark phases, respectively. **(E)** Mean (±SEM) of hemoglobin (g/dL), **(F)** mean (±SEM) of packed cell volume (%), and **(G)** mean (±SEM) of red blood cells in blood (millions/millimetre^3). Data are represented as mean ± SEM. Asterisk (*) over the line on the bar indicates a significant difference between *nMig*, *pMig*, and *Mig* states of buntings (*p < 0.05, Tukey's post-test).

suspended in tubes with equal amounts of PBS (phosphate buffer saline) containing 5 mM EDTA. Nearly 32–36 h after the first blood collection, in group 2, 50 µL of blood was drawn from the right brachial wing vein for hematocrit analysis.

Chemicals and other supplies

The anti CD71-PE, Annexin V-FITC, was purchased from Biolegend (San Diego, CA, United States). 5 (and 6) chloromethyl-2, 7-dichloro-dihydro-fluorescein diacetate (CM-H2DCFDA) and MitoTracker red (MTR) stain were procured from Molecular Probes, and Invitrogen (Eugene, OR, United States). All primers were commercially synthesized from Eurofins. Fetal bovine serum (FBS) was from Hyclone (South Logan, UT). For gene assay, PowerUp SYBR green, PCR Master Mix (Applied Biosystem, CA, United States), and RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific™) were used. Analytical reagents such as RPMI and HEPES reagent were procured from Sigma-Aldrich (India). TRIzol reagent was procured from Ambion. All other chemicals were of analytical grade.

Hematological analysis

Hemoglobin, PCV, and RBC (red blood cells) counts were assessed using standard procedures of spectrophotometry and calibration on Siemens ADVIA 2120 hematological analyzer and Sedgewick Rafter cells in a hemocytometer, respectively.

Flow cytometric analysis

Estimation of cell size, intracellular ROS, and mitochondrial membrane potential

Blood was collected in PBS containing 5 mM EDTA and washed three times with ice-cold saline containing HEPES buffer (10 mM, pH-7.4) and 1% FBS. Erythrocyte area and width were measured by analyzing forward scattering A (FSC-A) and width (FSC-W) using flow cytometry. ROS levels were analyzed by staining the erythrocytes with the CM-H2DCFDA stain. In brief, erythrocytes were suspended in pre-warmed PBS + 2% FBS and incubated with CM-H2DCFDA stain (5 µM). The fluorescent product of CM-H2DCFDA was analyzed immediately by flow cytometry (Bhardwaj and Saxena, 2014; 2015; Goodchild and DuRant, 2020; Rajaura et al., 2022). MMP was measured by staining with 200 nM Mitotracker Red (MTR), followed by flow cytometric analysis (Montgomery et al., 2012; Bhardwaj and Singh, 2018).

Analysis of reticulocytes and apoptotic cells

Reticulocyte proportion was estimated by staining the cells with anti-transferrin (CD71) monoclonal antibodies, followed by flow cytometric analysis (Schmidt et al., 1986; Bhardwaj and Saxena, 2013). For apoptotic cell analysis, erythrocytes were resuspended in PBS containing 2.5 mM calcium chloride and stained with Annexin V-FITC antibody for 20 min at room temperature (Graham et al., 2015; Bhardwaj et al., 2022). Cells were washed and resuspended in PBS with 2.5 mM calcium chloride ($CaCl_2$). After incubation (in the dark), cells were washed and analyzed using a FACSVerse flow cytometer and analyzed using Facsuite software. A minimum of 10,000 events (an event is the count of one cell) were analyzed using flow cytometry.

Relative expression of metabolic genes in blood

RNA isolation and preparation of cDNA

Total RNA was extracted from blood using TRIzol reagent (Ambion) as per the manufacturer's protocol. According to the TRIzol chloroform method, about 1×10^6 erythrocyte cells were used for RNA isolation. The aqueous state containing RNA was separated and then precipitated by isopropyl alcohol. The RNA pellet obtained at the bottom of the centrifuge tube was washed twice with 75% ethanol, followed by air drying at room temperature. Nuclease-free water was used for suspending the RNA pellet and its purity was estimated by studying the absorbance of the obtained samples at 260/280 and 260/230 nm wavelengths in a Nanodrop spectrophotometer. The integrity of the isolated RNA was checked by running 5 µg of RNA on 1.2% formaldehyde agarose gel. After assessing its purity and integrity, RNA was further used for cDNA synthesis. For RT-PCR, cDNA was synthesized by using 1 µg of RNA per reaction mixture. A total of 1 µg RNA was treated with RNase-free DNase (Promega M6101) and reverse transcribed to synthesize cDNA using a cDNA synthesis kit (Thermo Scientific, K1622). cDNA integrity was verified using beta actin amplification followed by agarose gel electrophoresis; a sharp band of 750bp confirmed cDNA integrity.

Specific gene expression using qPCR

The amplification of cDNA was carried out by RT-qPCR. A total of 7 genes related with the fatty acid metabolism (ATGL, DGAT2, and GOT2), transporter (CD36 and FABP3), and ROS metabolism (SOD1 and NOS2) were investigated in the present study. mRNA levels were measured by quantitative PCR (RT-qPCR) using Applied Biosystems QuantStudio3 and SYBR green chemistry as described earlier (Singh et al., 2013; Trivedi et al., 2014). Briefly, a total reaction volume of 10 µL contained 1 µL each of cDNA (10 ng/µL) and forward and reverse primers (100 nm), 5 µL of PowerUp SYBR Green master mix, and 2 µL nuclease free water. β-actin mRNA expressions served as reference control to calculate and present relative mRNA expression levels ($2^{-\Delta\Delta CT}$, Livak & Schmittgen, 2001).

Statistical analysis

Statistical data analysis was performed using GraphPad Prism software (Version 9). Data are shown as mean ± SEM (standard error of mean). Buntings are small birds, so values not detected due to less blood quantity were not included in analysis. Statistical significance was determined by two-way repeated measure ANOVA, followed by Tukey's post-analysis, for state-wise comparison or comparing *pMig* and *Mig* with *nMig*, respectively. A $p < 0.05$ was considered significant.

Results

All birds exhibited day activity, i.e., diurnal behavior, when placed under SD.

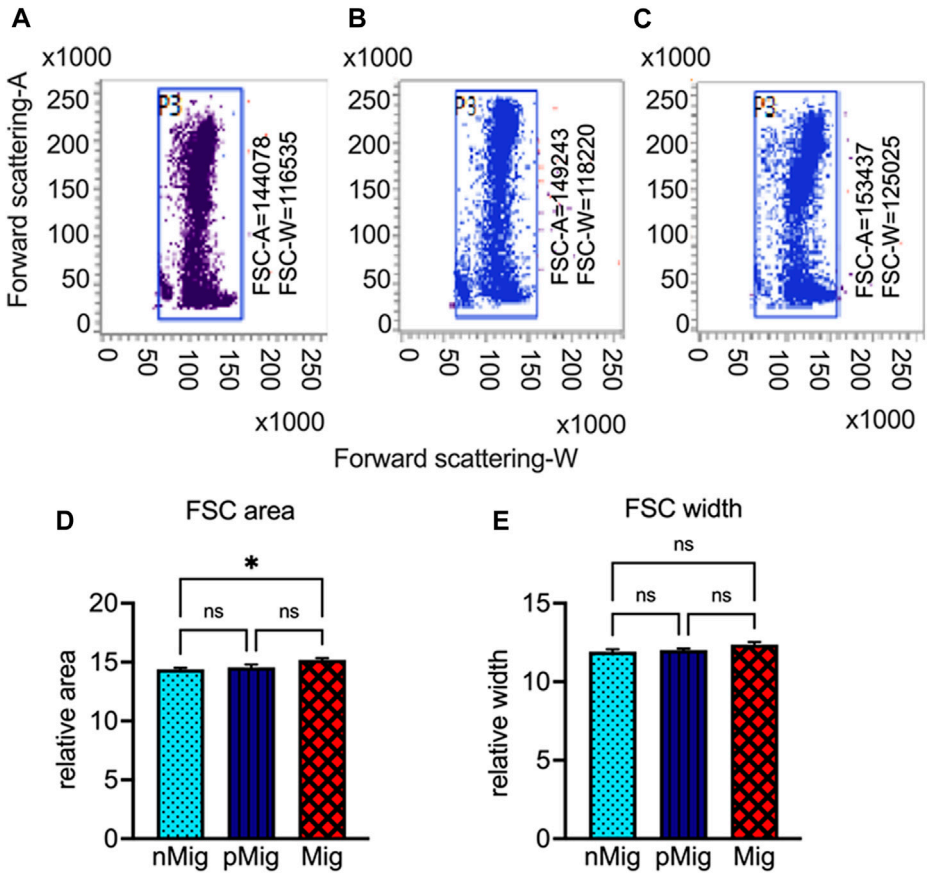

FIGURE 2
Changes in erythrocyte area and width during migration. Blood was collected from non-migratory (*nMig*, cyan blue), pre-migratory (*pMig*, blue), and migratory (*Mig*, red) red-headed buntings (n = 6) placed under SD/LD for 0, 7, and 28 days, and the mean area (forward scattering A) and width (forward scattering W) of erythrocytes were measured using flow cytometry. The dot-blots in Panel **(A–C)** show the FSC-A/FSC-W in non-migratory (*nMig*, A), pre-migratory (*pMig*, B), and migratory (*Mig*, C) states, respectively. The bar graphs in panels **(D,E)** depict the cumulative changes in erythrocyte area and width, respectively. The values in dot-plots indicate the mean FSC-A and FSC-W at different states. Data in **(D,E)** are represented as mean ± SEM. Asterisk (*) over the line on the bar indicates a significant difference between *nMig*, *pMig*, and *Mig* states of buntings (*p < 0.05, Tukey's post-test).

Behavioral and physiological differences between *nMig*, *pMig*, and *Mig* buntings

Diurnal behavior continued for up to 15 days after transfer to LD in buntings, following which birds exhibited a significant increase in body weight (two-way repeated measure ANOVA, followed by Tukey's post-hoc test revealed a difference, i.e., $F_{(2,\ 10)} = 18.28$; $p < 0.001$) (Figure 1B) and intense night activity (replication of *Zugunruhe* migratory activity in wild) (Figures 1C, D).

Erythrocyte area increases during simulated migration

A significant increase in hematocrit, i.e., hemoglobin ($F_{(2,\ 10)} = 23.23$; $p < 0.0005$, Figure 1E), packed cell volume ($F_{(2,\ 10)} = 7.003$; $p < 0.05$, Figure 1F), and RBC count ($F_{(2,\ 10)} = 7.064$; $p < 0.05$, Figure 1G), was observed during simulated migration. Also, the flow cytometric data showed enhanced erythrocyte area, but not width (Figures 2A–E), i.e., the mean erythrocyte area was 144,078 in *nMig* (Figures 2A, D), which increased to 149,243 in *pMig* (Figures 2B, D), with further enhancing to 153,437 during *Mig* (Figures 2C, D), showing 5.5% increase in the erythrocyte area. The erythrocyte width did not increase with change from *nMig* to *Mig* (Figure 2E).

The reticulocyte production and reactive oxygen species level increased during simulated migration

The simulated migration in bunting causes the increased production of reticulocytes in the blood. The proportions of reticulocytes ($CD71^{+ve}$) were 1.92% in *nMig* birds, which increased to 3.08% in the *pMig* state (Figures 3A, B). As the bird switches from *pMig* to *Mig* state, the reticulocyte production further increased more than two-fold as compared to the *pMig* state (Figures 3B, C). The cumulative data show a 4.8-fold enrichment of reticulocytes in

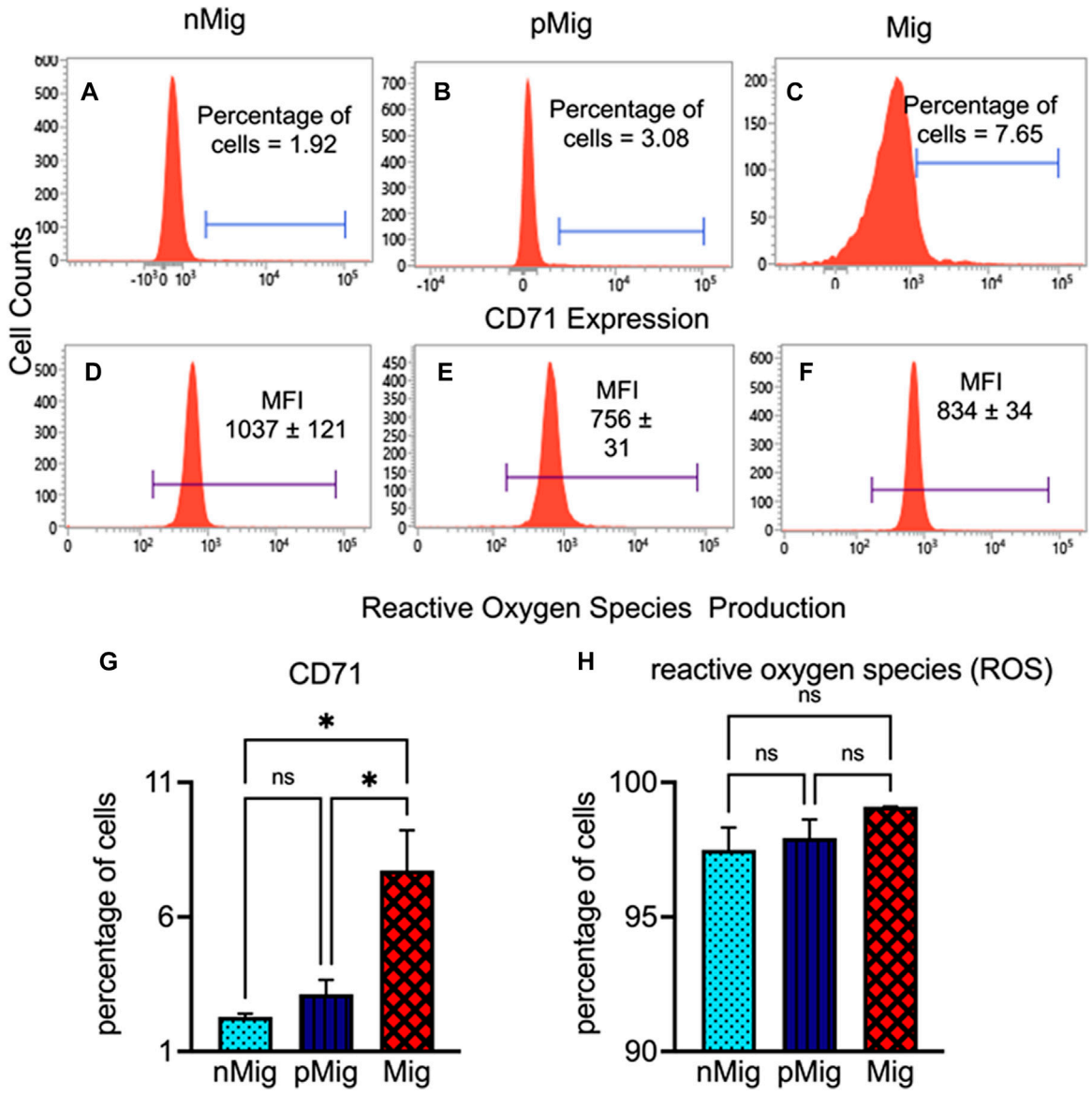

FIGURE 3
Modulation of reticulocyte production and ROS accumulation in peripheral blood circulation. The blood was isolated from non-migratory (*nMig*, cyan blue), pre-migratory (*pMig*, blue), and migratory (*Mig*, red) red-headed buntings (n = 6) placed under SD/LD for 0, 7, and 28 days. Newly formed reticulocytes were estimated by staining with anti-CD71-PE monoclonal antibody, and ROS accumulation was measured by staining with the CM-H2DCFDA stain. The flow cytometric histograms in Panel **(A–C)** show the reticulocyte proportion in *nMig*, *pMig* and *Mig* states, respectively. The ROS level in corresponding states has been depicted in panels **(D–F)**. The bar graph in panels **(G,H)** shows the cumulative alteration in the reticulocyte and ROS production. The horizontal blue line in panel **(A–C)** represents CD71-positive cells. In panels **(D–F)**, the blue lines correspond to the mean fluorescence intensity (MFI) of CM-H2DCFA stain. Data are represented as mean ± SEM. Asterisk (*) over the line on the bar indicates a significant difference between *nMig*, *pMig*, and *Mig* states of buntings (*p < 0.05, Tukey's post-test).

between *nMig* and *Mig* transition (Figure 3G) (two-way repeated measure ANOVA, followed by Tukey's post-hoc test revealed significant difference, i.e., $F_{(2, 8)} = 8.47$; $p < 0.05$).

In comparison to reticulocyte production, the ROS-positive cell percentages did not change significantly between *nMig*, *pMig*, and *Mig* (Figure 3H). However, the mean fluorescence intensity (MFI) showed a significant decline in the ROS production from *nMig* to *pMig* state transition. The ROS level was maximum in *nMig* groups, which decreased in *pMig* and *Mig* states. The flow cytometric histogram represents the MFI of ROS production in *nMig* was 1,037, which declined to 756 and 834 in the *pMig* and *Mig* groups, respectively (Figures 3D–F).

MMP and apoptosis in erythrocytes during simulated migration

MMP decreased as the bird switched from *nMig* to *pMig* and *Mig* states (Figures 4A–C). Histograms in Figure 4 show that the MFI of Mitotracker stain in *nMig* erythrocytes was 379, which declined to 261 in

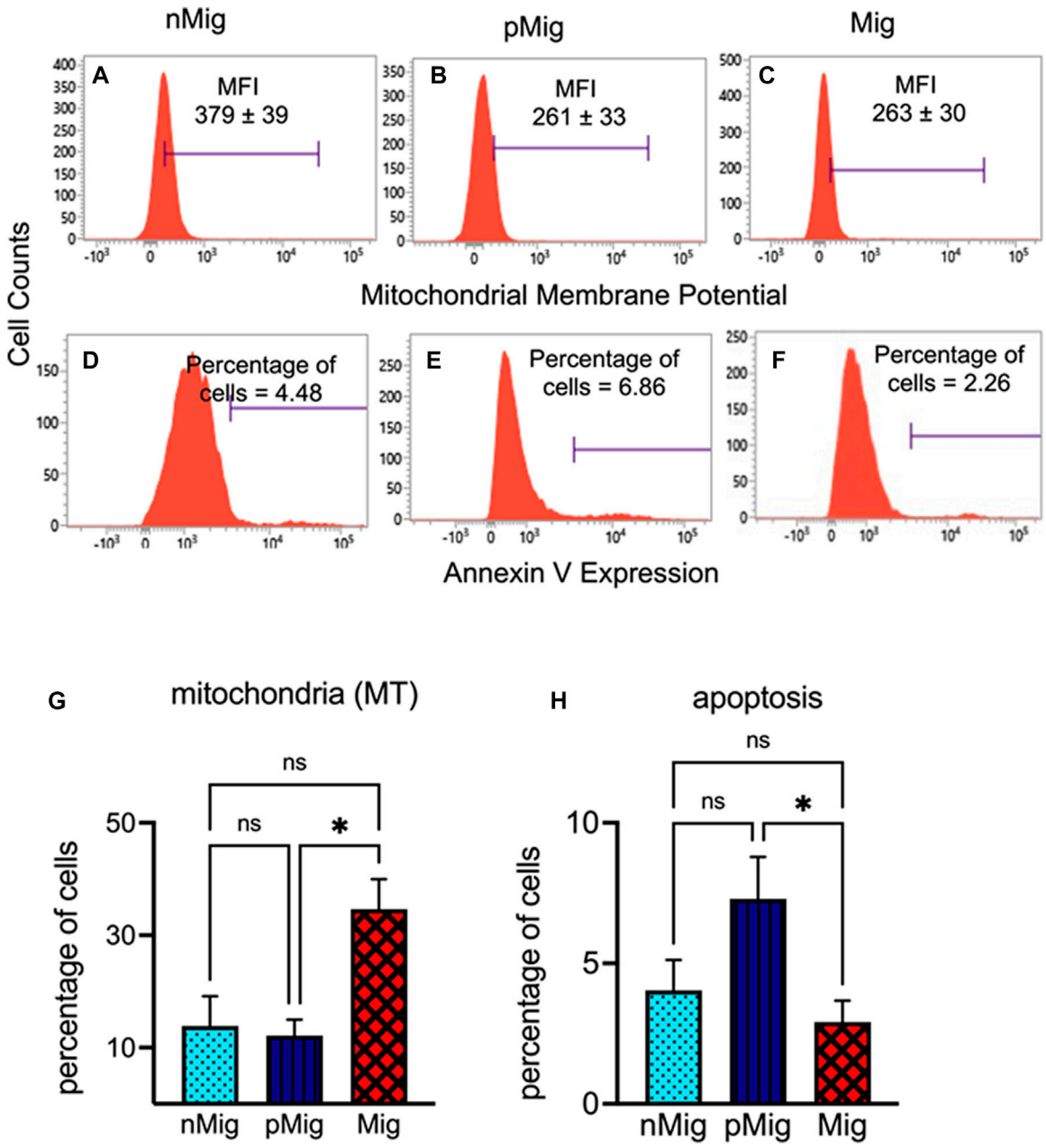

FIGURE 4
Alteration in the mitochondrial membrane potential (MMP) and apoptosis in erythrocytes in circulation. The blood was collected from non-migratory (*nMig*, cyan blue), pre-migratory (*pMig*, blue), and migratory (*Mig*, red) red-headed buntings (n = 6) placed under SD/LD for 0, 7, and 28 days. MMP erythrocytes were determined by staining with MitoTracker red dye. Apoptotic cells were quantified using Annexin V staining followed by flow cytometric analysis. Flow cytometric histograms in panels **(A–C)** show the MMP in *nMig*, *pMig*, and *Mig* states, respectively. The proportion of apoptotic cells in corresponding states has been depicted in panels **(D–F)**. The bar graph in panels **(G,H)** shows the cumulative changes in mitochondrial membrane potential and apoptotic cell proportion. The horizontal blue line in panel **(A–C)** represents the mean fluorescence intensity (MFI) of mitotracker stain. In panels **(D–F)**, it corresponds to the percentages of Annexin V-positive cells. Data are represented as mean ± SEM. Asterisk (*) over the line on the bar indicates a significant difference between *nMig*, *pMig*, and *Mig* states of buntings (*p < 0.05, Tukey's post-test).

the *pMig* state. Furthermore, as birds switched to simulated migration, the MFI level was almost similar to the *pMig* state. However, a significant increase in Mitotracker (MT)-positive cells proportion was seen during the *pMig* to *Mig* state transition but not in *nMig* to *pMig* transition (Figure 4G) (two-way repeated measure ANOVA, followed by Tukey's post-hoc test revealed significant difference, i.e., $F_{(2, 8)} = 5.62$; $p < 0.05$, between *nMig, pMig*, and *Mig*).

Changes in ROS and MMP may lead to the apoptosis of cells in the circulation. We calculated the apoptosis by staining the cells with Annexin V. The proportion of apoptotic cells reduced during the *Mig* state was 6.86%–2.26%) (Figures 4D–H). Two-way repeated measure ANOVA followed by Tukey's post-hoc test revealed significant ($F_{(2, 10)} = 6.06$; $p <$ 0.05) difference between *nMig*, *pMig*, and *Mig* states. There was 62% decline in apoptotic cell % in between *pMig* and *Mig* states.

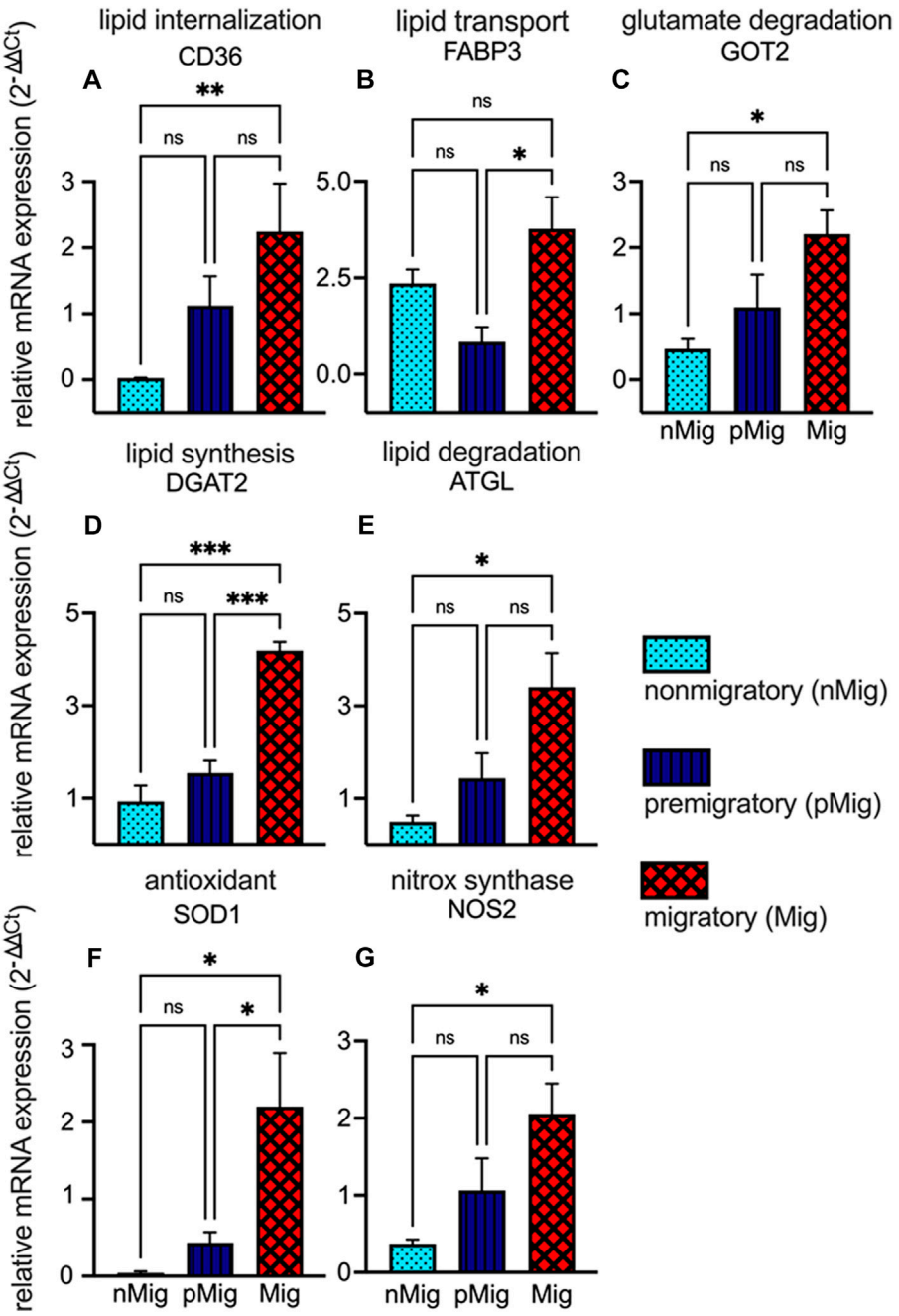

FIGURE 5
Changes in the relative expression of fatty acid transporters and antioxidant genes. The blood was collected from non-migratory (*nMig*, cyan blue), pre-migratory (*pMig*, blue), and migratory (*Mig*, red) red-headed buntings (n = 6) placed under SD/LD for 0, 7, and 28 days. RNA was isolated from 8L:16D/14L:10D exposed birds erythrocytes using TRIzol (TRI) reagents. The first strand of cDNA for isolated RNA was synthesized by using the First Strand cDNA Synthesis kit. The cDNA obtained was amplified using qRT-PCR. The RNA transcripts for different genes were quantified by SYBR Green method. Relative expression levels of fatty acid transporter genes CD36, FABP3, ATGL, DGAT2, and GOT2 have been shown in panels **(A–E)**. The expression levels of antioxidant genes SOD1 and NOS2 are shown in panels **(F,G)**. Expression levels of mRNA were normalized to β-actin levels using the $2^{-\Delta\Delta CT}$ method. Data are represented as mean ± SEM. Asterisk (*) over the line on the bar indicates a significant difference between *nMig*, *pMig*, and *Mig* states of buntings (*$p < 0.05$, Tukey's post-test).

Changes in the relative expression of metabolic genes in blood

The expression levels of antioxidant enzymes superoxide dismutase (SOD1), nitric oxide synthase (NOS2), fatty acid transporter (CD36), fatty acid binding protein (FABP3), adipose triglyceride lipase (ATGL), diacylglycerol acyl transferase (DGAT2), and glutamic-oxaloacetic transaminase (GOT2) were studied in different states. The mRNA expression levels of CD36 significantly increased ($F_{(2,\ 10)}$ = 9.06; $p < 0.005$, two-way repeated measure ANOVA, followed by Tukey's post-hoc test) from *nMig* to *Mig* states (Figure 5A). FABP3 mRNA expression was highest in the *Mig* state. Two-way repeated measure ANOVA followed by Tukey's post-hoc test revealed a significant ($F_{(2,6)}$ = 6.92; $p < 0.05$)

difference between *pMig* and *Mig* states with a 4.4-fold increase in the latter state (Figure 5B). GOT2 mRNA expression increased from *nMig* to *Mig* states. Two-way repeated measure ANOVA followed by Tukey's post-hoc test revealed significant ($F_{(2,7)} = 8.98$; $p < 0.05$) difference in GOT2 expression levels between *nMig* and *Mig* states with a 4.7-fold increase in the latter state (Figure 5C). DGAT2 mRNA expression increased from *nMig* to *Mig* states. Two-way repeated measure ANOVA followed by Tukey's post-Hoc test revealed a significant ($F_{(2,4)} = 118.4$; $p < 0.0005$) difference between *nMig* and *Mig* states with 4.4-fold increase in the latter state (Figure 5D). ATGL mRNA expression increased from *nMig* to *Mig* state. Two-way repeated measure ANOVA followed by Tukey's post-hoc test revealed a significant ($F_{(2,\ 7)} = 5.306$; $p < 0.05$) difference between *nMig* and *Mig* states with a 6.5-fold increase in the latter state (Figure 5E). SOD1 mRNA expression shows increase from *nMig* to *Mig* states. Two-way repeated measure ANOVA followed by Tukey's post-hoc test revealed significant ($F_{(2,\ 8)} = 7.668$; $p < 0.05$) change in SOD1 expression levels between *nMig* and *Mig* states with 31-fold change (Figure 5F). Changes in NOS2 expression were similar to those of SOD1. It was least in the *nMig* state and increased during transition to the *Mig* state. Two-way repeated measure ANOVA followed by Tukey's post-hoc test revealed significant difference between *nMig* and *Mig* states ($F_{(2,\ 5)} = 7.751$; $p < 0.05$) with a 5.5-fold change (Figure 5G).

Discussion

Erythrocytes are the major transporter of O_2 from the heart to various organs. They play a vital role in energy metabolism (Malkoc et al., 2021) in migratory birds. Migration is an energetically costly behavior that drives need-based erythrocyte modulation to deliver oxygen (Soulsbury et al., 2021). Molecular underpinnings of erythrocyte-associated cellular dynamics were assessed in night migratory buntings during different physiological states of spring migration, wherein physiological and behavioral changes of body weight and daily activity profiles conform to those reported in earlier studies.

Hematocrit and forward scattering area (FSC-A) and width (FSC-W) of erythrocytes in *nMig*, *pMig*, and *Mig* states corresponded to the energy requirements such that a significant increase in area during transition from *nMig* to *Mig* states occurred. Reasonably, increased cell area can accommodate more hemoglobin to enrich oxygen and CO_2 transport. Soulsbury et al. (2021) reviewed 631 bird species' data to conclude elongated erythrocytes during migration. Our estimation of $CD71^{+Ve}$ erythrocytes (reticulocytes) in different states of simulated migration highlights naive erythrocytes (reticulocytes) that freshly enter the circulation. The reticulocytes increased significantly from *nMig* to *Mig* states. The enhanced reticulocyte production might be a compensatory response to the metabolic demand and attrition due to oxidative stress (Jenni-Eiermann et al., 2014). We speculated that by the time buntings enter full-fledged migration, they adapt physiologically to recover from the erythrocyte deficiency through erythrocyte regeneration.

The relative level of ROS production in erythrocytes in *nMig*, *pMig*, and *Mig* states of simulated migration, despite extreme oxidative stress, did not vary as hypothesized. Our data suggest that though the ROS-positive cell percentages exhibited minor variations, the MFI of ROS significantly reduced in *pMig* and *Mig* states, compared to the *nMig*. In mammals, the erythrocyte production switches from bone marrow to spleen, facilitating resistance to oxidative stress (Bhardwaj and Saxena, 2014; 2015). Herein, a low ROS level in erythrocyte could be a strategy to combat oxidative stress during simulated migration, but this needs substantiation through further investigation.

Mitochondria are dually engaged in energy budgeting and ROS production. The proportion of MT-positive cells percentage increased significantly from *pMig* to *Mig* states. Increased energy demand justifies the increase in the percentage of MT cells. The MFI of MT, just like ROS MFI, declined in *pMig* birds, remaining almost similar until *Mig* transition. Erythrocyte ROS MFI aligned with MT MFI with simultaneous overexpression of SOD1 mRNA levels during migration (Dimayuga et al., 2007), suggesting intracellular redox signaling modulated the mitochondrial membrane potential.

The decline in MMP is also associated with increased mortality (Jeong and Seol, 2008; Wang and Youle, 2009). Studies relating mitochondrial activity to apoptosis in erythrocytes are limited to heat stress investigations in chickens, wherein higher temperatures caused morphological alterations and increased the activity of pro-apoptotic caspases in erythrocytes (Szabelak et al., 2021). Our investigation of erythrocyte apoptosis using Annexin V-positive cell percentages suggested altered apoptosis until migratory preparation, which stabilized during simulated migratory state, again suggesting birds' ability to maintain erythrocytes survival at the basal level.

Furthermore, we have also analyzed the molecular changes in ROS-related and fatty acid gene expression in various states. Erythrocytes contain a pool of antioxidant enzymes that scavenge free radicals. We observed a rise in erythrocyte SOD1 mRNA levels during simulated migration as also suggested in hypothalamus of buntings during migration (Sharma et al., 2021). Nitric oxide synthase (NOS2) is a ROS indicator. Nitric oxide (NO) is a small free radical with critical signaling roles in regulation of healthy vasculature performance, which, in humans, is supplemented by nitrite reduction pathways under hypoxia (Tejero et al., 2019). These suggestions improved the ability of bird vasculature to combat exercise stress. In birds also, NOS2 increase indicates increased proteolysis, which might be related to increased energy demands.

The expression of fat metabolism-related genes was also modulated in erythrocytes. The integral membrane glycoprotein CD36 is a fatty acid translocase (FAT), which plays an important role in the transportation of fatty acids (FAs) for energy production (Ibrahimi et al., 1999; Ibrahimi and Abumrad, 2002). CD36 mRNA expression increased from *nMig* to *Mig*. The increase in CD36 expression in erythrocytes might be due to energy accumulation, carrying fat droplets to adipose tissue (Araújo et al., 2022) as *Mig* preparation, which results from continuous pre-migratory hyperphagia (Jain and Kumar, 1995).

The fatty acid binding protein (FABP3) exhibits tissue-specific aspects of lipid and fatty acid metabolism. FABP3 is a fatty acid carrier in blood, whose mRNA expression began to alter during *pMig* but enhanced significantly from *pMig* to *Mig* states. The upregulation of FABP3 expression is correlated with the increased utilization of FA during migration (Gugleilmo, 2010; Gupta et al., 2020). The mRNA expression of ATGL, DGAT2, and GOT2 also increased from *nMig* to *Mig* transition with much overexpression during *Mig*. The increased ATGL expression indicated enhanced turnover of fatty acids. GOT2, a pyridoxal phosphate-dependent enzyme participating in the malate aspartate shuttle of electron transport chain, indicated more aerobic metabolism to produce energy for migration. The increased DGAT2 expression indicated

promotion of lipid internalization. To sum up, an increase in erythrocyte area, increased reticulocyte production, and adaptive changes in ROS and MMP occurred in erythrocytes during simulated migration. To support the metabolic efficiency of erythrocytes in avian migrants, the molecular data suggested increased expression of antioxidant and fat metabolizing genes *via nMig* to *Mig* transition.

Conclusion: taken together, nucleated and mitochondria-containing erythrocytes of red-headed buntings exhibit metabolic ability for enhanced energy metabolism and support ROS and apoptosis reduction by maintaining the 1) erythrocyte threshold through erythropoiesis, 2) adaptively reducing ROS consequences, and 3) modulating antioxidant function to support mitochondrial hyperactivity. This is the first study involving the study of erythrocyte ROS dynamics alongside molecular underpinnings of metabolic regulation in the blood of a long-distance obligate avian migrant.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Chaudhary Charan Singh University's Institutional Animal Ethical Committee (IAEC Approved Project Code: IAEC/2020/02 and IAEC/2022/09).

Author contributions

Conceived and designed the experiments: NG and NB. Performed the experiments: AK and NB. Analyzed the data: NG, NB, and AK. Contributed reagents/material/analysis tools: NG and NB. Contributed to the writing of manuscript: NG, NB, and AK.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2023.1111490/full#supplementary-material

References

Araújo, P. M., Viegas, I., Da Silva, L. P., Lopes, P. B., Tavares, L. C., and Ramos, J. A. (2022). Blood metabolites and profiling stored adipose tissue reveal the differential migratory strategies of eurasian reed and sedge warblers. *Birds* 3 (4), 359–373. doi:10.3390/birds3040024

Bańbura, J., Bańbura, M., Kaliński, A., Skwarska, J., Słomczyński, R., Wawrzyniak, J., et al. (2007). Habitat and year-to-year variation in haemoglobin concentration in nestling blue tits Cyanistes caeruleus. *Comp. Biochem. Physiology Part A Mol. Integr. Physiology* 148 (3), 572–577. doi:10.1016/j.cbpa.2007.07.008

Bhardwaj, N., and Saxena, R. K. (2014). Elimination of young erythrocytes from blood circulation and altered erythropoietic patterns during paraquat induced anemic phase in mice. *PLoS One* 9 (6), e99364. doi:10.1371/journal.pone.0099364

Bhardwaj, N., and Saxena, R. K. (2013). Heterogeneity of reticulocyte population in mouse peripheral blood. *Curr. Sci.* 105, 1611–1614.

Bhardwaj, N., and Saxena, R. K. (2015). Selective loss of younger erythrocytes from blood circulation and changes in erythropoietic patterns in bone marrow and spleen in mouse anemia induced by poly-dispersed single-walled carbon nanotubes. *Nanotoxicology* 9 (8), 1032–1040. doi:10.3109/17435390.2014.998307

Bhardwaj, N., Singh, A., Chandra, H., and Gupta, K. K. (2022). Paraquat treatment modulated the stress erythropoiesis response in bone marrow and liver of the splenectomized mice. *Chem. Biol. Lett.* 9 (2), 306.

Bhardwaj, N., and Singh, A. (2018). Paraquat treatment modulates integrin associated protein (CD47) and basigin (CD147) expression and mitochondrial potential on erythroid cells in mice. *Environ. Toxicol. Pharmacol.* 58, 37–44. doi:10.1016/j.etap.2017.12.007

Bredt, D. S., and Snyder, S. H. (1994). Nitric oxide: A physiologic messenger molecule. *Annu. Rev. Biochem.* 63, 175–195. doi:10.1146/annurev.bi.63.070194.001135

Butler, P. J. (2010). High fliers: The physiology of bar-headed geese. *Comp. Biochem. Physiology Part A Mol. Integr. Physiology* 156 (3), 325–329. doi:10.1016/j.cbpa.2010.01.016

Butler, P. J. (2016). The physiological basis of bird flight. *Philosophical Trans. R. Soc. B Biol. Sci.* 371 (1704), 20150384. doi:10.1098/rstb.2015.0384

Das, S., and Gupta, N. J. (2016). Seasonal modulation of diurnal food consumption in Indian songbirds. *Biol. Rhythm Res.* 47 (4), 621–629. doi:10.1080/09291016.2016.1178415

Dimayuga, F. O., Wang, C., Clark, J. M., Dimayuga, E. R., Dimayuga, V. M., and Bruce-Keller, A. J. (2007). SOD1 overexpression alters ROS production and reduces neurotoxic inflammatory signaling in microglial cells. *J. Neuroimmunol.* 182 (1-2), 89–99. doi:10.1016/j.jneuroim.2006.10.003

Dmitry, B., Juhaszova, M., and Sollot, S. (2014). Mitochondrial ROS-induced ROS release: An update and review. *Physiol. Rev.* 94, 909–950.

Fair, J., Whitaker, S., and Pearson, B. (2007). Sources of variation in haematocrit in birds. *Ibis* 149 (3), 535–552. doi:10.1111/j.1474-919x.2007.00680.x

Goodchild, C. G., and DuRant, S. E. (2020). Fluorescent heme degradation products are biomarkers of oxidative stress and linked to impaired membrane integrity in avian red blood cells. *Physiological Biochem. Zoology* 93 (2), 129–139. doi:10.1086/707920

Graham, J. E., Meola, D. M., Kini, N. R., and Hoffman, A. M. (2015). Comparison of the effects of glycerol, dimethyl sulfoxide, and hydroxyethyl starch solutions for cryopreservation of avian red blood cells. *Am. J. Veterinary Res.* 76 (6), 487–493. doi:10.2460/ajvr.76.6.487

Guglielmo, C. G. (2010). Move that fatty acid: Fuel selection and transport in migratory birds and bats. *Integr. Comp. Biol.* 50, 336. doi:10.1093/icb/icq097

Gupta, N. J., and Kumar, V. (2013). Testes play a role in termination but not in initiation of the spring migration in the night-migratory blackheaded bunting. *Anim. Biol. Leiden. Neth.* 63 (3), 321–329. doi:10.1163/15707563-00002415

Gupta, N. J., Nanda, R. K., Das, S., Das, M. K., and Arya, R. (2020). Night migratory songbirds exhibit metabolic ability to support high aerobic capacity during migration. *ACS omega* 5 (43), 28088–28095. doi:10.1021/acsomega.0c03691

Halliwell, B. (2011). Free radicals and antioxidants - quo vadis? *Trends Pharmacol. Sci.* 32, 125. doi:10.1016/j.tips.2010.12.002

Handby, T., Slezacek, J., Lupi, S., Colhoun, K., Harrison, X. A., and Bearhop, S. (2022). Changes in behaviour and proxies of physiology suggest individual variation in the building of migratory phenotypes in preparation for long distance flights. *Front. Ecol. Evol.* 10, 60. doi:10.3389/fevo.2022.749534

Ibrahimi, A., and Abumrad, N. A. (2002). Role of CD36 in membrane transport of long-chain fatty acids. *Curr. Opin. Clin. Nutr. Metabolic Care* 5 (2), 139–145. doi:10.1097/00075197-200203000-00004

Ibrahimi, A., Bonen, A., Blinn, W. D., Hajri, T., Li, X., Zhong, K., et al. (1999). Muscle-specific overexpression of FAT/CD36 enhances fatty acid oxidation by contracting muscle, reduces plasma triglycerides and fatty acids, and increases plasma glucose and insulin. *J. Biol. Chem.* 274 (38), 26761–26766. doi:10.1074/jbc.274.38.26761

Jain, N., and Kumar, V. (1995). Changes in food intake, body weight, gonads and plasma concentrations of thyroxine, luteinizing hormone and testosterone in captive male buntings exposed to natural daylengths at 29 N. *J. Biosci.* 20 (3), 417–426. doi:10.1007/bf02703845

Jenni, L., Müller, S., Spina, F., Kvist, A., and Lindström, Å. (2006). Effect of endurance flight on haematocrit in migrating birds. *J. Ornithol.* 147 (4), 531–542. doi:10.1007/s10336-006-0076-2

Jenni-Eiermann, S., Jenni, L., Smith, S., and Costantini, D. (2014). Oxidative stress in endurance flight: An unconsidered factor in bird migration. *Plos one* 9 (5), e97650. doi:10.1371/journal.pone.0097650

Jeong, S. Y., and Seol, D. W. (2008). The role of mitochondria in apoptosis. *BMB Rep.* 41, 11–22. doi:10.5483/bmbrep.2008.41.1.011

Kamata, H., Honda, S. I., Maeda, S., Chang, L., Hirata, H., and Karin, M. (2005). Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell* 120 (5), 649–661. doi:10.1016/j.cell.2004.12.041

Kostelecka-Myrcha, A. (1997). The ratio of amount of haemoglobin to total surface area of erythrocytes in birds in relation to body mass, age of nestlings, and season of the year. *Physiol. Zool.* 70 (3), 278–282. doi:10.1086/639597

Lavie, J., De Belvalet, H., Sonon, S., Ion, A. M., Dumon, E., Melser, S., et al. (2018). Ubiquitin-dependent degradation of mitochondrial proteins regulates energy metabolism. *Cell Rep.* 23 (10), 2852–2863. doi:10.1016/j.celrep.2018.05.013

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25 (4), 402–408. doi:10.1006/meth.2001.1262

Malkoc, K., Casagrande, S., and Hau, M. (2021). Inferring whole-organism metabolic rate from red blood cells in birds. *Front. Physiology* 12, 691633. doi:10.3389/fphys.2021.691633

McWilliams, S., Carter, W., Cooper-Mullin, C., DeMoranville, K., Frawley, A., Pierce, B., et al. (2021). How birds during migration maintain (oxidative) balance. *Front. Ecol. Evol.* 9, 742642. doi:10.3389/fevo.2021.742642

Merila, J., and Svensson, E. (1995). Fat reserves and health state in migrant Goldcrest Regulus regulus. *Funct. Ecol.* 9, 842–848. doi:10.2307/2389981

Minias, P. (2015). The use of haemoglobin concentrations to assess physiological condition in birds: A review. *Conserv. Physiol.* 3 (1), cov007. doi:10.1093/conphys/cov007

Montgomery, M. K., Hulbert, A. J., and Buttemer, W. A. (2012). Does the oxidative stress theory of aging explain longevity differences in birds? I. Mitochondrial ROS production. *Exp. Gerontol.* 47 (3), 203–210. doi:10.1016/j.exger.2011.11.006

Piersma, T., Everaarts, J. M., and Jukema, J. (1996). Build-up of red blood cells in refuelling bar-tailed godwits in relation to individual migratory quality. *Condor* 98 (2), 363–370. doi:10.2307/1369154

Price, E. R., Bauchinger, U., Zajac, D. M., Cerasale, D. J., McFarlan, J. T., Gerson, A. R., et al. (2011). Migration-and exercise-induced changes to flight muscle size in Migratory birds and association with IGF1 and myostatin mRNA expression. *J. Exp. Biol.* 214 (17), 2823–2831. doi:10.1242/jeb.057620

Rajaura, S., Chauhan, P., Chandra, H., and Bhardwaj, N. (2022). Aflatoxin B1 administration induces reactive oxygen species production and apoptosis of erythrocytes in mice. *Toxicon* 221, 106963. doi:10.1016/j.toxicon.2022.106963

Schmidt, J. A., Marshall, J., Hayman, M. J., and Beug, H. (1986). Primitive series embryonic chick erythrocytes express the transferrin receptor. *Exp. Cell Res.* 164 (1), 71–78. doi:10.1016/0014-4827(86)90455-6

Sharma, A., Singh, D., Gupta, P., Bhardwaj, S. K., Kaur, I., and Kumar, V. (2021). Molecular changes associated with migratory departure from wintering areas in obligate songbird migrants. *J. Exp. Biol.* 224 (11), jeb242153. doi:10.1242/jeb.242153

Singh, D., Rani, S., and Kumar, V. (2013). Daily expression of six clock genes in central and peripheral tissues of a night-migratory songbird: Evidence for tissue-specific circadian timing. *Chronobiology Int.* 30 (10), 1208–1217. doi:10.3109/07420528.2013.810632

Soulsbury, C. D., Dobson, J., Deeming, D. C., and Minias, P. (2021). Energetic lifestyle drives size and shape of avian erythrocytes. *Integr. Comp. Biol.* 62 (1), 71–80. doi:10.1093/icb/icab195

Stier, A., Bize, P., Schull, Q., Zoll, J., Singh, F., Geny, B., et al. (2013). Avian erythrocytes have functional mitochondria, opening novel perspectives for birds as animal models in the study of ageing. *Front. zoology* 10 (1), 33–39. doi:10.1186/1742-9994-10-33

Szabelak, A., Bownik, A., Knaga, S., and Kasperek, K. (2021). Early morphological and apoptotic responses of bird erythrocytes to thermal stress. *Biotech. Histochem.* 96 (3), 171–178. doi:10.1080/10520295.2020.1776897

Tejero, J., Shiva, S., and Gladwin, M. T. (2019). Sources of vascular nitric oxide and reactive oxygen species and their regulation. *Physiol. Rev.* 99 (1), 311–379. doi:10.1152/physrev.00036.2017

Trivedi, A. K., Kumar, J., Rani, S., and Kumar, V. (2014). Annual life history–dependent gene expression in the hypothalamus and liver of a migratory songbird: Insights into the molecular regulation of seasonal metabolism. *J. Biol. Rhythms* 29 (5), 332–345. doi:10.1177/0748730414549766

Wang, C., and Youle, R. J. (2009). The role of mitochondria in apoptosis. *Annu. Rev. Genet.* 43, 95–118. doi:10.1146/annurev-genet-102108-134850

Winterbourn, C. C. (2015). Are free radicals involved in thiol-based redox signaling? *Free Radic. Biol. Med.* 80, 164–170. doi:10.1016/j.freeradbiomed.2014.08.017

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Sandra G. Velleman,
The Ohio State University, United States

REVIEWED BY
Colin Guy Scanes,
University of Arkansas, United States
Francesca Soglia,
University of Bologna, Italy

*CORRESPONDENCE
Paul B. Siegel,
pbsiegel@vt.edu

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Broiler genetics and the future outlook

Paul B. Siegel*

School of Animal Sciences, Virginia Tech, Blacksburg, VA, United States

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While preparing this essay, the quote by Abner Kovner kept recurring.
"To Remember the Past.
To Live the Present.
To Trust the Future"

Hanging on the wall of my study is a plaque, dated 1948, that reads "The Chicken-of-Tomorrow Committee presents this Certificate of Quality to Paul Siegel for outstanding achievement in breeding and development of superior meat-type chickens." That was 75 years ago, and it is only during the past 100 years that the production of chickens for human meat consumption was no longer a by-product of the commercial egg industry. This comment may be surprising unless we recognize that the domestication of the chicken from its wild ancestry is recent in the context of human history (Smith and Daniel, 1975). Moreover, among domesticated farm animals, the chicken increased in size while mammals became smaller (Diamond, 1995). In an evolutionary context, the domestication of the chicken had not been great, as Jungle Fowl cross fully with domestic chickens (Sutherland et al., 2018).

There is a wealth of literature on the domestication of the chicken for religious, cultural, and sport reasons. Its origins and roles as a food source too was beautifully discussed essentially a century ago in the National Geographic magazine (Jull, 1927, 1930; Lewis, 1927). For broilers, examples of anthologies include Gordy (1974), Watts (1996), Cahaner and Siegel (1986), and Siegel (2014, 2018). These publications and others reveal that it is only during the past 100 years that the broiler ceased to be a by-product of the commercial egg industry, fostered by Cecile Steele, with a subsequent focus on meat (broilers and broiler genetics).

Initially, the process of producing broilers *via* broiler genetics involved the development of the brown egg "dual purpose" chicken. Males were still reared for meat and females for egg production. It was post World War II when the "Chicken-of-Tomorrow" program (Gordy, 1974) provided the impetus for the development of breeding programs explicitly genetically designed for the production of a commercial meat-type (broiler) chicken. The initial stocks, which consisted of line crosses, were distinct from that of dual purpose chickens. Thus, although the chicken was domesticated during Neolithic times, the development of genetic programs designed for broiler performance (meat) was a 20th century event. The rapid development of a broiler *per se* was based on available stocks and sound breeding principles based on development of qualitative and quantitative genetics, which were first demonstrated in animals early in the first decade of the 20th century [e.g., Bateson and Punnett, 1959 (1905–1908)]. Broiler genetics, although conceptually new, was founded on a solid biological background.

The plethora of literature on the reduction in time and feed required to produce today's "broiler" is a story well documented and beyond the scope of this essay. Yet, it is instructive to review the numerous traits that favored domestication of the chicken. Although some of these are no longer relevant in current broiler production due to human intervention, they are necessary to our understanding of why domestication of the chicken was not

complicated. They were small and did not migrate, there were social groupings of males and females, and they possessed behavior traits such as promiscuity and broodiness. Precocial young, with well-developed motor ability and auditory and tactile senses, contributed to an adaptation to a range of environments (Hale, 1989). The advent of electricity facilitated further human intervention on a larger scale *via* artificial incubation and brooding, which provided humans with tools to manipulate the photoperiod and thus maintain persistent egg production. The gasoline engine and railroad for transportation allowed for more interactions among geneticists, facilitating exchange of ideas. These, plus the emergence of vaccines and understanding of nutritional requirements for growth and reproduction, allowed for year round production and marketing of broilers. Thus, broiler genetics was becoming a specialty area *per se*.

As stated previously, with the rediscovery of Mendelism at the turn of the 20th century, the chicken, because of traits described earlier, became a model animal for genetic research. This fundamental knowledge led to an understanding for the development and application of breeding programs for meat traits that were quantitatively inherited. Broiler breeders had a fountain for broiler genetics research from publicly supported research as its basis, as well as a range of stocks developed by fanciers, many developed before the rediscovery of Mendelism. These, plus an appreciation for quantitatively inherited traits, genotype-environment interactions, genetic correlations, heterosis, and the concept of resource allocations facilitated development of the broiler *per se*, not as a spin-off from the genetics of egg production. Expansion of mass transportation and development of computer technology contributed to specialized breeding programs that capitalized on a short generation interval with mini-generations. The short generation interval (which is often overlooked), plus a moderate to high heritability for body weight, facilitated reduction in broilers reaching market weights at younger ages, which also improved feed efficiency. These are items that should not be ignored when discussing broiler genetics and improvements in broiler performance during the last 70 years.

Husbandry practices and high energy diets were contributing factors, but they were secondary to the dynamics of selection and crossing of specific male and female lines, i.e., breeding and genetics were the primary contributors (e.g., Havenstein et al., 2003). The financial investment was considerable, and thus it was essential for broiler breeders to have control of their parental lines. Basically, they were utilizing Mendelian genetics per segregation and recombination to protect their investments. Thus, while broiler genetics did not precede the founding of the broiler industry, without the genetic paradigm, the global industry would not be where it is today. Development of sophisticated breeding programs capitalized on the availability of science and technology. As stated previously, during the early phases of commercial broiler breeding, there was reliance on readily available science and technology and a broad gene pool. With a short generation interval, capital investments were necessary and considerable. The result was that only a handful of international groups survived. By producing a 4-way cross, they are able to protect their investments.

That a baby chick could survive for a few days on nourishment from the yolk, coupled with development of the fixed wing aircraft, allowed for global distribution of broiler stocks throughout the world. Broiler production is based on breeding programs (i.e., sound broiler genetics). Its shape is a V, where final product has a narrow base for the source of elite stocks. An analogy is the limited number of sources for long distance aircraft for the international airlines.

Broiler genetics has capitalized on a storehouse of genetic material coupled with science and technology developed over decades. It has allowed for application, which has allowed for an industry to provide an inexpensive meat product derived mainly from plant sources to a global consumer. The basic germplasm and research that allowed for the development of the broiler was derived mainly from public funding with little return to the science *per se* from which the programs were based.

The caveat is that the broiler industry (not unlike some other industries) is dependent on a few multinational groups for their basic product. Their main biological tool is the genetics of the broiler. Their goal is to provide a food product—the broiler—to a growing public. Yes, they should support and conduct fundamental research, but, that is, not their function. The timeline from pedigree to broiler covers several generations and considerable resources. It is important that elite broiler breeding programs rightfully are located at multiple sites. This is essential not only in the event of disease outbreaks, but also, for example, climatic disparities and geopolitical issues. Thus, technological advances in network security, cloud, cybersecurity, redundancy, big data, and business continuity have become ever more relevant to successful broiler genetics.

Globally, an ever emerging human population, with serious climate issues, suggests that there will be numerous challenges in the conversion of plant sources to broiler in the years to come (the production of laboratory meat is not within the realm of this essay). The major genetic changes in broiler breeding (e.g., Havenstein et al., 2003; Siegel, 2014) have been "cherry picked" from the availability of base populations, moderate to high heritability for important traits, and a short generation interval. Credit is given to those who took advantage of these items and realized that broiler breeding should be specific unto itself. Namely, broiler genetics is a subset of genetics *per se* and the broiler is the result of a complex biological system involving the life cycle of its genetic history.

The plateau in broiler genetics will not be for body weight and accompanying positively correlated traits. Body weight is a trait influenced by many genes with small effects (Lillie et al., 2018). It is multifaceted and thus an issue with its genetic variation (as we know from Darwin, the lifeblood of a breeding program is genetic variation) and how to use it. The challenge is from biological and economic constraints of allocation of resources. Because the broiler as the final product is immature when marketed, reproduction cannot be ignored. Broiler breeders have to produce fertile eggs. Biologically, there is competition for mesodermal, endodermal, and ectodermal branches of development. This balancing among resources and allocations are seen in neural and metabolic factors associated with skeletal (e.g., Siegel et al., 2019), muscular (e.g., Petracci et al., 2019), cardiovascular (e.g., Wideman et al., 2013), food consumption (e.g., te Pas et al., 2020), and additional (perhaps unseen) issues. An ironic example is the replacement of plant sources in broiler diets with insects, once considered a pest (van Huis and Gasco, 2023). In this context, not to be dismissed is the coevolution of the microbiome and the hologenome concept (Yang et al., 2017; Zhou et al., 2022a; Zhou

et al., 2022b). Such recent discoveries and technological advances provide new tools and challenges for the broiler breeder in the application of broiler genetics.

To address this dynamic for competition for biological resources will require greater interactions, recognizing the sensitivity of proprietary rights and access of information to the scientific community and general public. This interface will not occur "overnight", because public funding for broiler genetic research has declined. This has contributed to there being just a limited number of public institutions with the capability to train the next-generation of broiler geneticists, i.e., a basic understanding of the interface of the biology of avian species (poultry *per se*) with the technical skills necessary for the application of genetics in broiler breeding. Broiler genetics is the V of broiler breeding. Just as the distance from the primary breeder to the broiler *per se* is great, so is the distance from genotype to phenotype. This biological process is multifaceted, complex, and challenging. Be it broiler breeding or broiler genetics, the "kettle" is far from full. Thus, in concluding this essay, the quote from Eric Hoffer may be appropriate—"The only way to predict the future is to have the power to shape it."

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Bateson, W., and Punnett, R. C. (1959). "Experimental studies in the physiology of heredity," in *classical papers in genetics*. Editor J. A. Peters (Englewood, NJ, USA: Prentice-Hall), 42–59.

Cahaner, A., and Siegel, P. B. (1986). "Evaluation of industry breeding programs for meat-type chickens and turkeys," in Proceedings of the World Congress on Genetics Applied to Livestock Production, Lincoln, Nebraska, July 1986, 337–346.10

Diamond, J. (1995). *Guns, genes, and steel: The fates of human societies*. New York, NY, USA: W. W. Norton.

Gordy, J. F. (1974). "Broilers," in *American Poultry History. An anthology overview of 150 years*. Editor J. L. Skinner (Madison, WI, USA: Amer. Printing and Pub. Inc), 370–430.

Hale, E. B. (1989). "Domestication and evolution of behavior," in *The behavior of domestic animals*. Editor E. S. E. Hafez (Baltimore, MD, USA: Lippincott Williams and Wilkins), 22–42.

Havenstein, G. B., Ferket, P. R., and Qureshi, M. A. (2003). Growth, livability, and feed conversion of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. *Poult. Sci.* 82, 1500–1508. doi:10.1093/ps/82.10.1500

Jull, M. A. (1930). Fowls of the forests and streams tamed by man. *Nat. Geogr.* 54, 327–341.

Jull, M. A. (1927). The races of the domestic fowl. *Nat. Geogr.* 51, 349–452.

Lewis, H. R. (1927). America's debt to the hen. *Nat. Geogr.* 51, 453–468.

Lillie, M., Sheng, Z. Y., Honaker, C. F., Andersson, L., Siegel, P. B., and Carlborg, O. (2018). Genomic signatures of 60 years of bidirectional selection for 8-week body weight in chickens. *Poult. Sci.* 97, 781–790. doi:10.3382/ps/pex383

Petracci, M., Soglia, F., Madruga, M., Carvalho, L., Ida, E., and Estevez, M. (2019). Wooden-breast, white striping, and spaghetti meat: Causes, consequences and consumer perception of emerging broiler meat abnormalities. *Compr. Rev. Food Sci. Food Saf.* 18, 565–583. doi:10.1111/1541-4337.12431

Siegel, P. B., Barger, K., and Siewerdt, F. (2019). Limb health in broiler breeding: History using genetics to improve welfare. *J. Appl. Poult. Res.* 28, 785–790. doi:10.3382/japr/pfz052

Siegel, P. B. (2014). Evolution of the modern broiler and feed efficiency. *Ann. Rev. Anim. Biosci.* 2, 375–385. doi:10.1146/annurev-animal-022513-114132

Siegel, P. B. (2018). "Thoughts on the evolution of the domestic chicken as food for humans," in *Animals and human society*. Editors C. G. Scanes and S. R. Toukhsati (Cambridge, MA, USA: Academic P.), 167–169.

Smith, P., and Daniel, C. (1975). *The chicken book*. Boston, MA, USA: Little Brown & Co.

Sutherland, D. A. T., Honaker, C. F., Dorshorst, B., Andersson, L., Brisbane, I. L., Jr., et al. (2018). Growth patterns for three generations of an intercross between red junglefowl and chickens selected for low body weight. *J. Anim. Breed. Genet.* 135, 300–310. doi:10.1111/jbg.12336.te.Pas

te Pas, M. F. W., Borg, R., Buddiger, N. J. H., Wood, B. J., Rebel, J. M. J., van Krimpen, M. M., et al. (2020). Regulating appetite in broilers for improving body and muscular development - A review. *J. Anim. Physiol. Anim. Nutr.* 104, 1819–1834. doi:10.1111/jpn13407

van Huis, A., and Gasco, L. (2023). Insects as feed for livestock production. *Science* 379, 138–139. doi:10.1126/science.adc9165

Watts, G. B. (1996). "The broiler industry," in *American Poultry History 1974-1993. An anthology of the next 20 years*. Editor J. L. Skinner (Mt. Morris, IL: Watt Pub. Co), 110–115.

Wideman, R. F., Rhoads, D. D., Erf, G. F., and Anthony, N. B. (2013). Pulmonary arterial hypertension (ascites syndrome) in broilers: A review. *Poult. Sci.* 92, 64–83. doi:10.3382/ps.2012-02745

Yang, L., Liu, S., Ding, J., Dai, R., He, C., Xu, K., et al. (2017). Gut microbiota co-microevolution with selection for host humoral immunity. *Front. Microbiol.* 8, 1243. doi:10.3389/fmicb.2017.01243

Zhou, H., Yang, L., Ding, J., Dai, R., Chuan, H., Xu, K., et al. (2022). Intestinal microbiota and host cooperate for adaptation as a hologenome. *mSystems* 22, e0126121. doi:10.1128/msystems.01261-21

Zhou, H., Yang, L., Ding, J., Xu, K., Liu, J., Zhu, W., et al. (2022). Dynamics of small non-coding RNA profiles and the intestinal microbiome of high and low weight chickens. *Front. Microbiol.* 13, 916280. doi:10.3389/fmicb.2022.916280

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EDITED BY
Sandra G. Velleman,
The Ohio State University, United States

REVIEWED BY
Avigdor Cahaner,
Hebrew University of Jerusalem, Israel
Yuwares Malila,
National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand

*CORRESPONDENCE
Richard A. Bailey,
rbailey@aviagen.com

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Strategies and opportunities to control breast myopathies: An opinion paper

Richard A. Bailey*

Aviagen Ltd, Newbridge, United Kingdom

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Introduction

Chicken breast meat is one of the most sustainable and affordable sources of animal protein making it one of the most popular protein sources globally. As such, maintaining consistency in product quality is of the utmost importance. Over the last decade three novel myopathies have been identified in broilers (White striping (WS), Wooden breast (WB) and Spaghetti breast (SB)) (Soglia et al., 2019; Baldi et al., 2021; Barbut and Leishman, 2022); there has been a wealth of research across the poultry sector to understand their aetiology. These myopathies can be found together or individually in all broiler chicken breeds in all global regions (Lorenzi et al., 2014; Barbut, 2019; Soglia et al., 2019; Che et al., 2022a); the incidence and severity varies (Petracci et al., 2019; Che et al., 2022b) making them a complex area of study.

The exact aetiology of the myopathies is still not fully understood however a wide range of studies have used gene expression (Velleman and Clark, 2015; Zambonelli et al., 2016), proteomics (Kuttappan et al., 2017) and metabolomics (Boerboom et al., 2018) in an effort to characterise and understand the underlying biology. These studies have shown that muscle affected by the myopathies have an increased expression of genes associated with a range of metabolic, anatomical, and structural biological processes. Whilst the three myopathies are distinct from each other, the current understanding indicates a common root in hypoxia and oxidative stress resulting in disturbed growth and development within in the muscle (Petracci et al., 2019; Soglia et al., 2021). Whilst these studies indicate what is occurring in the affected muscle at the point of sampling it is still not clear what the initial triggers are.

Current opportunities for control strategies

Genetic selection for broiler performance traits such as bodyweight (BW) and breast yield (BY) has been a core theory as a cause of the myopathies. Published data of large populations of broiler pure lines have estimated low genetic correlation between the three myopathies and performance traits (BY and BW), this indicates there is little shared genetic background between the myopathies and broiler performance traits (Bailey et al., 2015; 2020). Heritabilities for the myopathies were also estimated in these studies and found to be low to moderate (0.04 for SB, 0.024–0.097 for WB and 0.185–0.338 for WS). Alnahhas et al. (2016)reported a higher heritability for WS (0.65), where WS was measured on an underlying continuous scale rather than a categorical scale as per Bailey et al. (2015, 2020). According to Dempster and Lerner (1950) this can result in a higher heritability estimate; a heritability of 0.65 on the continuous scale would correspond to a lower heritability of 0.41 on the observed categorical scale. Another key difference is the fitting of the effect of the common maternal environment as done by Bailey et al. (2015, 2020).

Alnahhas et al. (2016) did not fit this effect therefore this environmental effect is included in the genetic variance, thus over-estimating the heritability. The low to moderate heritabilities indicate that there is a genetic component to myopathy development but it is not the major contributing factor. Nevertheless, the genetic component can be used to select against the genetic propensity for developing the myopathies (Bailey et al., 2015). Empirical testing has shown that genetic selection for WB potentially reduces the relative incidence of WB by around 9.2% (Bailey et al., 2020), whilst at the same time continuing to improve breast yield through balanced breeding. Even though improvements can be made through genetic selection, these improvements are slow due to the low heritabilites of the myopathies and thus must be viewed as a long term strategy. The non-genetic factors should not be dismissed as they offer a more impactful and more immediate opportunity to reduce the incidence of myopathies as they have a significantly greater influence than the genetic factors.

There are many non-genetic factors that can influence broiler growth rates such as incubation, brooding, nutrition, temperature and ventilation (Bartov, 1987; Leksrisompong et al., 2009; Baracho et al., 2019). A key aspect of muscle growth and development are the satellite cells which drive growth and repair of muscle (Moss and Leblond, 1970), and play an important role in meat quality traits (Velleman, 2022). Incubation conditions influence early satellite cell development and can influence meat quality traits and may play a role in myopathy development (Oviedo-Rondón et al., 2020b; Halevy, 2020). During the first week post hatch these cells are most active and their population increases rapidly (Mann et al., 2011; Daughtry et al., 2017; Halevy, 2020). Satellite cell number and activity are negatively impacted upon if conditions during brooding are not optimal, e.g., elevated temperatures (Patael et al., 2019) or suboptimal early nutrition (Harthan et al., 2014; Powell et al., 2014; 2016; Velleman et al., 2014). It is therefore essential that the development of satellite cells is supported to maximize their potential to support optimal muscle development to reduce the risk of a myopathy occurring.

Oxidative stress and hypoxia have been highlighted as a key feature of all three myopathies (Soglia et al., 2021) therefore it is important to ensure optimal management though the whole life of the bird. Poor ventilation leading to poor oxygen availability or heat stress can lead to oxidative stress in the muscle increasing the risk of myopathies (Ain Baziz et al., 1996; Livingston et al., 2019a; Patael et al., 2019; Zaboli et al., 2019; Emami et al., 2021). With this in mind it is important to ensure that the environmental conditions within the broiler shed are in line with the breeder recommendations. Excessive build-up of carbon dioxide (>3000ppm, for example,) during brooding has been associated with an increased mortality and impaired cardiovascular function (McGovern et al., 2001; Olanrewaju et al., 2008) which will undoubtedly influence oxygen supply to the muscle.

Whilst genetic correlations indicate that there are no significant links between the myopathies and bird growth at the genetic level, phenotypically it is often the larger birds in a flock which express the myopathies. This phenotypic relationship is not always the case however, as some studies report that WS and WB are not linked to bird weight (Lorenzi et al., 2014; Trocino et al., 2015). Wooden breast and white striping do not occur spontaneously; chronological studies have shown that disruption to the breast meat at the cellular level can start as early as 2 weeks of age (Brothers et al., 2019; Chen et al., 2019) indicating that this could be a key time point to influence muscle development to reduce myopathy risk especially as growth rates start to increase from around 3 weeks of age (Aviagen, 2022). With this in mind, an important part of the strategy to control the myopathies could be to look at the growth trajectory of the birds and their breast muscles. Characterising the manner in which an individual bird reaches its final bodyweight and/or breast yield over time rather than the ultimate value may offer more insight into myopathy development and guide management strategies. Demand on the muscle for growth increases as the broiler reaches mid-phase growth; thus if there has been insufficient satellite cell development during early growth there may be an increased risk of myopathies occurring. In practical terms, any potential for accelerated growth later in life of the flock (e.g., following partial depopulation) could place increased demand on the muscle and pose a risk for myopathy development, particularly if early bird growth and satellite cell development was suboptimal.

One approach to influencing growth is reducing nutrient intake by diluting or limiting the availability of feed; these methods ultimately impact upon the efficiency of production through poor bird growth or the birds compensate by eating more food and thus do not offer a suitable solution (Meloche et al., 2018a; 2018b; Livingston et al., 2019b). By targeting specific amino acid levels or ratios the broiler growth curve can be influenced in a more elegant manner. Lysine is a key amino acid for muscle growth—when levels are reduced by 15% during mid-phase growth, WS and WB incidence is significantly reduced without impacting upon performance (Meloche et al., 2018c). Reduced incidence of WS and WB were also seen when the level of histidine (Lackner et al., 2022) or arginine (Zampiga et al., 2019) was increased relative to lysine. A theory for the success of altering the growth curve through mid-phase could be that it allows for the muscle support structures (e.g., the vascular system and connective tissues) to reach equilibrium with the muscle fibres prior to the next stage of growth.

Adoption of all-plant based diets has been considered by some to be a cause for the increase in myopathies due to reduced intake of dietary creatine which is found in diets containing animal by-products (Khan and Cowen, 1977; Ringel et al., 2007). Creatine supports muscle function by providing an alternative energy source to ATP/ADP (Wyss and Kaddurah-Daouk, 2000). Birds naturally produce creatine from arginine and glycine *via* the intermediate guanidinoacetic acid (GAA) (Portocarero and Braun, 2021) but this may divert these important amino acids away from other biologically important processes in the muscle such as blood vessel and connective tissue development (Oviedo-Rondón et al., 2020a). Exogenous GAA can be supplemented in the feed; when administered to broilers fed all-plant based diets it has been found to reduce the incidence of myopathies and increase breast meat yield (Córdova-Noboa et al., 2018a; 2018b).

Increasing dietary antioxidants such as vitamin E and selenium have been used to reduce oxidative stress and myopathies but results have been mixed and may depend upon the quality of fat in the diet (Guetchom et al., 2012; Kuttappan et al., 2012; 2021; Vieira et al., 2021). A novel approach to increase antioxidant levels was taken by increasing dietary phytase (Greene et al., 2019). Phytase breaks

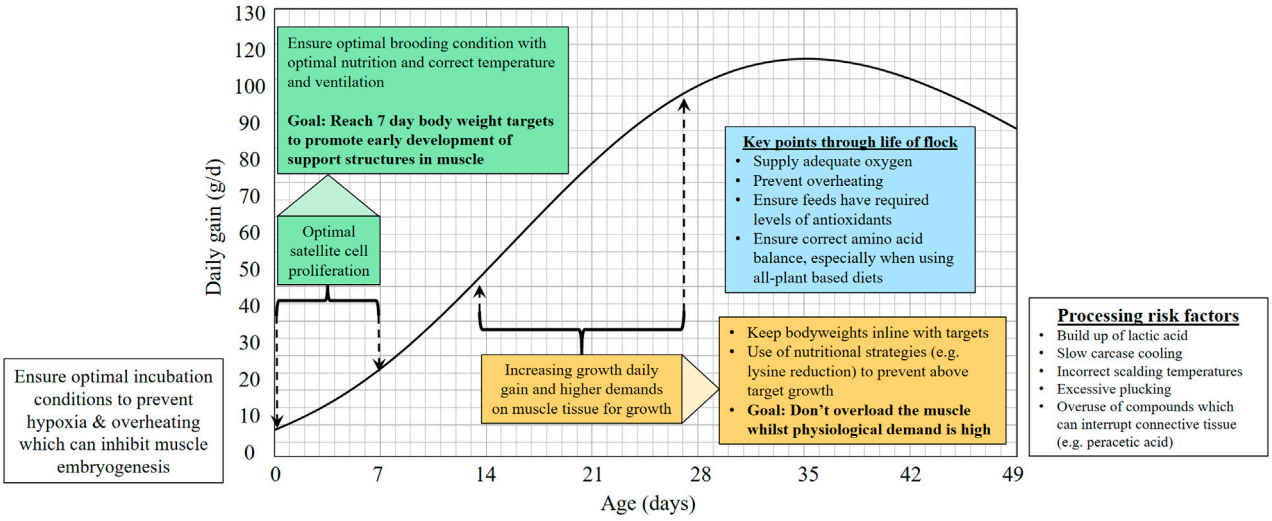

FIGURE 1
Graph proposing critical stages of broiler lifecycle where management may be critical for reducing myopathies.

down phytate in the feed and releases inositol which is absorbed by the bird and taken up by the myoctyes. Greene et al. (2019), demonstrated that "super dosing" of phytase at a level of 2000 FTU significantly reduced the WB incidence and severity broilers, and also showed through metabolomics that it acted as an antioxidant with modulation of genes associated with oxygen homeostasis linking with a reduction in oxidative stress.

Spaghetti breast is probably the least understood myopathy (Baldi et al., 2021) and its incidence appears to be more sporadic than WB and WS making it more difficult to study. This myopathy is characterised by a loss of integrity of the muscle tissue which could indicate an insufficiency in the connective tissue in the muscle (Baldi et al., 2018; 2021; Soglia et al., 2021). Interestingly, in contrast to WB and WS, it is more likely to be found in female birds rather than males (Druyan et al., 2019; Pascual et al., 2020) which may offer avenues to understand its aetiology. In a study by Griffin et al. (2018), photographs of carcases of birds euthanised on farm were used to map the development of the three myopathies over time. Whilst WB and WS were easy to detect immediately *post mortem*, the authors stated that SB was not and thus not described fully due to the uncertainty; this raises the question of whether SB is present in the live bird (Petracci et al., 2019) or only detectable following *post mortem* change in the muscle. Immediately *post mortem*, muscle pH drops as a result of lactic acid production which is accompanied by the release of proteolytic enzymes (Etherington, 1984; Soglia et al., 2018; Lilburn et al., 2019). This process can ultimately soften connective tissue in the muscle (Etherington, 1984; Shi et al., 2021) and, in the event of an insufficiency in the connective tissue, could potentially cause SB to manifest. With that in mind it is possible that processes in the slaughter house could exacerbate the impact of *post mortem* changes in the muscle and thus increase SB incidence in a flock. The rate of cooling of carcases *post mortem* has an influence on the rate of lactic acid production and the activity of the proteolytic enzymes (Etherington, 1984; Mir et al., 2017; Shi et al., 2021)—the slower the rate of cooling the greater the opportunity for degradation of muscle (Huang et al., 2016). The use of compounds such as peracetic acid as part of meat hygiene measures may also impact connective tissue in a similar way to lactic acid so may play a role in the manifestation of SB. During plucking there is a manipulation of the carcases by the fingers on the pluckers — this physical interaction on the breast meat of the bird could disrupt the integrity of the connective tissue. As SB incidence remains highly variable these factors could offer areas to reduce incidence at the slaughter plant whilst the underlying aetiology is further investigated.

Conclusion

Breast myopathies remain an important focus for the poultry industry and the poultry science community, and it is clear that there is still a lot to understand. The reduction in breast myopathies relies on a holistic approach to control: Balanced breeding by poultry breeders can target the genetic component but the larger influence from non-genetic factors remains an important focus area. Understanding the biological needs of the muscle and ongoing physiology in the modern broiler provides key time-points for strategies to reduce the myopathies and gain more insight into their aetiology (Figure 1).

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

Conflict of interest

Author RB was employed by Aviagen Ltd.

Publisher's note

References

Ain Baziz, H., Geraert, P. A., Padilha, J. C. F., and Guillaumin, S. (1996). Chronic heat exposure enhances fat deposition and modifies muscle and fat partition in broiler carcasses. *Poult. Sci.* 75, 505–513. doi:10.3382/ps.0750505

Alnahhas, N., Berri, C., Chabault, M., Chartrin, P., Boulay, M., Bourin, M. C., et al. (2016). Genetic parameters of white striping in relation to body weight, carcass composition, and meat quality traits in two broiler lines divergently selected for the ultimate pH of the pectoralis major muscle. *BMC Genet.* 17, 61. doi:10.1186/S12863-016-0369-2

Aviagen (2022). *Ross 308 performance objectives 2022*, 3–5. Available at: www.aviagen.com.

Bailey, R. A., Souza, E., and Avendano, S. (2020). Characterising the influence of genetics on breast muscle myopathies in broiler chickens. *Front. Physiol.* 11, 1041. doi:10.3389/fphys.2020.01041

Bailey, R. A., Watson, K. A., Bilgili, S. F., and Avendano, S. (2015). The genetic basis of pectoralis major myopathies in modern broiler chicken lines. *Poult. Sci.* 94, 2870–2879. doi:10.3382/PS/PEV304

Baldi, G., Soglia, F., Mazzoni, M., Sirri, F., Canonico, L., Babini, E., et al. (2018). Implications of white striping and spaghetti meat abnormalities on meat quality and histological features in broilers. *Animal* 12, 164–173. doi:10.1017/S1751731117001069

Baldi, G., Soglia, F., and Petracci, M. (2021). Spaghetti meat abnormality in broilers: Current understanding and future research directions. *Front. Physiol.* 12, 715. doi:10.3389/fphys.2021.684497

Baracho, M. S., Nääs, I. de A., Lima, N. D. S., Cordeiro, A. F. S., and Moura, D. J. (2019). Factors affecting broiler production: A meta-analysis. *Braz. J. Poult. Sci.* 21, 1052. eRBCA-2019. doi:10.1590/1806-9061-2019-1052

Barbut, S., and Leishman, E. M. (2022). Quality and processability of modern poultry meat. *Anim. (Basel)* 12. doi:10.3390/ANI12202766

Barbut, S. (2019). Recent myopathies in broiler's breast meat fillets. *Worlds Poult. Sci. J.* 75, 559–582. doi:10.1017/S0043933919000436

Bartov, I. (1987). Effect of early nutrition on fattening and growth of broiler chicks at 7 weeks of age. *Br. Poult. Sci.* 28, 507–518. doi:10.1080/00071668708416984

Boerboom, G., Van Kempen, T., Navarro-Villa, A., and Pérez-Bonilla, A. (2018). Unraveling the cause of white striping in broilers using metabolomics. *Poult. Sci.* 97, 3977–3986. doi:10.3382/PS/PEY266

Brothers, B., Zhuo, Z., Papah, M. B., and Abasht, B. (2019). RNA-seq analysis reveals spatial and sex differences in pectoralis major muscle of broiler chickens contributing to difference in susceptibility to wooden breast disease. *Front. Physiol.* 10, 764. doi:10.3389/FPHYS.2019.00764

Che, S., Wang, C., Iverson, M., Varga, C., Barbut, S., Bienzle, D., et al. (2022a). Characteristics of broiler chicken breast myopathies (spaghetti meat, woody breast, white striping) in Ontario, Canada. *Poult. Sci.* 101, 101747. doi:10.1016/J.PSJ.2022.101747

Che, S., Wang, C., Varga, C., Barbut, S., and Susta, L. (2022b). Prevalence of breast muscle myopathies (spaghetti meat, woody breast, white striping) and associated risk factors in broiler chickens from Ontario Canada. *PLoS One* 17, e0267019. doi:10.1371/journal.pone.0267019

Chen, L. R., Suyemoto, M. M., Sarsour, A. H., Cordova, H. A., Oviedo-Rondón, E. O., Wineland, M., et al. (2019). Temporal characterization of wooden breast myopathy ("woody breast") severity and correlation with growth rate and lymphocytic phlebitis in three commercial broiler strains and a random-bred broiler strain. *Avian Pathol.* 48, 319–328. doi:10.1080/03079457.2019.1598541

Córdova-Noboa, H. A., Oviedo-Rondón, E. O., Sarsour, A. H., Barnes, J., Ferzola, P., Rademacher-Heilshorn, M., et al. (2018a). Performance, meat quality, and pectoral myopathies of broilers fed either corn or sorghum based diets supplemented with guanidinoacetic acid. *Poult. Sci.* 97, 2479–2493. doi:10.3382/ps/pey096

Córdova-Noboa, H. A., Oviedo-Rondón, E. O., Sarsour, A. H., Barnes, J., Sapcota, D., López, D., et al. (2018b). Effect of guanidinoacetic acid supplementation on live performance, meat quality, pectoral myopathies and blood parameters of male broilers fed corn-based diets with or without poultry by-products. *Poult. Sci.* 97, 2494–2505. doi:10.3382/PS/PEY097

Daughtry, M. R., Berio, E., Shen, Z., Suess, E. J. R., Shah, N., Geiger, A. E., et al. (2017). Satellite cell-mediated breast muscle regeneration decreases with broiler size. *Poult. Sci.* 96, 3457–3464. doi:10.3382/PS/PEX068

Dempster, E. R., and Lerner, I. M. (1950). Heritability of threshold characters. *Genetics* 35, 212–236. doi:10.1093/GENETICS/35.2.212

Druyan, S., Olowe, R., Haron, A., Shinder, D., Horowitz, T., and Cahaner, A. (2019). "Studying the factors involved in the induction of breast muscle myopathies in commercial broiler flocks," in XVIII European symposium on the quality of eggs and egg products, Çeşme, Turkey, 158–159. Available at: http://wpsa.com/images/downloads/tables_of_contents/eggmeat_2019_BoA.pdf.

Emami, N. K., Cauble, R. N., Dhamad, A. E., Greene, E. S., Coy, C. S., Velleman, S. G., et al. (2021). Hypoxia further exacerbates woody breast myopathy in broilers via alteration of satellite cell fate. *Poult. Sci.* 100, 101167. doi:10.1016/J.PSJ.2021.101167

Etherington, D. J. (1984). The contribution of proteolytic enzymes to postmortem changes in muscle. *J. Anim. Sci.* 59, 1644–1650. doi:10.2527/JAS1984.5961644X

Greene, E., Flees, J., Dadgar, S., Mallmann, B., Orlowski, S., Dhamad, A., et al. (2019). Quantum blue reduces the severity of woody breast myopathy via modulation of oxygen homeostasis-related genes in broiler chickens. *Front. Physiol.* 10, 1251. doi:10.3389/FPHYS.2019.01251

Griffin, J. R., Moraes, L., Wick, M., and Lilburn, M. S. (2018). Onset of white striping and progression into wooden breast as defined by myopathic changes underlying Pectoralis major growth. Estimation of growth parameters as predictors for stage of myopathy progression. *Avian Pathol.* 47, 2–13. doi:10.1080/03079457.2017.1356908

Guetchom, B., Venne, D., Chénier, S., and Chorfi, Y. (2012). Effect of extra dietary vitamin E on preventing nutritional myopathy in broiler chickens. *J. Appl. Poult. Res.* 21, 548–555. doi:10.3382/japr.2011-00440

Halevy, O. (2020). Timing is everything—the high sensitivity of avian satellite cells to thermal conditions during embryonic and posthatch periods. *Front. Physiol.* 11, 235. doi:10.3389/fphys.2020.00235

Harthan, L. B., McFarland, D. C., and Velleman, S. G. (2014). The effect of nutritional status and myogenic satellite cell age on Turkey satellite cell proliferation, differentiation, and expression of myogenic transcriptional regulatory factors and heparan sulfate proteoglycans syndecan-4 and glypican-1. *Poult. Sci.* 93, 174–186. doi:10.3382/PS.2013-03570

Huang, J. C., Yang, J., Huang, F., Huang, M., Chen, K. J., Xu, X. L., et al. (2016). Effect of fast pH decline during the early postmortem period on calpain activity and cytoskeletal protein degradation of broiler M. pectoralis major. *Poult. Sci.* 95, 2455–2463. doi:10.3382/ps/pew206

Khan, A. W., and Cowen, D. C. (1977). Rapid estimation of muscle proteins in beef–vegetable protein mixtures. *J. Agric. Food Chem.* 25, 236–238. doi:10.1021/JF60210A029

Kuttappan, V. A., Bottje, W., Ramnathan, R., Hartson, S. D., Coon, C. N., Kong, B. W., et al. (2017). Proteomic analysis reveals changes in carbohydrate and protein metabolism associated with broiler breast myopathy. *Poult. Sci.* 96, 2992–2999. doi:10.3382/PS/PEX069

Kuttappan, V. A., Goodgame, S. D., Bradley, C. D., Mauromoustakos, A., Hargis, B. M., Waldroup, P. W., et al. (2012). Effect of different levels of dietary vitamin E (DL-α-tocopherol acetate) on the occurrence of various degrees of white striping on broiler breast fillets. *Poult. Sci.* 91, 3230–3235. doi:10.3382/ps.2012-02397

Kuttappan, V. A., Manangi, M., Bekker, M., Chen, J., and Vazquez-Anon, M. (2021). Nutritional intervention strategies using dietary antioxidants and organic trace minerals to reduce the incidence of wooden breast and other carcass quality defects in broiler birds. *Front. Physiol.* 12, 663409. doi:10.3389/fphys.2021.663409

Lackner, J., Hess, V., Stef, L., and Sauerwein, H. (2022). Effects of feeding different histidine to lysine ratios on performance, meat quality, and the occurrence of breast myopathies in broiler chickens. *Poult. Sci.* 101, 101568. doi:10.1016/J.PSJ.2021.101568

Leksrisompong, N., Romero-Sanchez, H., Plumstead, P. W., Brannan, K. E., Yahav, S., and Brake, J. (2009). Broiler incubation. 2. Interaction of incubation and brooding temperatures on broiler chick feed consumption and growth. *Poult. Sci.* 88, 1321–1329. doi:10.3382/PS.2008-00412

Lilburn, M. S., Griffin, J. R., and Wick, M. (2019). From muscle to food: Oxidative challenges and developmental anomalies in poultry breast muscle. *Poult. Sci.* 98, 4255–4260. doi:10.3382/PS/PEY409

Livingston, M. L., Ferket, P. R., Brake, J., and Livingston, K. A. (2019a). Dietary amino acids under hypoxic conditions exacerbates muscle myopathies including wooden breast and white stripping. *Poult. Sci.* 98, 1517–1527. doi:10.3382/ps/pey463

Livingston, M. L., Landon, C., Barnes, H. J., and Brake, J. (2019b). White striping and wooden breast myopathies of broiler breast muscle is affected by time-limited feeding, genetic background, and egg storage. *Poult. Sci.* 98, 217–226. doi:10.3382/ps/pey333

Lorenzi, M., Mudalal, S., Cavani, C., and Petracci, M. (2014). Incidence of white striping under commercial conditions in medium and heavy broiler chickens in Italy. *J. Appl. Poult. Res.* 23, 754–758. doi:10.3382/JAPR.2014-00968

Mann, C. J., Perdiguero, E., Kharraz, Y., Aguilar, S., Pessina, P., Serrano, A. L., et al. (2011). Aberrant repair and fibrosis development in skeletal muscle. *Skelet. Muscle* 1, 21. doi:10.1186/2044-5040-1-21

McGovern, R. H., Feddes, J., Zuidhof, M., and Robinson, J. (2001). Growth performance, heart characteristics and the incidence of ascites in broilers in response to carbon dioxide and oxygen concentrations. *Can. Biosyst. Eng.* 43, 4.1–4.6. doi:10.7939/R3319SG48

Meloche, K. J., Fancher, B. I., Emmerson, D. A., Bilgili, S. F., and Dozier, W. A. (2018a). Effects of quantitative nutrient allocation on myopathies of the Pectoralis major muscles in broiler chickens at 32, 43, and 50 days of age. *Poult. Sci.* 97, 1786–1793. doi:10.3382/PS/PEX453

Meloche, K. J., Fancher, B. I., Emmerson, D. A., Bilgili, S. F., and Dozier, W. A. (2018b). Effects of reduced dietary energy and amino acid density on Pectoralis major myopathies in broiler chickens at 36 and 49 days of age. *Poult. Sci.* 97, 1794–1807. doi:10.3382/PS/PEX454

Meloche, K. J., Fancher, B. I., Emmerson, D. A., Bilgili, S. F., and Dozier, W. A. (2018c). Effects of reduced digestible lysine density on myopathies of the Pectoralis major muscles in broiler chickens at 48 and 62 days of age. *Poult. Sci.* 97, 3311–3324. doi:10.3382/PS/PEY171

Mir, N. A., Rafiq, A., Kumar, F., Singh, V., and Shukla, V. (2017). Determinants of broiler chicken meat quality and factors affecting them: A review. *J. Food Sci. Technol.* 54, 2997–3009. doi:10.1007/s13197-017-2789-z

Moss, F., and Leblond, C. P. (1970). Nature of dividing nuclei in skeletal muscle of growing rats. *J. Cell Biol.* 44, 459–462. doi:10.1083/JCB.44.2.459

Olanrewaju, H. A., Dozier, W. A., Purswell, J. L., Branton, S. L., Miles, D. M., Lott, B. D., et al. (2008). Growth performance and physiological variables for broiler chickens subjected to short-term elevated carbon dioxide concentrations. *Int. J. Poult. Sci.* 7, 738–742. doi:10.3923/IJPS.2008.738.742

Oviedo-Rondón, E. O., Córdova-Noboa, H. A., and Wineland, M. J. (2020a). The role of incubation conditions in the onset of avian myopathies. *Front. Physiol.* 11, 545045. doi:10.3389/fphys.2020.545045

Oviedo-Rondón, E. O., Velleman, S. G., and Wineland, M. J. (2020b). The role of incubation conditions in the onset of avian myopathies. *Front. Physiol.* 11, 1160. doi:10.3389/fphys.2020.545045

Pascual, A., Trocino, A., Birolo, M., Cardazzo, B., Bordignon, F., Ballarin, C., et al. (2020). *Dietary supplementation with sodium butyrate: Growth, gut response at different ages, and meat quality of female and male broiler chickens*, 1135–1146. doi:10.1080/1828051X.2020.1824590

Patael, T., Piestun, Y., Soffer, A., Mordechay, S., Yahav, S., Velleman, S. G., et al. (2019). Early posthatch thermal stress causes long-term adverse effects on pectoralis muscle development in broilers. *Poult. Sci.* 98, 3268–3277. doi:10.3382/PS/PEZ123

Petracci, M., Soglia, F., Madruga, M., Carvalho, L., Ida, E., and Estévez, M. (2019). Wooden-breast, white striping, and spaghetti meat: Causes, consequences and consumer perception of emerging broiler meat abnormalities. *Compr. Rev. Food Sci. Food Saf.* 18, 565–583. doi:10.1111/1541-4337.12431

Portocarero, N., and Braun, U. (2021). The physiological role of guanidinoacetic acid and its relationship with arginine in broiler chickens. *Poult. Sci.* 100, 101203. doi:10.1016/j.psj.2021.101203

Powell, D. J., McFarland, D. C., Cowieson, A. J., Muir, W. I., and Velleman, S. G. (2014). The effect of nutritional status on myogenic gene expression of satellite cells derived from different muscle types. *Poult. Sci.* 93, 2278–2288. doi:10.3382/PS.2013-03810

Powell, D. J., Velleman, S. G., Cowieson, A. J., Singh, M., and Muir, W. I. (2016). Influence of chick hatch time and access to feed on broiler muscle development. *Poult. Sci.* 95, 1433–1448. doi:10.3382/ps/pew047

Ringel, J., Lemme, A., Knox, A., Mc Nab, J., and Redshaw, M. S. (2007). "Effects of graded levels of creatine and guanidino acetic acid in vegetable-based diets on performance and biochemical parameters in muscle tissue," in *16th European symposium on poultry nutrition*, 387–390.

Shi, H., Shahidi, F., Wang, J., Huang, Y., Zou, Y., Xu, W., et al. (2021). Techniques for postmortem tenderisation in meat processing: Effectiveness, application and possible mechanisms. *Food Prod. Process. Nutr.* 3, 21–26. doi:10.1186/S43014-021-00062-0

Soglia, F., Mazzoni, M., and Petracci, M. (2019). Spotlight on avian pathology: Current growth-related breast meat abnormalities in broilers. *Avian Pathol.* 48, 1–3. doi:10.1080/03079457.2018.1508821

Soglia, F., Petracci, M., Davoli, R., and Zappaterra, M. (2021). A critical review of the mechanisms involved in the occurrence of growth-related abnormalities affecting broiler chicken breast muscles. *Poult. Sci.* 100, 101180. doi:10.1016/J.PSJ.2021.101180

Soglia, F., Zeng, Z., Gao, J., Puolanne, E., Cavani, C., Petracci, M., et al. (2018). Evolution of proteolytic indicators during storage of broiler wooden breast meat. *Poult. Sci.* 97, 1448–1455. doi:10.3382/PS/PEX398

Trocino, A., Piccirillo, A., Birolo, M., Radaelli, G., Bertotto, D., Filiou, E., et al. (2015). Effect of genotype, gender and feed restriction on growth, meat quality and the occurrence of white striping and wooden breast in broiler chickens. *Poult. Sci.* 94, 2996–3004. doi:10.3382/PS/PEV296

Velleman, S. G., and Clark, D. L. (2015). Histopathologic and myogenic gene expression changes associated with wooden breast in broiler breast muscles. *Avian Dis.* 59, 410–418. doi:10.1637/11097-042015-REG.1

Velleman, S. G., Coy, C. S., and Emmerson, D. A. (2014). Effect of the timing of posthatch feed restrictions on broiler breast muscle development and muscle transcriptional regulatory factor gene expression. *Poult. Sci.* 93, 1484–1494. doi:10.3382/PS.2013-03813

Velleman, S. G. (2022). Why breast muscle satellite cell heterogeneity is an issue of importance for the poultry industry: An opinion paper. *Front. Physiol.* 13, 1640. doi:10.3389/fphys.2022.987883

Vieira, V., Marx, F. O., Bassi, L. S., Santos, M. C., Oba, A., de Oliveira, S. G., et al. (2021). Effect of age and different doses of dietary vitamin E on breast meat qualitative characteristics of finishing broilers. *Anim. Nutr.* 7, 163–167. doi:10.1016/J.ANINU.2020.08.004

Wyss, M., and Kaddurah-Daouk, R. (2000). Creatine and creatinine metabolism. *Physiol. Rev.* 80, 1107–1213. doi:10.1152/PHYSREV.2000.80.3.1107

Zaboli, G., Huang, X., Feng, X., and Ahn, D. U. (2019). How can heat stress affect chicken meat quality? - a review. *Poult. Sci.* 98, 1551–1556. doi:10.3382/PS/PEY399

Zambonelli, P., Zappaterra, M., Soglia, F., Petracci, M., Sirri, F., Cavani, C., et al. (2016). Detection of differentially expressed genes in broiler pectoralis major muscle affected by White Striping - wooden Breast myopathies. *Poult. Sci.* 95, 2771–2785. doi:10.3382/PS/PEW268

Zampiga, M., Soglia, F., Petracci, M., Meluzzi, A., and Sirri, F. (2019). Effect of different arginine-to-lysine ratios in broiler chicken diets on the occurrence of breast myopathies and meat quality attributes. *Poult. Sci.* 98, 2691–2697. doi:10.3382/PS/PEY608

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EDITED BY
Sandra G. Velleman,
The Ohio State University, United States

REVIEWED BY
Monika Proszkowiec-Weglarz,
Agricultural Research Service,
United States Department of Agriculture,
United States
Nabeel Alnahhas,
Laval University, Canada

*CORRESPONDENCE
Grégoy Y. Bédécarrats,
gbedecar@uoguelph.ca

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Impact of growth trajectory on sexual maturation in layer chickens

Mohammad A. Bahry[1], Charlene Hanlon[1,2], Clara J. Ziezold[1], Sierra Schaus[1] and Grégoy Y. Bédécarrats[1]*

[1]Department of Animal Biosciences, University of Guelph, Guelph, ON, Canada, [2]Department of Poultry Science, College of Agriculture, Auburn University, Auburn, AL, United States

Recent studies showed that apart from photostimulation, metabolic triggers may independently activate sexual maturation and egg production in chickens. However, the origin, mode of action, and specific target(s) of this metabolic control remain unknown. Beyond body weight (BW), we hypothesize that body composition (BC) and associated specific metabolic signals are involved. Thus, this study was conducted to determine the BW and BC thresholds triggering spontaneous sexual maturation in layer pullets under different growth trajectories. Day-old Lohman LSL lite and Lohman brown lite chicks (n = 210 each) raised in brooding cages under *ad libitum* (AL) feeding until 8 weeks of age were randomly allocated into individual cages and assigned to one of 3 experimental growth profiles; AL, breeder's target (T), restricted 20% below target (R), (n = 70 birds/profile/strain). Birds had free access to water throughout the trial. All hens were maintained on 10 h of light (10 lux) throughout the rest of the study. Blood and tissue samples were collected throughout the study to measure plasma estradiol (E_2) concentrations and organ weights, respectively. Furthermore, carcasses were subjected to Dual-energy X-ray absorptiometry (DEXA) analyses. All analyses were completed with SAS using the MIXED procedure. Results show that R treatment slowed ($p < 0.001$) growth, delayed age at first egg (FE) and egg production ($p < 0.001$) and resulted in lower BW at FE ($p < 0.001$), lower ovary weight and number of follicles ($p < 0.001$) compared to AL in both strains, whereas, the strain significantly impacted body weight ($p < 0.0001$), ovary weight ($p < 0.001$), BW at FE ($p < 0.001$), age at FE ($p < 0.001$), egg production ($p < 0.0001$), E_2 ($p < 0.0001$) and body composition ($p < 0.05$). For DEXA, AL feeding ($p < 0.001$) increased fat deposition compared to R. Furthermore, there was a positive correlation between plasma E_2 and bone mineral content ($p < 0.01$) and bone mineral density ($p < 0.01$). In conclusion, feed allocation impacted growth and BC in a strain dependent manner which resulted in differing age at sexual maturation and egg production. Furthermore, a body fat threshold between 10% to 15% appears to be required for the occurrence of spontaneously sexual maturation in laying hens.

KEYWORDS

layers, body weight, body composition, sexual maturation, egg production

1 Introduction

Chickens are seasonal breeders, relying on changes in photoperiod to initiate and terminate reproduction. However, it has also been shown that apart from photostimulation, metabolic triggers can independently activate sexual maturation and egg production in both layers and broiler breeders (van der Klein et al., 2018a; Baxter and Bédécarrats, 2019; Hanlon et al., 2021). Thus, it is evident that growth trajectories can influence the initiation of reproduction, possibly at all levels of the hypothalamic-pituitary-ovarian axis. However, the origin, mode of action, and specific target(s) of this metabolic control remain to be determined. Beyond body weight (**BW**), body composition (**BC**) may be directly related to metabolic input on sexual maturation in chickens. Studies in broiler breeders and quail revealed that specific BW and BC are required to initiate maturation (Bornstein et al., 1984; Yang et al., 2013). Altering growth trajectories (van Emous et al., 2013), feed allocation (Robinson et al., 2007), or dietary composition (Spratt and Leeson, 1987) have all been proven management strategies that can alter BC. Specifically, Heijmans et al. (2021) reported that birds on a 15% elevated growth curve demonstrated greater abdominal fat accumulation. Interestingly, van der Klein et al. (2018a) showed that a fat pad of 2.5% of BW or higher was required for broiler breeder hens to enter lay, as hens with a lower fat pad (1.5%) did not enter lay before 55 weeks of age (**woa**). Since the abdominal fat pad was proposed as an accurate indicator of overall fat accumulations in chickens (Sato et al., 2009), signalling from adipose tissue is likely behind the metabolic threshold involved in initiating sexual maturation. Therefore, achieving a critical BC threshold during the juvenile stage may be required to support egg formation throughout a laying cycle (Hanlon et al., 2020). Thus, by controlling for BW and hence BC, the first aim of this study was to determine the carcass fat and lean thresholds associated with spontaneous sexual maturation in brown (**B**) and white (**W**) strains of modern commercial layers.

The hypothalamic-pituitary-gonadal (HPG) axis controls reproduction in chickens through a tight balance between stimulatory (chicken gonadotropin-releasing hormone I—cGnRH-I) and inhibitory (gonadotropin inhibitory hormone—GnIH) inputs (Bédécarrats, 2015; Hanlon et al., 2020). In addition to its reproductive function, GnIH also possesses orexigenic effects that stimulate feed intake in chickens (Tachibana et al., 2005; Chowdhury et al., 2012). Increased levels of GnIH directly inhibit cGnRH-I (Bentley et al., 2003; Bentley et al., 2008) within the hypothalamus and reduce the release of gonadotropins (luteinizing hormone; LH and follicle-stimulating hormone; FSH; Tsutsui et al., 2000; Ciccone et al., 2004; Ikemoto and Park, 2005; Ubuka et al., 2006) by the anterior pituitary gland. Once released into the systemic circulation, LH and FSH initiate the maturation of small white follicles (**SWFs**), which gradually increase their production of estradiol (E_2; Williams and Sharp, 1978; Robinson and Etches, 1986). These SWFs will continue to progress and develop, undergo cyclic recruitment into the hierarchy (F1-F6; Johnson, 1993), and eventually result in the daily ovulation of the largest follicle (F1) (Johnson et al., 1996). In broiler breeders, the number of hierarchical follicles increases with elevations in BW under *ad libitum* feeding (Hocking, 1993), resulting in a double hierarchy which can be controlled through feed restriction (Hocking et al., 1989). However, the link between the feeding paradigm and follicular maturation remains unclear in laying hens. Non-etheless, we hypothesize that metabolic status will impact the timing of ovarian maturation. Thus, the second aim of this study was to investigate whether induced changes in BW and composition can alter ovarian follicular development and maturation, and therefore E_2 profiles.

Additionally, during sexual maturation, skeletal development shifts from longitudinal growth to the medullary formation (Miller and Bowman, 1981; Dacke et al., 1993; Whitehead and Fleming, 2000), providing a mineral reservoir to supplement dietary calcium (**Ca**) for eggshell formation (Mueller et al., 1964; Miller, 1977). This occurs concurrently with the rise in E_2, and studies in quail showed that the development of medullary bone and an elevation in total plasma Ca is triggered 72–120 h following estradiol valerate administration (Miller and Bowman, 1981). The authors said that the changes in plasma E_2 concentration at the onset of sexual maturation affect bone physiology in high-producing quail to provide enough Ca required in the egg-production process. Eusemann et al. (2020) reported that bone mineral density (**BMD**) was higher in non-laying hens compared to laying hens. Furthermore, exogenous E_2 administration increased BMD and reduced the risk of fracture in non-laying hens, while it increased the risk of fracture in laying hens. It was found that the radiographic bone density was higher in traditional breeds with lower egg production compared to the high-producing birds (Hocking et al., 2003). This was supported by Habig et al. (2017), who reported that hens with higher laying performance showed a lower BMD than those with moderate laying performance. Thus, the final aim of this study was to investigate the impact of strain and differing growth curves on laying status, plasma E_2, bone mineral content (**BMC**) and BMD.

TABLE 1 Lighting schedule (no photostimulation).

Program start (day)	Photoperiod (h)	Intensity (lux)
0	16	40
4	16	35
7	14	30
12	13	25
19	12	20
26	11	15
33 to end (25 woa)	10	10

woa, weeks of age.

2 Materials and methods

2.1 Animals and experimental design

Day-old Lohmann LSL lite (**W**) and Lohmann brown lite (**B**) *Gallus gallus domesticus* (n = 210 each) were purchased from a local hatchery (Archers Hatchery, Brighton, ON) and housed at the

Arkell Poultry Research Station (University of Guelph, Guelph, ON) in colony brooding cages (76 cm wide × 66 cm deep × 40 cm tall; n = 7 cages; 30 chicks/cage) for the first 4 woa, then density was reduced to 14 chicks/cage (n = 15 cages) until 8 woa. Birds were fed *ad libitum* and maintained under the photo schedule according to the breeder's management guide for North America (Table 1). At 8 woa, pullets were randomly allocated into individual cages across two rooms (n = 210 cages/room) and assigned to one of 3 experimental growth profiles, *ad libitum* (**AL**), breeder's recommended target (**T**), and feed restricted to achieve a BW 20% below target (**R**) (n = 70 birds/profile/strain). From 8 woa, individual BWs were recorded weekly and feed allocation for T and R birds was determined individually based on actual BWs ($\frac{Target\ bodyweight \times Target\ feed\ intake}{Actual\ bodyweight}$). All hens were fed the same commercial diet (Floradale Feed Mill Limited, Floradale, Ontario, Canada) formulated to meet or exceed the NRC requirements (National Research Council, 1994), including a starter crumble diet (0–6 woa with 21% energy), pullet grower crumble diet (7–16 woa with 18% energy), and layer breeder (17 woa to end of the trial). All hens were maintained on a 10 h photoperiod (10 lux) throughout the rest of the study to avoid confounding photostimulatory with metabolic triggers. This experiment was approved by the Animal Care Committee at the University of Guelph, and all procedures and management followed the guidelines from the Canadian Council for Animal Care (CCAC, 2009).

2.2 Growth and production performance

The growth trajectory was determined by calculating weekly weight gain. Individual egg production was recorded daily and expressed as a weekly hen-day production percentage. Furthermore, BW and age at first egg (**AFE**) were recorded to determine the timing of sexual maturation for each hen. At 14, 16, 18, 20, 22, and 25 woa, 6 birds per treatment and strain were weighed and approximately 3 mL of blood was collected from the brachial vein and placed in sodium heparin tubes for later processing. Birds were then euthanized *via* cervical dislocation. Ovaries were weighed without large yellow follicles (**LYFs**; follicles larger than 8 mm) and the total number of SWFs and LYFs from each ovary were counted. Ovary weight was expressed relative to BW.

2.3 Body composition

After removing the head and ovary, BC was analyzed from the same carcasses at 14, 16, 18, 20, 22, and 25 woa (n = 6 birds/treatment/strain) *via* Dual Energy X-ray Absorptiometry (DXA). After euthanasia, carcasses were placed at 4°C to slowly decrease the core temperature before being stored at −20°C until analysis. Before DXA scanning, all carcasses were placed at 4°C for 48 h and then at room temperature until thawed. Scans were performed using a Lunar Prodigy Advance DXA scanner (GE Healthcare, Madison, WI) with the enCore software version 16. A quality assurance block was conducted daily prior to the scans to calibrate the machine. Carcasses were placed on the scanning bed in a uniform lateral position. DXA scans were run using the small animal setting in small mode according to Swennen et al. (2004) to measure, BMC (g), total bone area (cm^2), fat tissue weight (g), lean tissue weight (g) and total tissue weight (g). Fat % and lean % were calculated as $(\frac{fat\ or\ lean\ weight}{Tissue\ weight}) \times 100$ and BMD (g/cm^2) was calculated as $(\frac{BMC}{total\ bone\ area}) \times 100$.

2.4 Estradiol (E2) analysis

In addition to the blood sample collected from sacrificed birds described in Section 2.2, repeated blood samples were also collected from focal birds (n = 10 birds/treatment/strain) every 2 weeks from 10–14 woa and weekly from 14 to 25 woa. Plasma was recovered by centrifugation (Centrifuge J6-MI, Beckman Coulter, Inc., United States) for 15 min at 900 × g at 4°C and stored at −20°C until extractions and assays were completed as described by Hanlon et al. (2021). Briefly, plasma samples underwent a fat extraction using the cold ethanol extraction protocol described by Baxter et al. (2014). The DetectX commercial estradiol ELISA was used to measure E_2 concentration following the manufacturer's protocol (DetectX 17β-estradiol enzyme immunoassay #K030-H5, Arbor Assays, Ann Arbor, Michigan). Samples were assayed in duplicates, with optical densities measured at 450 nm using a microplate spectrophotometer (Model 550, Bio-Rad, CA, United States). Data were analyzed using the MyAssays software (www.myassays.com/arbor-assays-estradiol-eia-kit.assay) with a four-parameter logistic curve and the intra-assay and inter-assay coefficient of variance (CV) were <15%.

2.5 Statistical analyses

All statistical analyses were performed using SAS v9.4 (SAS Institute, Cary, NC). BW and egg production parameters, as well as E_2 concentrations from focal birds, were analyzed by a three-way ANOVA (PROC MIXED) with respect to strain, treatment, age, and their interactions. All other parameters, including measures of BC, E_2 concentrations from sacrificed birds, relative ovary weight, and the number of follicles were analyzed using a two-way ANOVA (PROC MIXED), whereby strain, treatment and their interactions were independent variables. Random effects for all measures included room, tier, and their interaction. Means were separated by the least squares means of fixed effects (LSMEANS), the test of least significant differences (PDIFF) and Tukey's multiple comparisons. When appropriate, repeated measures on individual hens over time were included in the model with respect to hen ID. Pearson correlations (PROC CORR) were performed within treatments across variables, including, E_2, BMC and BMD. Statements of significance were based on $p < 0.05$ and data were expressed as mean ± SEM.

3 Results

3.1 Body weight

As shown in Figure 1, the feeding strategy successfully generated 3 separate growth trajectories in both strains. The significant

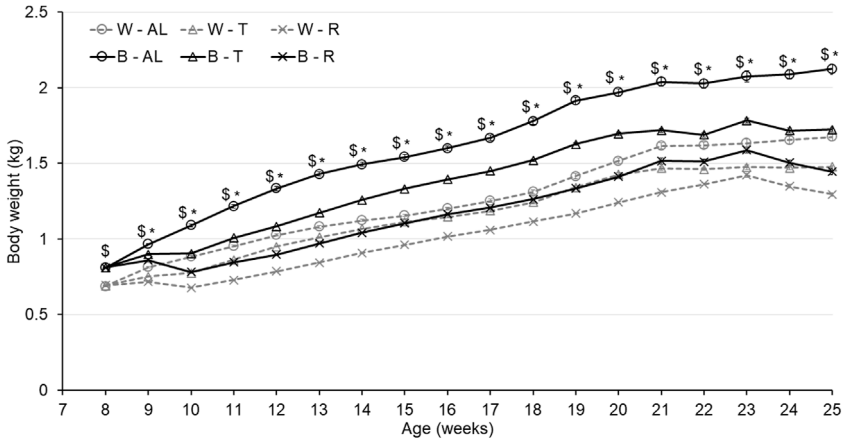

FIGURE 1
Weekly body weight of Lohmann LSL lite (W) birds and Lohmann Brown lite (B) birds reared under 3 feeding trajectories: *ad libitum* (AL), breeder's recommended target (T) and Restricted (R). Values correspond to the mean ± SEM. *p*-values for the sources of variation: Age, $p < 0.0001$; Treatment, $p < 0.0001$; Strain, $p < 0.0001$; Age*Treatment, $p < 0.0001$; Age*Strain, $p < 0.0001$; Age*Strain*Treatment, $p < 0.0001$. The "$" symbols indicate significant differences between strain at specific time points while "*" indicates significant differences between treatment at specific time points.

TABLE 2 Age at first egg (AFE) and body weight at first egg (BW-FE) of Lohmann LSL lite (W) and Lohmann Brown lite (B) birds reared under 3 feeding trajectories: *ad libitum* (AL), breeder's recommended target (T) and Restricted (R). Values correspond to the mean ± SEM. Different letters in different rows indicate significant differences between groups ($p < 0.05$).

Strain	Treatment	AFE	SEM	BW-FE	SEM
		days		g	
W	AL	142^{b}	1.0	1590^{c}	0.017
	T	140^{b}	1.0	1480^{d}	0.018
	R	152^{a}	1.1	1400^{e}	0.019
B	AL	135^{c}	1.0	2000^{a}	0.017
	T	138^{bc}	1.0	1740^{b}	0.017
	R	152^{a}	1.1	1570^{c}	0.019
Source of variation		***p*-value**			
Strain		<0.001		<0.001	
Treatment		<0.001		<0.001	
Strain*Treatment		<0.05		<0.001	

interaction between treatment and strain resulted in B birds displaying increased body weight gain compared to W birds from the beginning of treatment (8 woa) until the end of the trial ($p < 0.0001$). Furthermore, the significant interaction between age and treatment resulted in R birds displaying lower body weights regardless of strain ($p < 0.0001$) to achieve the expected 20% reduction in BW, while birds reared under AL feeding conditions were significantly heavier than birds reared under T ($p < 0.0001$). A three-way interaction between strain, treatment, and age was also observed ($p < 0.0001$) resulting in the weight of B-AL birds significantly higher than B-T from 10 to 25 woa (end of study). Interestingly, the BW of W-AL birds was initially higher than W-T from 10 to 12 woa, but this difference was no longer observed from 13 to 19 woa. However, starting at 20 woa, W-AL birds were significantly heavier than W-T and this difference remained throughout the end of the study. Ultimately, this resulted in AL birds being 400-g and 200-g heavier than T birds in the B and W strains, respectively, demonstrating a more prominent impact of treatment in B birds.

3.2 Reproduction

Regarding AFE, there was an interaction between strain and treatment ($p < 0.0001$). While B-R birds were delayed by 16.7 days compared to B-AL, W-R birds were only delayed by 10.7 days compared to their AL counterparts. Interestingly, AFE for T birds did not differ from AL in either strain (Table 2). The aforementioned delay in AFE resulted in a significant interaction of strain, treatment, and age on egg production ($p < 0.05$). At 19 woa, B-AL hens had the highest production, with B-R and W-R hens demonstrating the lowest. However, by 20–22 woa, AL and T feeding resulted in higher production rates than R hens, regardless of strain (Figure 2). Despite lower production rates and a 2-week delay in AFE, 50% of R birds entered lay at around 21 woa which was delayed approximately by 1.5 weeks compared to W-AL and W-T, and 2.5 weeks compared to B-AL and B-T. However, all R hens entered lay by 23 woa, which was 1 week later than the AL and T hens (Figure 3). Furthermore, treatment and strain also affected BW at first egg (BW-FE; $p < 0.0001$), with B-AL birds displaying the highest BW-FE (2.00-kg) and W-R birds the smallest (1.40-kg; Table 2). However, there were no differences in BW-FE between B-R and W-AL.

Plasma concentration of E_2 was used as an indirect measure of the activation of the reproductive axis. In the absence of photostimulation, a single defined E_2 peak was not observed among the focal birds (Figure 4). There was an interaction between strain, age, and treatment ($p < 0.0001$), with W-AL and

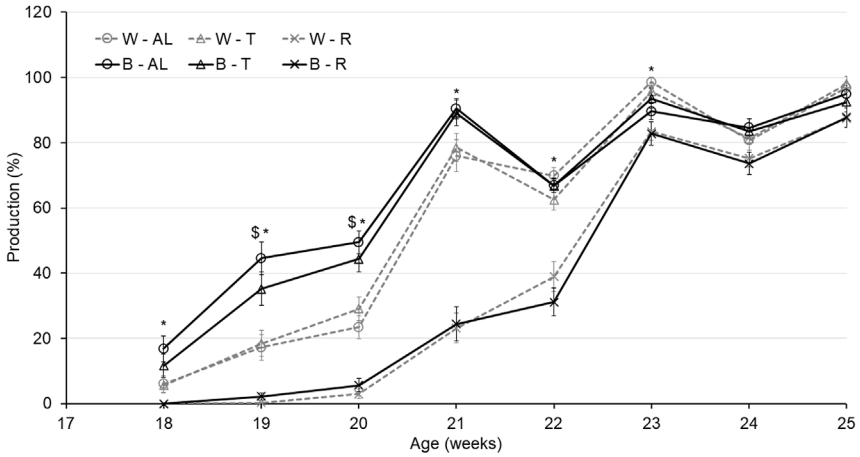

FIGURE 2
Egg production of Lohmann LSL lite (W) and Brown lite (B) birds reared under 3 feeding trajectories: *ad libitum* (AL), breeder's recommended target (T) and Restricted (R). Data are expressed as egg/per housed hen on a weekly basis from 18 weeks of age to egg to 25 weeks of age. Values correspond to the means ± SEM. *p*-values for the sources of variation: Age, $p < 0.0001$; treatment, $p < 0.0001$; strain, $p < 0.0001$; Age*Treatment, $p < 0.0001$; Age*Strain, $p < 0.0001$; Age*Strain*Treatment, $p < 0.05$. The "$" symbols indicate significant differences between strain at specific time points while "*" indicates significant differences between treatment at specific time points.

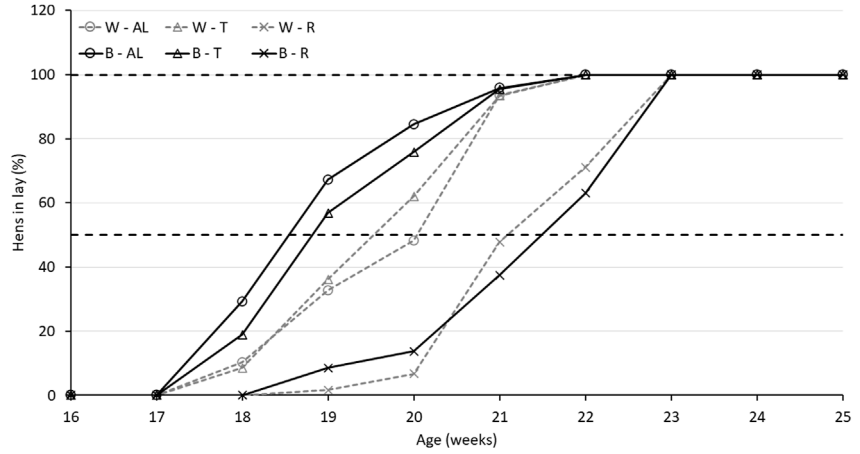

FIGURE 3
Percentage of Lohmann LSL lite (W) and Brown lite (B) birds reared under 3 feeding trajectories: *ad libitum* (AL), breeder's recommended target (T) and Restricted (R) in lay.

W-R demonstrating higher E_2 concentrations than B-AL and B-R, respectively, at 19 and 20 woa. Interestingly, at 21 and 22 woa regardless of strain, R birds had the highest E_2 concentrations. Relative ovary weight, the number of SWFs and the number of LYFs further illustrate the differences in sexual maturation (Table 3). The interaction between age and treatment ($p < 0.0001$) indicates that relative ovary weights were higher in W birds starting from 20 woa until the end of the trial while B hens had heavier ovaries only at 20 and 22 woa. The significant interaction between age and treatment resulted in R birds showing the lowest number of LYFs regardless of strain ($p < 0.0001$) starting from 20 woa. The interaction between age and treatment also revealed that AL hens displayed a higher number of SWFs compared to R birds ($p < 0.0001$) starting from 22 woa and, the significant interaction between strain and age ($p < 0.0001$) resulted in more numerous SWFs in B hens at the beginning of maturation (18 woa). However, this difference dissipated between 20 and 22 woa. Interestingly, at 25 woa when all W birds were in lay, the number of SWFs was increased in W compared to B hens, suggesting a higher reproductive capacity (Table 3).

3.3 Body composition and correlations

Changes in BC including relative fat percentage, lean percentage, BMC and BMD are shown in Table3, 4. Treatment impacted the percentage of fat ($p < 0.0001$), with AL feeding increasing the overall

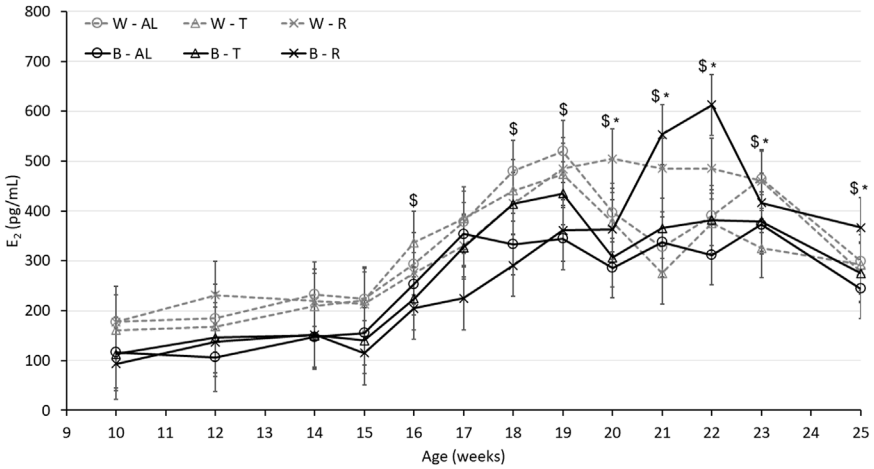

FIGURE 4
Plasma estradiol (E_2) concentration of Lohmann LSL lite (W) and Brown lite (B) birds reared under 3 feeding trajectories: *ad libitum* (AL), breeder's recommended target (T) and Restricted (R). Values are expressed as mean ± SEM of 10 birds/treatment/strain. *p*-values for the sources of variation: Age, $p < 0.0001$; treatment, $p < 0.05$; strain, $p < 0.0001$; Age*Treatment, $p < 0.0001$; Age*Strain, $p < 0.0001$; Age*Strain*Treatment, $p < 0.0001$. The "$" symbols indicate significant differences between strain at specific time points while "*" indicates significant differences between treatment at specific time points.

relative fat deposition, while it was reduced for R and T ($p < 0.0001$), independent of strain (Table 3). Accordingly, AL feeding resulted in a lower lean tissue percentage compared to T and R ($p < 0.0001$; Table 3). Bone parameters were also impacted by the growth trajectories. The interaction between age and treatment indicates that BMD was higher ($p < 0.05$) in AL compared to R at 18 woa and was attenuated at 25 woa irrespective of the strains. R birds showed a lower BMC ($p < 0.05$) at 18 woa compared to T birds and had a higher BMC ($p < 0.05$) compared to AL at 25 woa. Furthermore, there was a moderate positive correlation between plasma E_2 from the sampled birds and BMC ($r = 0.467$; $p < 0.0001$) and BMD ($r = 0.415$, $p < 0.001$) in R birds and a weak correlation in AL and T birds (Table 4). In addition, BMC had a strong ($p < 0.0001$) correlation with BMD irrespective of the treatment (Table 4).

4 Discussion

Beyond photostimulation, BW and BC play an important role in sexual maturation and egg production in chickens (Gous and Cherry, 2004; van der Klein et al., 2018b; van Emous et al., 2018; Zuidhof, 2018; Baxter and Bédécarrats, 2019; Hanlon et al., 2020; Hanlon et al., 2021). Therefore, this study aimed to investigate whether body growth and composition could impact spontaneous sexual maturation in brown and white strains of commercial layer hens. We hypothesized that differential growth trajectories would alter BC, and thus metabolic status. Specifically, a certain degree of body fat appears to be required to allow for sexual maturation to proceed in broiler breeders and quail (Bornstein et al., 1984; Yang et al., 2013). It was previously suggested that initiation of sexual maturation is governed not only by photoperiod (Chaney and Fuller, 1975) and age, but also by BW (Brody et al., 1980; Brody et al., 1984; Hanlon et al., 2021), body fat (Bornstein et al., 1984; Kwakkel et al., 1995), and lean body mass (Soller et al., 1984). As anticipated, R feeding reduced BW, while AL increased it, and this effect was more pronounced in B birds. This suggests that the recommended BW target in the management guidelines for B may not allow hens to reach satiety when housed individually, while guidelines for W do.

Changes in BW are positively associated with changes in BC (Heijmans et al., 2021). In line with this finding, our results from DXA indicate that the BC was strongly affected by the feeding regimens. Feed restriction resulted in the lowest percentage of body fat and this effect was more pronounced in B birds irrespective of the maturation status. It was reported that feed-restricted layers had a lower BW and abdominal fat pad compared to AL due to the distribution of nutrients available for maintenance, production, and storage (Murugesan and Persia, 2013). A 2.6-fold reduction in fat deposition was further observed in time-restricted laying hens due to an 11.7% reduction in feed intake and a 9.6% reduction in BW compared to control birds which had free access to feed throughout the light period (Saibaba et al., 2021). In addition, studies in broiler breeders revealed that relaxing feed restriction results in increased BW with a higher abdominal fat pad (Renema et al., 2001; Robinson et al., 2007; Van Emous et al., 2013; Van der Klein et al., 2018a; Salas et al., 2019; Heijmans et al., 2021). A relationship between BW and body fat mass during the rearing stage was established in broiler breeders (Sun and Coon, 2005; Van Emous et al., 2013; Salas et al., 2019), with BC positively correlated with feed allocation (Robinson et al., 2007). In the current study, the increase in fat content suggests that a body fat threshold between 10% to 15% is required to spontaneously initiate sexual maturation. The importance of such a fat threshold on sexual maturation was further studied in broiler breeders (van der Klein et al., 2018a; Zuidhof, 2018; Hadinia et al., 2020) and quail (Reddish et al., 2003). For instance, van der Klein et al. (2018a) reported that birds with a high BW had a higher fat pad proportion (2.2%) compared to the standard BW (1.6%), and the authors speculated that a fat pad mass threshold played a critical role on the process of sexual maturation. A similar concept was also

TABLE 3 Plasma estradiol concentrations, ovary weight, number of follicles, and body composition of Lohmann LSL lite (W) and Lohmann Brown lite (B) birds reared under 3 feeding trajectories: *ad libitum* (AL), breeder's recommended target (T) and Restricted (R) at 14, 16, 18, 20, 22, and 25 weeks of age. Values correspond to the mean ± SEM (n = 6 birds/treatment/strain). NS, not significant; ND, not detected, E_2, estradiol; LYFs, large yellow follicles; SWFs, small white follicles; BMC, bone mineral content; BMD, bone mineral density. Different letters indicate significant differences in the appropriate age.

Strain	Age (wk)	Treatment	Follicles								Tissue				Bone			
			E_2	SEM	Ovary	SEM	LYFs	SEM	SWFs	SEM	Fat	SEM	Lean	SEM	BMC	SEM	BMD	SEM
			pg/mL		% BW						%		%		g		g/cm^2	
W	14	AL	249	52.6	0.06	0.024	ND	—	ND	—	13.6	1.45	86.4	1.52	24.1	2.57	0.22	0.011
		T	176	52.5	0.06	0.024	ND	—	ND	—	11.7	1.45	88.3	1.52	25.8	2.57	0.23	0.011
		R	96	52.5	0.06	0.024	ND	—	ND	—	8.5	1.45	91.6	1.52	24.1	2.57	0.23	0.011
	16	AL	265	52.6	0.06	0.024	ND	—	ND	—	12.3	1.45	87.8	1.52	25.3	2.57	0.27	0.011
		T	258	52.5	0.06	0.024	ND	—	ND	—	9.7	1.45	90.3	1.52	27.4	2.57	0.28	0.011
		R	202	52.5	0.06	0.024	ND	—	ND	—	6.6	1.45	93.4	1.52	27.2	2.57	0.26	0.011
	18	AL	485	57.5	0.12	0.026	2.2	0.83	108	45.3	19.7	1.59	80.4	1.85	31.8	3.14	0.29	0.012
		T	460	52.6	0.11	0.024	1.7	0.76	100	42.0	16.6	1.45	83.5	1.52	35.4	2.57	0.31	0.011
		R	352	52.6	0.07	0.024	0.1	0.76	43	41.8	11.4	1.45	88.6	1.52	31.1	2.57	0.27	0.011
	20	AL	364	57.6	0.27^{a}	0.024	6.3^{a}	0.76	202	41.8	19.9	1.45	80.1	1.52	33.3	2.57	0.29	0.012
		T	393	52.5	0.25 ^{ab}	0.024	5.1^{a}	0.76	154	41.7	17.6	1.45	82.4	1.52	40.0	2.57	0.31	0.011
		R	461	52.5	0.13^{b}	0.024	1.7^{b}	0.76	117	41.7	15.9	1.45	84.1	1.52	36.2	2.57	0.29	0.011
	22	AL	449	52.6	0.38^{a}	0.024	7.6^{a}	0.76	456^{a}	41.8	13.2	1.46	86.8	1.52	36.8	2.58	0.25	0.011
		T	401	52.6	0.39^{a}	0.026	7.2 ^{ab}	0.83	367 ^{ab}	45.5	8.6	1.45	91.4	1.52	30.2	2.57	0.25	0.011
		R	499	52.6	0.21^{b}	0.024	3.9^{b}	0.76	211^{b}	41.8	15.5	1.45	84.4	1.52	29.5	2.57	0.25	0.011
	25	AL	302	52.6	0.55^{a}	0.024	7.0	0.76	729^{a}	41.8	26.3	1.45	73.7	1.52	35.3	2.57	0.26	0.011
		T	251	52.6	0.52 ^{ab}	0.024	6.4	0.76	559 ^{ab}	42.0	20.7	1.45	79.3	1.52	32.3	2.57	0.28	0.011
		R	308	52.6	0.50^{b}	0.024	5.6	0.76	378^{b}	41.8	19.1	1.45	81.0	1.52	39.2	2.57	0.27	0.011
B	14	AL	142	52.6	0.06	0.024	ND	—	ND	—	18.4	1.45	81.6	1.52	29.2	2.57	0.25	0.011
		T	119	52.5	0.05	0.024	ND	—	ND	—	11.4	1.59	88.5	1.66	27.8	2.58	0.25	0.011
		R	77	52.6	0.05	0.024	ND	—	ND	—	11.1	1.59	88.9	1.66	27.2	2.57	0.23	0.011
	16	AL	237	57.5	0.07	0.024	ND	—	ND	—	17.3	1.45	82.7	1.52	33.2	2.57	0.28	0.012
		T	312	52.6	0.07	0.024	ND	—	ND	—	14.7	1.59	89.1	1.52	34.9	2.57	0.28	0.011

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TABLE 3 (*Continued*) Plasma estradiol concentrations, ovary weight, number of follicles, and body composition of Lohmann LSL lite (W) and Lohmann Brown lite (B) birds reared under 3 feeding trajectories: *ad libitum* (AL), breeder's recommended target (T) and Restricted (R) at 14, 16, 18, 20, 22, and 25 weeks of age. Values correspond to the mean ± SEM ($n = 6$ birds/treatment/strain). NS, not significant; ND, not detected, E_2, estradiol; LYFs, large yellow follicles; SWFs, small white follicles; BMC, bone mineral content; BMD, bone mineral density. Different letters indicate significant differences in the appropriate age.

Strain	Age (wk)	Treatment	Follicles								Tissue				Bone			
			E_2	SEM	Ovary	SEM	LYFs	SEM	SWFs	SEM	Fat	SEM	Lean	SEM	BMC	SEM	BMD	SEM
			pg/mL		% BW						%		%		g		g/cm^2	
		R	114	52.6	0.05	0.024	ND	—	ND	—	9.2	1.59	90.7	1.66	34.5	2.57	0.28	0.011
	18	AL	490	52.5	0.16	0.024	0.7	0.76	204	41.7	17.4	1.45	82.6	1.52	38.3	2.57	0.31	0.011
		T	431	52.6	0.09	0.026	1.4	0.83	216	42.0	14.3	1.45	85.2	1.66	45.2	2.81	0.30	0.011
		R	269	52.6	0.06	0.024	0.0	0.76	134	42.0	10.6	3.53	89.4	3.70	32.1	2.57	0.26	0.011
	20	AL	330	52.6	0.27^{a}	0.024	7.7^{a}	0.76	290	41.8	18.3	1.45	81.7	1.52	38.9	2.57	0.28	0.011
		T	520	52.5	0.26^{a}	0.024	7.0 ^{ab}	0.76	297	41.7	12.8	1.45	87.3	1.52	42.8	2.57	0.30	0.011
		R	543	52.6	0.15^{b}	0.024	3.8^{b}	0.76	123	42.0	9.6	1.77	90.4	2.14	39.6	2.81	0.32	0.011
	22	AL	355	52.6	0.31^{a}	0.024	8.2^{a}	0.76	329	41.8	15.0	1.45	85.0	1.52	32.7	2.57	0.26	0.011
		T	430	52.6	0.33^{a}	0.024	7.8^{a}	0.76	256	41.8	16.0	1.45	84.0	1.52	35.0	2.57	0.28	0.011
		R	556	57.5	0.25^{b}	0.026	3.4^{b}	0.83	223	45.3	10.6	1.77	89.8	1.66	39.6	2.81	0.27	0.012
	25	AL	288	52.6	0.32	0.024	7.3	0.76	438	41.8	15.1	1.45	84.9	1.52	26.8^{b}	2.57	0.28	0.011
		T	294	52.5	0.35	0.024	6.4	0.76	387	41.7	12.4	1.45	87.6	1.52	38.6 ^{ab}	2.57	0.30	0.011
		R	387	52.6	0.31	0.024	5.7	0.76	303	42.0	11.1	1.59	88.9	1.66	43.1^{a}	2.57	0.30	0.011

Source of variation	*p-value*							
Age	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Treatment	NS	<0.001	<0.001	<0.001	<0.001	<0.001	<0.05	<0.05
Strain	NS	<0.001	NS	NS	<0.05	<0.05	<0.001	<0.01
Age*Treatment	<0.001	<0.001	<0.001	NS	NS	NS	<0.01	NS
Age*Strain	NS	<0.001	<0.05	<0.001	<0.001	<0.001	NS	NS
Treatment*Strain	NS	NS	NS	NS	NS	NS	NS	NS
Age*Treatment*Strain	NS	NS	NS	NS	<0.05	<0.05	NS	NS

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TABLE 4 Correlation between plasma estradiol concentrations and bone parameters of Lohmann LSL lite (W) and Lohmann Brown lite (B) birds reared under 3 feeding trajectories: *ad libitum* (AL), breeder's recommended target (T) and Restricted (R) before and after maturation. E_2, estradiol; BMC, bone mineral content; BMD, bone mineral density. *$p < 0.05$;** $p < 0.001$;*** $p < 0.0001$.

	Ad Libitum			Target			Restricted		
Trait	E_2	BMC	BMD	E_2	BMC	BMD	E_2	BMC	BMD
E_2	1	0.33*	0.366*	1	0.342**	0.321**	1	0.467***	0.415**
BMC		1	0.667***		1	0.745***		1	0.781***
BMD			1			1			1

proposed in quail (Zelenka et al., 1984; Yannakopoulos et al., 1995) and Yannakopoulos et al. (1995) specified that quail with lower body fat content displayed a delayed AFE, as they had not yet met the minimum body fat threshold. Lean tissue weight, representing protein levels in the body, was increased for AL and T treatments. However, rather than inducing sexual maturation, it may correspond to the development of reproductive organs, especially the oviduct. We further found that the ovary weight was higher in AL and T birds compared to R. Kwakkel et al. (1993) speculated that protein deposition in white leghorn pullets after 11 woa could be due to sexual maturity and reflect the development of the reproductive tract. In our case, total lean weight was higher in AL birds, but a concurrent elevation in fat deposition in this treatment resulted in an overall lower lean percentage compared to T and R hens. Eitan et al. (2014) emphasized the importance of the lean mass on sexual maturation in broiler breeders. The authors suggested that a body lean threshold is required to regulate sexual maturation rather than body fat content. However, our data suggest that rather than fat and lean content, the ratio (%) may be more important when a minimum BW has been achieved in layers.

Our results show that AL birds had a higher BW at first egg and displayed advanced maturations by 10.7 days in W and 16.7 days in B birds compared to those reared under R. Additionally, within B hens, AFE was further advanced by 2.9 days between T and AL. Interestingly, this corresponded to the observed increased growth trajectory and fat deposition for these birds under AL feeding. Surprisingly, the narrow 64 g BW threshold associated with sexual maturation reported by Hanlon et al. (2021) in white Leghorns derivatives, including the same W strain, was not observed in the current study. Though, in the present study, all W hens entered lay between 1.40 and 1.59 kg depending on treatment, which is comparable to the 1.43–1.49 kg BW range reported by Hanlon et al. (2021). Interestingly, the impact of treatment on BW-FE observed for B birds was much larger than for W suggesting that the possible BW threshold proposed by Hanlon et al. (2021) may depend on the strain (W *versus* B) and the growth trajectory. However, the impact of BC may be more consistent across strain and growth trajectory as we report that 10%–15% fat deposition is necessary for the onset of sexual maturations.

Beyond AFE, AL feeding was associated with improved development of reproductive organs with birds in the AL groups showing a higher ovary weight and the number of LYFs which resulted in higher egg productions irrespective of the strain. Furthermore, Time restricted feeding in laying hens resulted in lower BW, lighter egg weight, reduced egg production by 3.8% and persistency of egg laying by an average of 2.1% (Saibaba et al., 2021). In addition, previous studies in broiler breeders showed that high feed intake during the pubertal period elevates the number of hierarchal follicles (Hocking et al., 1987; Katanbaf et al., 1989; Yu et al., 1992) and accelerates the sexual maturation process (Wilson and Harms, 1986; Yu et al., 1992). Hocking (1993) reported that birds with a higher BW had more LYFs and a positive linear connection between the number of LYFs, BW and food intake was observed.

One key process during sexual maturation is the production of E_2 by SWFs. In turn, E_2 prepares the hen for active egg laying by switching the physiology and nutrient partitioning toward the synthesis of egg components (Hanlon et al., 2022) through the alteration of liver and bone physiology. During sexual maturation, E_2 stimulates genes responsible for egg yolk and egg white production by binding to its receptor (ERα) in the liver (Bergink and Wallace, 1974; Kirchgessner et al., 1987; Flouriot et al., 1996) and transporting these components to the oviduct (Ratna et al., 2016). We report here that birds in the W group had an overall higher plasma E_2 than those in B. However, the lack of photostimulation most likely resulted in a lack of synchronization within the treatment and a distinct identifiable peak in E_2 could not be observed in the focal birds. Non-etheless, in the sampled birds, the increase in E_2 concentration was delayed by a couple of weeks in R birds. The delay in egg production in R birds in the current study could be due to the delay in the production of E_2 irrespective of the strains as it was reported in broiler breeders that birds receiving R feed during the rearing period had delayed E_2 production followed by later AFE (Onagbesan et al., 2006), but higher cumulative egg production (van der Klein et al., 2018b). Unfortunately, this study only focused on the period leading up to peak production, and further studies should consider the impact on the remainder of the laying cycle.

In addition to lean and fat carcass content, the bone health of laying hens is critical to reproductive success. Due to the amount of Ca required for eggshell formation, high-producing strains of layer chickens are prone to Ca imbalance, which can result in osteoporosis near the end of the laying cycle (Webster, 2004). The onset of this disorder is said to be dependent on both laying performance and plasma E_2 concentrations (Eusemann et al., 2022). The rise in plasma E_2 at the initiation of the laying cycle shifts skeletal development from longitudinal growth to medullary formation, which results in calcium storage (Miller and Bowman, 1981; Dacke et al., 1993; Whitehead and Fleming, 2000) to support eggshell formation. Thus, exogenous administration of E_2 in laying hens and roosters resulted in weaker bone strength

compared to the untreated birds (Urist and Deutsch, 1960; Chen et al., 2014). E_2 also elevates the levels of calcitriol, the active form of vitamin D, receptors in the intestine mucosa to enhance the uptake of dietary sources of Ca (for review: Hanlon et al., 2022). Hanlon et al. (2021), Hanlon et al. (2022) reported that the modern strains of layers exhibit recurrent elevations of E_2 throughout the laying cycle, which may trigger the medullary bone formation to help provide an adequate Ca source during the laying period. They further suggested that the recurrent elevations of E_2 positively correlated with medullary BMD and negatively correlated with cortical BMD.

However, although DXA provides an overall BMC and BMD value, our results demonstrated that B birds had higher BMC and BMD compared to W birds. BMC and BMD were lower at 18 woa and higher at 25 woa in R birds. Thus, the increased levels of BMC and BMD in both strains under R compared to AL birds could result from the elevations of plasma E_2 concentrations at 22 woa. Furthermore, the results of the current study revealed a positive correlation between E_2 and BMC and BMD which could be due to the development of medullary bone formation to support egg production as proposed by Hanlon et al. (2022), demonstrating a positive correlation between E_2 and medullary BMC and BMD through a 100-week laying cycle of hens to provide enough amount of Ca for the support of egg production.

5 Conclusion

Feed allocation impacted growth and BC in a strain-dependent manner, resulting in differing sexual maturation and egg production. Specifically, higher BW, body fat percentage, and plasma E_2 concentrations increased egg production and advanced AFE in birds which were reared under AL feeding conditions, which was more pronounced in B. Therefore, a body fat threshold between 10% to 15% appears to be required for the occurrence of spontaneously sexual maturation in laying hens. Furthermore, the positive correlation of plasma E_2 with BMC and BMD highlights the importance of E_2 and BC on sexual maturation in laying hens.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the University of Guelph Animal Care Committee.

Author contributions

GB designed the study and secured funding. MB, CH, CZ, SS, and GB all participated in the execution of the study. MB was the primary writer and conducted the analyses with the support of CZ and GB. GB and CH participated in the redaction and editing of the manuscript. GB supported MB throughout the project as the supervisor. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Baxter, M., and Bédécarrats, G. Y. (2019). Evaluation of the impact of light source on reproductive parameters in laying hens housed in individual cages. *J. Poult. Sci.* 56, 148–158. doi:10.2141/jpsa.0180054

Baxter, M., Joseph, N., Osborne, V. R., and Bédécarrats, G. Y. (2014). Red light is necessary to activate the reproductive axis in chickens independently of the retina of the eye. *Poult. Sci.* 93, 1289–1297. doi:10.3382/ps.2013-03799

Bédécarrats, G. Y. (2015). Control of the reproductive axis: Balancing act between stimulatory and inhibitory inputs. *Poult. Sci.* 94, 810–815. doi:10.3382/ps/peu042

Bentley, G. E., Perfito, N., Ukena, K., Tsutsui, K., and Wingfield, J. C. (2003). Gonadotropin-inhibitory peptide in song sparrows (Melospiza melodia) in different reproductive conditions, and in house sparrows (*Passer domesticus*) relative to chicken-gonadotropin-releasing hormone. *J. Neuroendocrinol.* 15, 794–802. doi:10.1046/j.1365-2826.2003.01062.x

Bentley, G. E., Ubuka, T., McGuire, N. L., Chowdhury, V. S., Morita, Y., Yano, T., et al. (2008). Gonadotropin-inhibitory hormone and its receptor in the avian reproductive system. *Gen. Comp. Endocrinol.* 156, 34–43. doi:10.1016/j.ygcen.2007.10.003

Bergink, E. W., and Wallace, R. A. (1974). Precursor product relationship between amphibian vitellogenin and the yolk proteins, lipovitellin and phosvitin. *J. Biol. Chem.* 249, 2897–2903. doi:10.1016/s0021-9258(19)42715-4

Bornstein, S., Plavnik, I., and Lev, Y. (1984). Body weight and/or fatness as potential determinants of the onset of egg production in broiler breeder hens. *Br. Poult. Sci.* 25, 323–341. doi:10.1080/00071668408454873

Brody, T. B., Siegel, P. B., and Cherry, J. A. (1984). Age, body weight and body composition requirements for the onset of sexual maturity of dwarf and normal chickens. *Br. Poult. Sci.* 25, 245–252. doi:10.1080/00071668408454863

Brody, T., Eitan, Y., Soller, M., Nir, I., and Nitsan, Z. (1980). Compensatory growth and sexual maturity in broiler females reared under severe food restriction from day of hatching. *Br. Poult. Sci.* 21, 437–446. doi:10.1080/00071668008416694

Canadian Council on Animal Care (2009). *CCAC guidelines on: The care and use of farm animals in research, teaching, and testing*. Berlin, Germany: Springer.

Chaney, L. W., and Fuller, H. L. (1975). The relation of obesity to egg production in broiler breeders. *Poult. Sci.* 54, 200–208. doi:10.3382/ps.0540200

Chen, S. Y., Li, T. Y., Tsai, C. H., Lo, D. Y., and Chen, K. L. (2014). Gender, caponization and exogenous estrogen effects on lipids, bone and blood characteristics in Taiwan country chickens. *Anim. Sci. J.* 85, 305–312. doi:10.1111/asj.12147

Chowdhury, V. S., Tomonaga, S., Nishimura, S., Tabata, S., Cockrem, J. F., Tsutsui, K., et al. (2012). Hypothalamic gonadotropin-inhibitory hormone precursor mRNA is increased during depressed food intake in heat-exposed chicks. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 162, 227–233. doi:10.1016/j.cbpa.2012.03.009

Ciccone, N. A., Dunn, I. C., Boswell, T., Tsutsui, K., Ubuka, T., Ukena, K., et al. (2004). Gonadotrophin inhibitory hormone depresses gonadotrophin α and follicle-stimulating hormone β subunit expression in the pituitary of the domestic chicken. *J. Neuroendocrinol.* 16, 999–1006. doi:10.1111/j.1365-2826.2005.01260.x

Dacke, C. G., Arkle, S., Cook, D. J., Wormstone, I. M., Jones, S., Zaidi, M., et al. (1993). Medullary bone and avian calcium regulation. *J. Exp. Biol.* 88, 63–88. doi:10.1242/jeb.184.1.63

Eitan, Y., Lipkin, E., and Soller, M. (2014). Body composition and reproductive performance at entry into lay of anno 1980 versus anno 2000 broiler breeder females under fast and slow release from feed restriction. *Poult. Sci.* 93, 1227–1235. doi:10.3382/ps.2013-03239

Eusemann, B. K., Patt, A., Schrader, L., Weigend, S., Thöne Reineke, C., and Petow, S. (2020). The role of egg production in the etiology of keel bone damage in laying hens. *Front. Vet. Sci.* 7, 81. doi:10.3389/fvets.2020.00081

Eusemann, B. K., Ulrich, R., Sanchez-Rodriguez, E., Benavides-Reyes, C., Dominguez-Gasca, N., Rodriguez-Navarro, A. B., et al. (2022). Bone quality and composition are influenced by egg production, layer line, and oestradiol-17ß in laying hens. *Avian Pathol.* 51, 267–282. doi:10.1080/03079457.2022.2050671

Flouriot, G., Pakdel, F., and Valotaire, Y. (1996). Transcriptional and post-transcriptional regulation of rainbow trout estrogen receptor and vitellogenin gene expression. *Mol. Cell Endocrinol.* 124, 173–183. doi:10.1016/s0303-7207(96)03960-3

Gous, R. M., and Cherry, P. (2004). Effects of body weight at, and lighting regimen and growth curve to, 20 weeks on laying performance in broiler breeders. *Br. Poult. Sci.* 45, 445–452. doi:10.1080/00071660400001256

Habig, C., Baulain, U., Henning, M., Scholz, A. M., Sharifi, A. R., Janisch, S., et al. (2017). How bone stability in laying hens is affected by phylogenetic background and performance level. *Eur. Poult. Sci.* 81, 200. doi:10.1399/eps.2017.200

Hadinia, S. H., Carneiro, P. R. O., Fitzsimmons, C. J., Bédécarrats, G. Y., and Zuidhof, M. J. (2020). Post-photostimulation energy intake accelerated pubertal development in broiler breeder pullets. *Poult. Sci.* 99, 2215–2229. doi:10.1016/j.psj.2019.11.065

Hanlon, C., Ramachandran, R., Zuidhof, M. J., and Bédécarrats, G. Y. (2020). Should I lay or should I grow: Photoperiodic versus metabolic cues in chickens. *Front. Physiol.* 11, 707. doi:10.3389/fphys.2020.00707

Hanlon, C., Takeshima, K., and Bédécarrats, G. Y. (2021). Changes in the control of the hypothalamic-pituitary gonadal Axis across three differentially selected strains of laying hens (Gallus gallus domesticus). *Front. Physiol.* 12, 651491. doi:10.3389/fphys.2021.651491

Hanlon, C., Takeshima, K., Kiarie, E. G., and Bédécarrats, G. Y. (2022). Bone and eggshell quality throughout an extended laying cycle in three strainsof layers spanning 50 years of selection. *Poult. Sci.* 101, 101672. doi:10.1016/j.psj.2021.101672

Heijmans, J., Duijster, M., Gerrits, W. J. J., Kemp, B., Kwakkel, R. P., and van den Brand, H. (2021). Impact of growth curve and dietary energy-to-protein ratio on productive performance of broiler breeders. *Poult. Sci.* 100, 101131. doi:10.1016/j.psj.2021.101131

Hocking, P. M., Bain, M., Channing, C. E., Fleming, R., and Wilson, S. (2003). Genetic variation for egg production, egg quality and bone strength in selected and traditional breeds of laying fowl. *Br. Poult. Sci.* 44, 365–373. doi:10.1080/0007166031000085535

Hocking, P. M. (1993). Effects of body weight at sexual maturity and the degree and age of restriction during rearing on the ovarian follicular hierarchy of broiler breeder females. *Br. Poult. Sci.* 34, 793–801. doi:10.1080/00071669308417638

Hocking, P. M., Gilbert, A. B., Walker, M., and Waddington, D. (1987). Ovarian follicular structure of white leghorns fed ad libitum and dwarf and normal broiler breeders fed ad libitum or restricted until point of lay. *Br. Poult. Sci.* 28, 493–506. doi:10.1080/00071668708416983

Hocking, P. M., Waddington, D., Walker, M. A., and Gilbert, A. B. (1989). Control of the development of the ovarian follicular hierarchy in broiler breeder pullets by food restriction during rearing. *Br. Poult. Sci.* 30, 161–173. doi:10.1080/00071668908417134

Ikemoto, T., and Park, M. K. (2005). Chicken RFamide-related peptide (GnIH) and two distinct receptor subtypes: Identification, molecular characterization, and evolutionary considerations. *J. Reprod. Dev.* 51, 359–377. doi:10.1262/jrd.16087

Johnson, A. L., Bridgham, J. T., Witty, J. P., and Tilly, J. L. (1996). Susceptibility of avian ovarian granulosa cells to apoptosis is dependent upon stage of follicle development and is related to endogenous levels of bcl-xlong gene expression. *Endocrinology* 137 (5), 2059–2066. doi:10.1210/endo.137.5.8612548

Johnson, A. L. (1993). Regulation of follicle differentiation by gonadotropins and growth factors. *Poult. Sci.* 72, 867–873. doi:10.3382/ps.0720867

Katanbaf, M. N., Dunnington, E. A., and Siegel, P. B. (1989). Restricted feeding in early and late-feathering chickens. 2. reproductive responses. *Poult. Sci.* 68, 352–358. doi:10.3382/ps.0680352

Kirchgessner, T. G., Heinzmann, C., Svenson, K. L., Gordonb, D. A., Nicosiab, M., Lebherz', H. G., et al. (1987). Regulation of chicken apolipoprotein B: Cloning, tissue distribution, and estrogen induction of mRNA. *Gene* 9, 241–251. doi:10.1016/0378-1119(87)90332-5

Kwakkel, R. P., Ducro, B. J., and Koops, W. J. (1993). Multiphasic analysis of growth of the body and its chemical components in white leghorn pullets. *Poult. Sci.* 72, 1421–1432. doi:10.3382/ps.0721421

Kwakkel, R. P., van Esch, J. A. W., Ducro, B. J., and Koops, W. J. (1995). Onset of lay related to multiphasic growth and body composition in white leghorn pullets provided ad libitum and restricted diets. *Poult. Sci.* 74, 821–832. doi:10.3382/ps.0740821

Miller, S. C., and Bowman, B. M. (1981). Medullary bone osteogenesis following estrogen administration to mature male Japanese quail. *Devl. Biol.* 87, 52–63. doi:10.1016/0012-1606(81)90060-9

Miller, S. C. (1977). Osteoclast cell-surface changes during the egg laying cycle in Japanese quail. *J. Cell Biol.* 75, 104–118. doi:10.1083/jcb.75.1.104

Mueller, W. J., Schraer, R., and Schraer, H. (1964). Calcium metabolism and skeletal dynamics of laying pullets. *J. Nutr.* 84, 20–26. doi:10.1093/jn/84.1.20

Murugesan, G. R., and Persia, M. E. (2013). Validation of the effects of small differences in dietary metabolizable energy and feed restriction in first-cycle laying hens. *Poult. Sci.* 92, 1238–1243. doi:10.3382/ps.2012-02719

National Research Council (1994). *Nutrient requirements of poultry*. 9th Edn. Washington, DC: National Academy Press.

Onagbesan, O. M., Metayer, S., Tona, K., Williams, J., Decuypere, E., and Bruggeman, V. (2006). Effects of genotype and feed allowance on plasma luteinizing hormones, follicle-stimulating hormones, progesterone, estradiol levels, follicle differentiation, and egg production rates of broiler breeder hens. *Poult. Sci.* 85, 1245–1258. doi:10.1093/ps/85.7.1245

Ratna, W. N., Bhatt, V. D., Chaudhary, K., Ariff, A., Bavadekar, S. A., and Ratna, H. N. (2016). Estrogen-responsive genes encoding egg yolk proteins vitellogenin and apolipoprotein II in chicken are differentially regulated by selective estrogen receptor modulators. *Theriogenology* 85, 376–383. doi:10.1016/j.theriogenology.2015.08.015

Reddish, J. M., Nestor, K. E., and Lilburn, M. S. (2003). Effect of selection for growth on onset of sexual maturity in randombred and growth-selected lines of Japanese quail. *Poult. Sci.* 82, 187–191. doi:10.1093/ps/82.2.187

Renema, R. A., Robinson, F. E., and Goerzen, P. R. (2001). Effects of altering growth curve and age at photostimulation in female broiler breeders. 1. Reproductive development. *Can. J. Anim. Sci.* 81 (4), 467–476. doi:10.4141/A00-109

Robinson, F. E., and Etches, R. J. (1986). Ovarian steroidogenesis during follicular maturation in the domestic fowl (*Gallus domesticus*). *Biol. Reprod.* 35, 1096–1105. doi:10.1095/biolreprod35.5.1096

Robinson, F. E., Zuidhof, M. J., and Renema, R. A. (2007). Reproductive efficiency and metabolism of female broiler breeders as affected by genotype, feed allocation, and age at photostimulation. 1. Pullet growth and development. *Poult. Sci.* 86, 2256–2266. doi:10.1093/ps/86.10.2256

Saibaba, G., Ruzal, M., Shinder, D., Yosefi, S., Druyan, S., Arazi, H., et al. (2021). Time-restricted feeding in commercial layer chickens improves egg quality in old age and points to lack of adipostat activity in chickens. *Front. Physiol.* 12, 651738. doi:10.3389/fphys.2021.651738

Salas, C., Ekmay, R. D., England, J., Cerrate, S., and Coon, C. N. (2019). Effect of body weight and energy intake on body composition analysis of broiler breeder hens. *Poult. Sci.* 98, 796–802. doi:10.3382/ps/pey377

Sato, K., Abe, H., Kono, T., Yamazaki, M., Nakashima, K., Kamada, T., et al. (2009). Changes in peroxisome proliferator-activated receptor gamma gene expression of chicken abdominal adipose tissue with different age, sex and genotype. *Anim. Sci. J.* 80, 322–327. doi:10.1111/j.1740-0929.2009.00639.x

Soller, M., Eitan, Y., and Brody, T. (1984). Effect of diet and early quantitative feed restriction on the minimum weight requirement for onset of sexual maturity in white rock broiler breeders. *Poult. Sci.* 63, 1255–1261. doi:10.3382/ps.0631255

Spratt, R. S., and Leeson, S. (1987). Broiler breeder performance in response to diet protein and energy. *Poult. Sci.* 66, 683–693. doi:10.3382/ps.0660683

Sun, J., and Coon, C. N. (2005). The effects of body weight, dietary fat, and feed withdrawal rate on the performance of broiler breeders. *J. Appl. Poult. Res.* 14, 728–739. doi:10.1093/japr/14.4.728

Swennen, Q., Janssens, G. P. J., Geers, R., Decuypere, E., and Buyse, J. (2004). Validation of dual-energy x-ray absorptiometry for determining *in vivo* body composition of chickens. *Poult. Sci.* 83, 1348–1357. doi:10.1093/ps/83.8.1348

Tachibana, T., Sato, M., Takahashi, H., Ukena, K., Tsutsui, K., and Furuse, M. (2005). Gonadotropin-inhibiting hormone stimulates feeding behavior in chicks. *Brain Res.* 1050, 94–100. doi:10.1016/j.brainres.2005.05.035

Tsutsui, K., Saigoh, E., Ukena, K., Teranishi, H., Fujisawa, Y., Kikuchi, M., et al. (2000). A novel avian hypothalamic peptide inhibiting gonadotropin release. *Biochem. Biophys. Res. Commun.* 275, 661–667. doi:10.1006/bbrc.2000.3350

Ubuka, T., Ukena, K., Sharp, P. J., Bentley, G. E., and Tsutsui, K. (2006). Gonadotropin-inhibitory hormone inhibits gonadal development and maintenance by decreasing gonadotropin synthesis and release in male quail. *Endocrinology* 147, 1187–1194. doi:10.1210/en.2005-1178

Urist, M. R., and Deutsch, N. M. (1960). Osteoporosis in the laying hen. *Endocrinology* 66, 377–391. doi:10.1210/endo-66-3-377

van der Klein, S. A. S., Bédécarrats, G. Y., Robinson, F. E., and Zuidhof, M. J. (2018b). Early photostimulation at the recommended body weight reduced broiler breeder performance. *Poult. Sci.* 97, 3736–3745. doi:10.3382/ps/pey215

van der Klein, S. A. S., Bédécarrats, G. Y., and Zuidhof, M. J. (2018a). The effect of rearing photoperiod on broiler breeder reproductive performance depended on body weight. *Poult. Sci.* 97, 3286–3294. doi:10.3382/ps/pey199

van Emous, R. A., de La Cruz, C. E., and Naranjo, V. D. (2018). Effects of dietary protein level and age at photo stimulation on reproduction traits of broiler breeders and progeny performance. *Poult. Sci.* 97, 1968–1979. doi:10.3382/ps/pey053

van Emous, R. A., Kwakkel, R. P., van Krimpen, M. M., and Hendriksf, W. H. (2013). Effects of growth patterns and dietary crude protein levels during rearing on body composition and performance in broiler breeder females during the rearing and laying period. *Poult. Sci.* 92, 2091–2100. doi:10.3382/ps.2012-02987

Webster, A. B. (2004). Welfare implications of avian osteoporosis. *Poult. Sci.* 83, 184–192. doi:10.1093/ps/83.2.184

Whitehead, C. C., and Fleming, R. H. (2000). Osteoporosis in cage layers. *Poult. Sci.* 79, 1033–1041. doi:10.1093/ps/79.7.1033

Williams, J. B., and Sharp, P. J. (1978). Ovarian morphology and rates of ovarian follicular development in laying broiler breeders and commercial egg-producing hens. *Br. Poult. Sci.* 19, 387–395. doi:10.1080/00071667808416490

Wilson, H. R., and Harms, R. H. (1986). Performance of broiler breeders as affected by body weight during the breeding season. *Poult. Sci.* 65 (6), 1052–1057. doi:10.3382/ps.0651052

Yang, S., Suh, Y., Choi, Y. M., Shin, S., Han, J. Y., and Lee, K. (2013). Loss of fat with increased adipose triglyceride lipase-mediated lipolysis in adipose tissue during laying stages in quail. *Lipids* 48, 13–21. doi:10.1007/s11745-012-3742-6

Yannakopoulos, A. L., Christaki, E., and Florou-Paneri, P. (1995). Effect of age and carcase composition on the onset of sexual maturity in quail under normal feeding regimens. *Br. Poult. Sci.* 36, 771–777. doi:10.1080/00071669508417821

Yu, M. W., Robinson, F. E., and Robblee, A. R. (1992). Effect of feed allowance during rearing and breeding on female broiler breeders. 1. growth and carcass characteristics. *Poult. Sci.* 71, 1739–1749. doi:10.3382/ps.0711739

Zelenka, D. J., Cherry, J. A., Nir, I., and Siegel, P. B. (1984). Bodyweight and composition of Japanese quail (Coturnix coturnixjaponica) at sexual maturity. *Growth* 48, 16–28.

Zuidhof, M. J. (2018). Lifetime productivity of conventionally and precision-fed broiler breeders. *Poult. Sci.* 97, 3921–3937. doi:10.3382/ps/pey252

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EDITED BY
Andras Csillag,
Semmelweis University, Hungary

REVIEWED BY
Loreta Medina,
Universitat de Lleida, Spain
Koichi J. Homma,
Teikyo University, Japan
Diego Echevarria,
Miguel Hernández University of Elche, Spain

*CORRESPONDENCE
Krystyna Pierzchała-Koziec,
rzkoziec@cyf-kr.edu.pl
Colin G. Scanes,
scanes@uwm.edu

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Avian opioid peptides: evolutionary considerations, functional roles and a challenge to address critical questions

Krystyna Pierzchała-Koziec[1]* and Colin G. Scanes[2]*

[1]Department of Animal Physiology and Endocrinology, University of Agriculture in Krakow, Kraków, Poland, [2]Colin G. Scanes, Department of Biological Science, University of Wisconsin Milwaukee, Milwaukee, WI, United States

The present review considers the putative hormonal opioid peptides in birds. In birds and all other vertebrates, there are four opioid related genes encoding a series of peptides. These genes are, respectively, proenkephalin (PENK), prodynorphin (PDYN), pronociceptin (PNOC) and proopiomelanocortin (POMC). Proenkephalin (PENK) encodes Met- and Leu-enkephalin together with peptides containing met enkephalin motifs in birds, mammals and reptiles. Proopiomelanocortin (POMC) encodes β endorphin together with adrenocorticotropic hormone (ACTH), and melanocyte stimulating hormone (MSH). Prodynorphin (PDYN) encoding dynorphins A and B with α- and β-neoendorphins together intermediate polypeptides across the vertebrates. Pronociceptin (PNOC) encodes nociceptin together with possibly putative avian nocistatin and a non-opioid peptide derived from the C terminal of pronociceptin. There is a high degree of identity in the sequences of enkephalin peptides, dynorphin-A and B and nociceptin in birds and, to a less extent, across vertebrates. The opioid peptides exert effects related to pain together with other biological actions such as growth/development acting via a series of opioid receptors. What is unclear, particularly in birds, is the biological roles and interactions (additivity, antagonistic and synergistic) for the individual opioid peptides, the processing of the prohormones in different tissues and the physiological relevance of the different peptides and, particularly, of the circulating forms.

KEYWORDS

enkephalin, dynorphin, nociceptin, birds, chicken

1 Introduction

This review will consider the neuropeptides from PENK, PDYN, and PNOC focusing on these in birds. With one exception, these peptides contain an enkephalin motif; this being an tetrapeptide with tyrosine–glycine—glycine–phenylalanine residues (YGGF). Moreover, a series of questions will be asked. It is noted that we have previous reviewed the peptides from POMC and, hence, these will not be discussed in the present discussion (Scanes and Pierzchała -Koziec, 2018; Scanes and Pierzchała -Koziec, 2021).

Four genes have been identified that encode opioid peptides. These are the following:

- Proenkephalin (PENK) encoding Met- and Leu-enkephalin,
- Proopiomelanocortin (POMC) encoding β-endorphin, adrenocorticotropic hormone (ACTH), and melanocyte stimulating hormone (MSH),

- Prodynorphin (PDYN) encoding Dynorphin-A and B together with α-Neoendorphin and β-Neoendorphin,
- Pronociceptin (PNOC) encoding Nociceptin/Orphanin FQ together with a putative avian nocistatin and a possibly biologically active C terminal peptide (reviewed: Bu et al., 2020; Dhaliwal and Gupta, 2022).

There is some evidence for other opioid like peptides. For example, Zadina and colleagues (1997) reported two peptides with opioid activity, namely, endomorphin-1 (YPWF-NH_2) and endomorphin-2 (YPFF-NH_2). However, a gene(s) encoding endomorphin-1 and endomorphin-2 has not been yet identified. Other endomorphin-like peptides have been reported, specifically mexneurin 1 (Mx 1), Mx 2, and Mx 3. These are encoded by prepromexneurin (Matus-Ortega et al., 2017).

There are four major types of opioid receptors. These G protein-coupled receptors include the following:

- Delta opioid receptors (DOR) binding Met- and Leu-enkephalin,
  - o Sub-types
    - Delta 1
    - Delta 2
- Mu opioid receptors (MOR) binding β endorphin together with both endomorphin 1 and 2,
  - o Sub-types
    - Mu 1
    - Mu 2
    - Mu 3
- Kappa opioid receptors (KOR) binding dynorphin-A and B,
  - o Sub-types
    - Kappa 1
    - Kappa 2
    - Kappa 3
- Nociceptin receptors (NOR) (naloxone insensitive) binding nociceptin

(reviewed: Bu et al., 2020; Dhaliwal and Gupta, 2022). In addition, there is a zeta opioid receptor (reviewed Dhaliwal and Gupta, 2022).

1.1 Evolution of PENK, PDYN, PNOC, and POMC genes

Proenkephalin, prodynorphin, pronociceptin, and proopiomelanocortin (POMC) not only share enkephalin motifs but also cysteine residues at similar points in their sequences (reviewed: Fricker et al., 2022). The basis for four opioid genes and, also, the four receptors are two separate gene duplication early in vertebrate evolution (Sundström et al., 2010).

1.2 Converting enzymes

Proenkephalin, prodynorphin, and pronociceptin can be cleaved by a series of cysteine proteases/thiol proteases/convertases acting at both monobasic and dibasic sites. This generates a series of neuropeptides depending on the presence and specificity of the convertases. Different tissues can have different expression levels of proenkephalin and/or prodynorphin and/or pronociceptin and different convertases generating a series of neuropeptides (Day et al., 1998).

2 Proenkephalin (PENK) and the derived enkephalin neuropeptides

2.1 Structures and processing of proenkephalin and enkephalin neuropeptides derived from proenkephalin

Met-enkephalin is a pentapeptide with the following sequence of amino-acid residues: tyrosine–glycine-glycine- phenylalanine–methionine (YGGFM). Similarly, Leu-enkephalin is a pentapeptide with the following sequence of amino-acid residues: tyrosine–glycine-glycine- phenylalanine–leucine (YGGFL).

There are seven enkephalin motifs (YGGFM/L with two basic amino-acid residues adjacent to the ends of the motif) in avian proenkephalin (see Figure 1; Supplementary Figure S1). Proenkephalin can processed into the following:

- YGGFM x 4 [sites 1, 2, and 4 (Figure 1)]
- YGGFL [Site 5 (Figure 1)]
- YGGFMRF or YGGFMR [Site 6 (Figure 1)].

The possibility of additional biologically active products of proenkephalin are discussed below under "Biological activity of Met-enkephalin and other proenkephalin derived peptides".

There are also both glycosylation and phosphorylation sites within proenkephalin (reviewed: Fricker et al., 2022).

2.2 Evolution of enkephalin peptides

Four enkephalin peptides (YGGFM x 4, YGGFL, and YGGFMRF/YGGFMR), have identical sequences in birds, mammals and reptiles. Moreover, there are the same flanking basic amino acid pairs (Table 1). In contrast, the peptide (YGGFMRSI or YGGFMRSV) in birds and reptiles differs from that in mammals (Supplementary Figure S1). The regions of proenkephalin that are not part of enkephalin peptides show little variation across the class Aves and in reptiles (see Supplementary Figure S1).

It is noted that there are degenerate enkephalin motifs and/or the absence of flanking pairs of basic amino acid residues in both boney and cartilaginous fish (see Table 2; Supplementary Figure S1). Leu-enkephalin is not present in non-tetrapod sarcopterygians, actinopterygian fish or cartilaginous (Chondrichthyes) fish with Met-enkephalin replacing it (Table 2).

2.3 Converting enzymes and proenkephalin

There are multiple converting enzymes generating neuropeptides from proenkephalin (see Figure 1). For instance, cathepsin L in secretory vesicles in chromaffin granules converts proenkephalin to enkephalins (Yasothornsrikul et al., 2003).

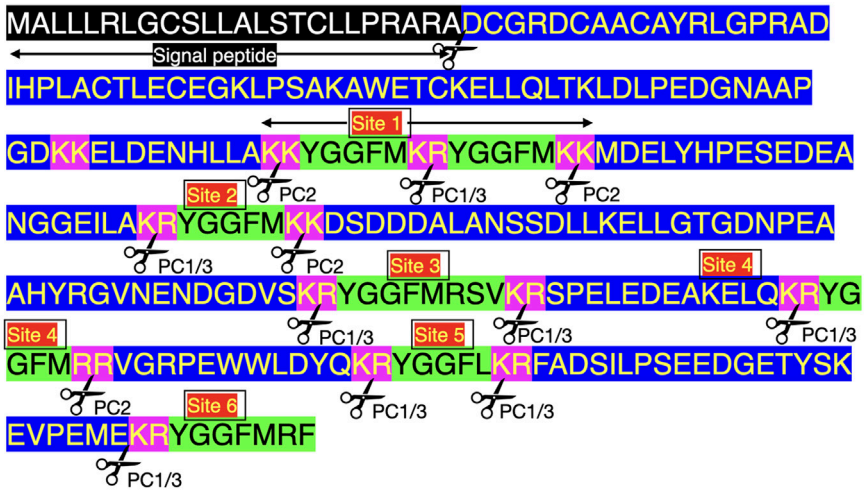

FIGURE 1
Structure of chicken preproenkephalin (deduced from mRNA Genbank XM_040664746). Key: PC prohormone convertase Pink highlighted indicates pairs of basic amino acid residues Green highlighted indicates enkephalin motifs Black highlighted indicates signal peptide Blue highlight other amino acid residues.

TABLE 1 Enkephalin peptides with flanking pairs of basic amino acid residues.

	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6
Enkephalin						
Aves	YGGFM x 2	YGGFM	YGGFMRSI	YGGFM	YGGFL	YGGFMRF
Mammalia			YGGFMRGL or YGGFMKSA			
Reptilia			YGGFMRSI			
Amphibia			YGGFMRDY or YGGFMRGS		YGGFM	
Non-tetrapod Sarcopterygii			YGGFMRSL			
Actinopterygii	YGGFM & YGGFT or YGGFMI	YGGFI or absent	e.g., YGGFM		Absent	YGGFMGY or YGGFMD
Chondrichthyes	YGGFM x 2	YGGFM	YGGFMNGF		YGGFM	YGGFMRI
Pairs of flanking basic amino acid residues						
Aves	KK & KK with KR between 2 motifs	KR & KK	KR & KR	KR & RR	KR & KR	KR
Mammalia						
Reptilia						
Amphibia					KR & RR	
Non-tetrapod Sarcopterygii	KR & KK with KR between 2 motifs					
Actinopterygii	KK & KK with KR between 2 motifs				Not applicable	
Chondrichthyes		KR & KK			KR & KR	

TABLE 2 Comparison of enkephalin motifs in proenkephalin and prodynorphin in vertebrate classes.

	Enkephalin motifs[a]	Met-enkephalin Motifs[b]	Leu-enkephalin Motifs[c]	Degenerate motifs[d]
Proenkephalin				
Mammalia	7	6	1	0
Aves	7	6	1	0
Reptilia	7	6	1	0
Amphibia	7	6	1	0
Non-tetrapod sarcopterygians	7	7	0	0
Actinopterygii	5	5	0	2
Chondrichthyes	7	7	0	0
Prodynorphin				
Mammalia	3	0	3	0
Aves	4	2	1	1
Reptilia	5	3[5]	1	0
Amphibia	4	2	2	0
Non-tetrapod sarcopterygians	4	0[5]	3	0
Actinopterygii	5	1[6]	2–3	1–2
Chondrichthyes	5	1–3	1	1–2

[a]YGGFM/L.
[b]K/RK/RYGGFMK/RK/R.
[c]K/RK/RYGGFLK/RK/R.
[d]GGFM, or YGGF, or GGF, with or without flanking pairs of basic amino-acid residues.
[e]Plus YGGFF.
[f]Plus YGGFI.

2.4 Biological activity of Met-enkephalin and other proenkephalin derived peptides

It is reasonable to assume that Met- and Leu-enkephalin play a role in reducing pain and associated responses in mammals (Cullen and Cascella, 2022; Dhaliwal and Gupta, 2022) and birds (Scanes and Pierzchała-Koziec, 2018).

There are also negative effects of Met-enkephalin on growth and development. For instance, Met-enkephalin inhibits angiogenesis in the chorioallantoic membrane of chick embryo with the effect reduced in the presence of naltrexone (Blebea et al., 2000). Moreover, Met-enkephalin exerts an anti-proliferative effect on cultured adrenocortical cells (rat: Malendowicz et al., 2005). Furthermore, Met-enkephalin depresses proliferation of peripheral blood T cells based in the elevated proliferation following application of anti-sense oligonucleotide (humans: Kamphuis et al., 1998). In contrast, Met-enkephalin stimulated proliferation by human peripheral lymphocytes (Hucklebridge et al., 1989).

It is assumed that proenkephalin derived peptides act via δ opioid receptors; with met- and leu-enkephalin having similar activities in both a cortical acetylcholine release assay in rats (Jhamandas and Sutak, 1980) and evoking a response with chicken δ opioid receptors (Bu et al., 2020). While this is probably the case with Met-and Leu-enkephalin, there is evidence that other proenkephalin derived peptides act via κ or μ opioid receptors. Fragments of proenkephalin have been isolated from bovine adrenal medullary tissue; these containing at least one enkephalin motif (Mizuno et al., 1980a; Mizuno et al., 1980b; Kilpatrick et al., 1981) (Table 3). Superficially, it would be thought that these endogenous fragments that are smaller and closer to the enkephalin would have greater biological activity. However, that is not the case. In fact, the longer the fragments, the greater their activities are in a guinea pig ileum assay (Table 3) (Kilpatrick et al., 1981). These might be dismissed as irrelevant to a discussion of avian opioids. What suggests that these peptides are important to avian physiology is that the sequences of these putative fragments in chickens as an exemplar bird, the chicken, are identical to those in cattle (Table 3; Figure 3; Supplementary Figure S1). Were these not to be functional, random mutations would have been expected in 315 million years since the last common ancestor of birds and mammals (Irisarri et al., 2017). Without selective pressure, these would be incorporated to proenkephalin.

2.5 Circulating and tissue concentrations of Met-enkephalin

Table 4 summarizes plasma and tissue concentrations of Met-enkephalin in chickens. Plasma concentrations of native Met-enkephalin (free, five amino acids peptide) were similar in male

TABLE 3 Biological activity of a series of cattle opioid peptides in the guinea pig ileum assay (data calculated from Kilpatrick et al., 1981).

Opioid peptides	Mammalian peptide	Potency in guinea pig ileum assay[a]
Met-enkephalin	YGGFM	1.0
Leu-enkephalin	YGGFL	0.071
β-endorphin	YGGFMTSEKSQTPLVTLFKNAIIKNAHKKGQ[b]	1.16
Dynorphin A 1–13	YGGFLRRIRPKLK	69.2
Extended enkephalin containing peptides (mammalian)		
BAM12[c]	YGGFMRRVGRPE	2.3
BAM20[c]	YGGFMRRVGRPEWWMDYEKR	16.4
BAM22[c]	YGGFMRRVGRPEWWMDYEKRYG	27.7
Peptide E	YGGFMRRVGRPEWWMDYEKRYGGFL	37.5

[a]Acting via κ opioid receptors.
[b]GeneBank accession XM_019970607.
[c]Mizuno et al., 1980a; Mizuno et al., 1980b

and female chickens (Table 4). Plasma concentrations of cryptic Met-enkephalin (total, Met-enkephalin released from proenkephalin by enzymatic hydrolysis) were 15.7 fold higher than those of native Met-enkephalin (Table 4).

What are the possibilities? It may be a proenkephalin, or fragments of proenkephalin (as in Table 1) or intermediate forms in the processing of proenkephalin as is seen with dynorphin (see section below—Converting Enzymes and Prodynorphin).

The highest concentrations of Met-enkephalin in chickens are in the hypothalamus, adrenal gland and anterior pituitary gland (see Table 4) (Pierzchała-Koziec and Mazurkiewicz-Karasińska, 2016).

2.6 Stress and circulating concentrations of Met-enkephalin

In mammals, there is strong evidence that plasma concentration of immunoreactive Met-enkephalin are elevated by stresses such as insulin induced hypoglycemia in sheep (Owens et al., 1988), induction of diabetes in rats (Kolta et al., 1992), acute induction of hypotension in anesthetized dogs (Mason et al., 1987) and restraint stressed rats (Barron et al., 1990).

Similarly, both plasma concentrations of native Met-enkephalin and adrenal expression of PENK (proopiomelanocortin) were elevated in female chickens subjected to restraint. Water deprivation did not affect either native or cryptic Met-enkephalin but depressed adrenal concentrations of Met-enkephalin. Plasma concentrations of native but not cryptic Met-enkephalin were increased in feed deprived immature female chickens. Morphine challenge was followed by depressed plasma and adrenal concentrations of both native and cryptic Met-enkephalin together with decrease adrenal expression of PENK in female chickens (Pierzchała-Koziec and Mazurkiewicz-Karasińska, 2016). Plasma concentrations of native Met-enkephalin were increased in young chickens stressed by crowding while adrenal concentrations of Met-enkephalin were depressed by crowding.

It is generally assumed that plasma is the compartment of blood in which hormones are found. However, in mammals, Met enkephalin is reported to be produced by leukocytes (human: Kraemer et al., 2013). Similarly, Met-enkephalin is reported to be synthesized by peripheral blood T cells and monocytes (humans: Kamphuis et al., 1998). Production of Met-enkephalin by monocytes was increased in the presence of lipopolysaccharide (humans: Kamphuis et al., 1998). An additional possibility is that fragments of proenkephalin and/or Met- or Leu-enkephalin are generated at the target tissue level.

2.7 Differential processing and release of proenkephalin derived neuropeptides

There is strong evidence that basal and stressed induced circulating concentrations of proenkephalin derived neuropeptides can be and, often are, different. The late fetal increases in circulating concentrations of Met-enkephalin were smaller than those for Met-enkephalin-arginine-phenylalanine (MERF) (Simonetta et al., 1993). Moreover, induction of hypotension in fetal sheep was accompanied by markedly greater increase in circulating concentrations of MERF than those of Met-enkephalin (Mateo et al., 1995). Similarly, asphyxia is followed by increases in circulating concentrations of MERF in fetal sheep (Coulter et al., 1990). Furthermore, there were increases circulating concentrations of MERF but not Met-enkephalin in hypoxic fetal sheep (Simonetta et al., 1996). There is not information on the differential release of different enkephalin peptides in birds.

2.8 Questions

What is not known are the following:

1. The extent to which different enkephalin peptides are released and whether this varies with different tissues.

TABLE 4 Plasma and tissue concentrations of Met-enkephalin in 14 weeks old chickens (based on data in Pierzchala- Koziec and Mazurkiewicz-Karasińska, 2016).

Tissue	Mean ± SEM	
	Female	Male
Plasma (pmoles L^{-1})		
Native Met-enkephalin	50 ± 7.9	50 ± 8.4
Cryptic Met-enkephalin	813 ± 98	758 ± 61
Tissue native Met enkephalin concentrations (pmoles g^{-1})		
Anterior pituitary gland	797 ± 119^{d}	986 ± 128^{c}
Hypothalamus	329 ± 49^{c}	101 ± 11^{b}**
Adrenal gland	135 ± 15^{b}	144 ± 20.0^{b}
Heart atria	3.6 ± 0.1^{a}	5.2 ± 1.1^{a}
Heart ventricles	2.4 ± 0.6^{a}	3.6 ± 0.1^{a}
Kidney	1.7 ± 0.2^{a}	2.9 ± 0.6^{a}

^{a,b,c,d} Different superscript letter indicates difference $*p < 0.05$ between tissues by one way ANOVA, and Tukey's test.
Sex difference $**p < 0.01$.

2. The biological activities of different proenkephalin peptides to avian opioid receptors and exert agonist or even antagonist effects on different derived neuropeptides in avian tissues. In particular, are any of the peptides derived from proenkephalin capable of activating avian μ opioid receptors.
2. Whether cryptic Met-enkephalin represents intermediates in the proteolytic cleavage and/or proenkephalin.
3. Whether circulating concentration of enkephalin reflects hormonal mode of action or do they reflect "spill over" from neural, paracrine or autocrine effects?
4. The physiological control of the release of enkephalin and other products of cleavage of proenkephalin in birds.
5. Whether avian leukocytes or, for that matter, erythrocytes and thrombocytes, produce Met-enkephalin or other products of cleavage of proenkephalin and contribute to plasma concentrations of enkephalin neuropeptides.

3 Prodynorphin (PDYN) and the derived neuropeptides

3.1 Structure of neuropeptides derived from prodynorphin

There are three enkephalin motifs together with one putative or degenerate enkephalin motif in prodynorphin in chickens (Figure 2) and other birds (Supplementary Figure S2). Similarly, there are four enkephalin motifs/degenerate enkephalin motifs in birds (Supplementary Figure S2). There are five enkephalin motifs in prodynorphin in reptiles and the lungfish (Supplementary Figure S2). Moreover, there are five enkephalin motifs/degenerate enkephalin motifs in boney fish (Supplementary Figure S2). In contrast, there are only three enkephalin motifs in mammals (Supplementary Figure S2). It is suggested that in five enkephalin motifs represents the ancestral form with motifs and/or the flanking basic amino acid residue pairs lost during tetrapod evolution. In contrast, there are seven enkephalin motifs in proenkephalin (Figure 1; Supplementary Figure S1).

Figure 3 summarizes the processing of prodynorphin. In mammals, there are three enkephalin motifs with flanking basic amino acid residues. These are processed into opioid neuropeptides: α- and β - neoendorphins and dynorphins A and B (Figures 3, 4; Supplementary Figure S2).

In chickens, other avian species, and cold-blooded vertebrates, there are sequences of amino acid residues with flanking pairs of basic amino-acid residues that could generate both dynorphin A and dynorphin B (see Figures 4; Supplementary Figure S2).

Despite the degree of identity between dynorphin A and B in their homologues across tetrapods, the regions of prodynorphin that are not part of dynorphin neuropeptides exhibit considerable variation even across the class Aves (Figure 4; Supplementary Figure S2).

3.2 Evolution of dynorphin

The sequences of the neuropeptides dynorphin A and dynorphin B, are remarkably conservative across vertebrate classes (see Figure 3; Supplementary Figure S2). The enkephalin motifs in proenkephalin and dynorphin across the vertebrates are compared in Table 2. In contrast, there are marked differences in the sequences between avian α- (or β-) neoendorphin and mammalian or reptilian α- (or β-) neoendorphin (Figure 4).

Dynorphin A has an identical structure in eutherian and placental mammals, reptiles, birds and amphibians (see Figure 3; Supplementary Figure S2). There are single substitutions in monotremes and lungfish (see Figure 3; Supplementary Figure S2).

Dynorphin B, there is an identical structure in some avian species including chickens, ducks and pigeons together with reptiles and amphibians (see Figures 3, 4; Supplementary Figure S2). This suggested that this is the ancestral form of dynorphin B in tetrapods. Compared to the structure of dynorphin B in many

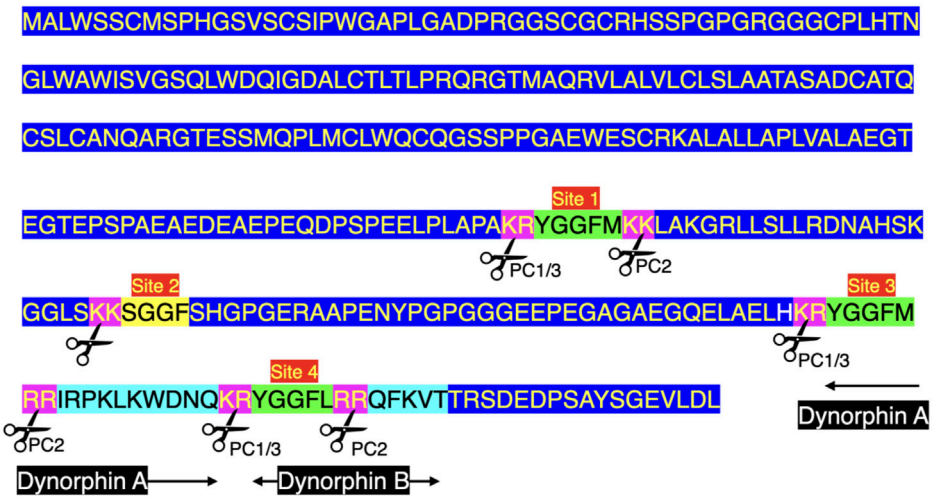

FIGURE 2
Structure of chicken preprodynorphin (deduced from mRNA Genbank XM_040650978). Key: PC prohormone convertase Green highlight indicates enkephalin motif Light blue highlight indicates peptide sequences found in neuropeptides along with enkephalin motif Pink highlight indicates flanking basic amino-acid residues pairs Yellow highlight indicates degenerate enkephalin motif e.g., lacking two basic amino-acid residue pairs on either N or C sides of the enkephalin motif or lacking enkephalin motif Blue highlight other amino acid residues.

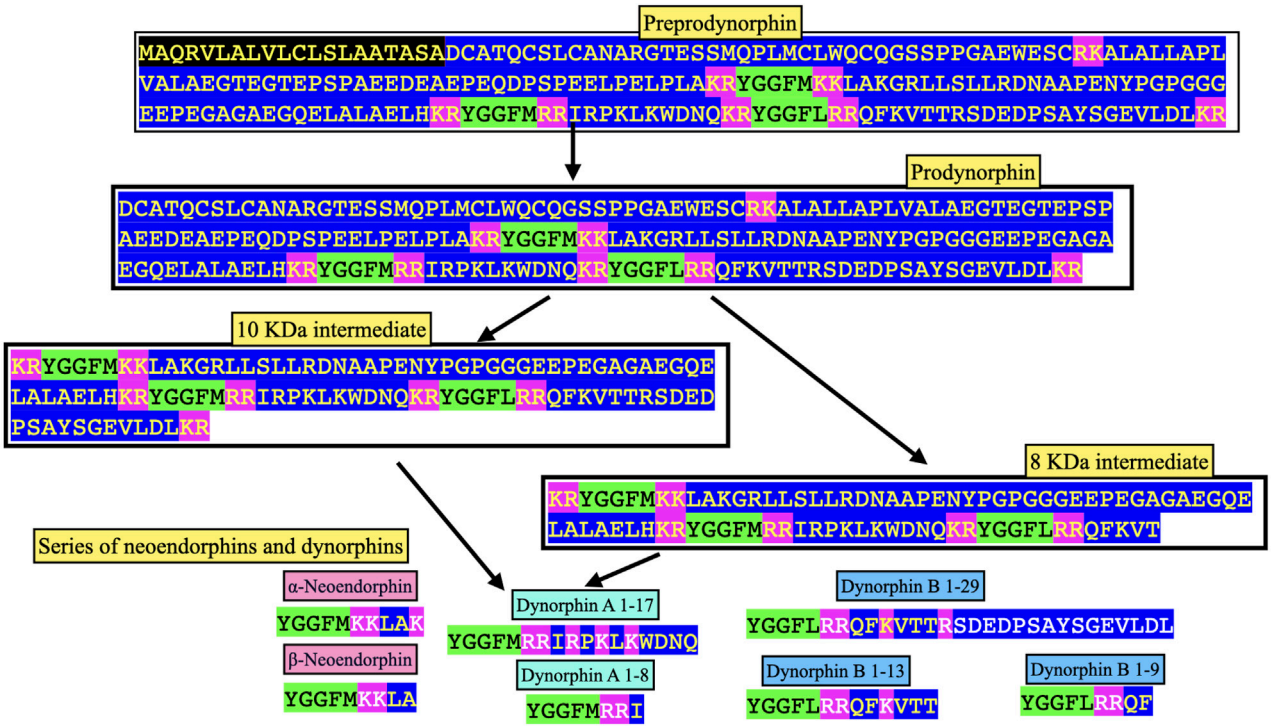

FIGURE 3
Processing of chicken prodynorphin. Key: Green highlight indicates enkephalin motif Pink highlight indicates amino-acid residue pairs Black highlight indicates signal peptide Blue highlight other amino acid residues.

neognath birds, there are only single amino-acid residue substitutions (but not the same one) with those in eutherian and placental mammals and also in birds of the Infra-class *Paleognathae*, (see Figure 4; Supplementary Figure S2). There are three differences of the 13 amino-acid residues between lungfish (YGGFLRRHFKITV) compared to those of tetrapods.

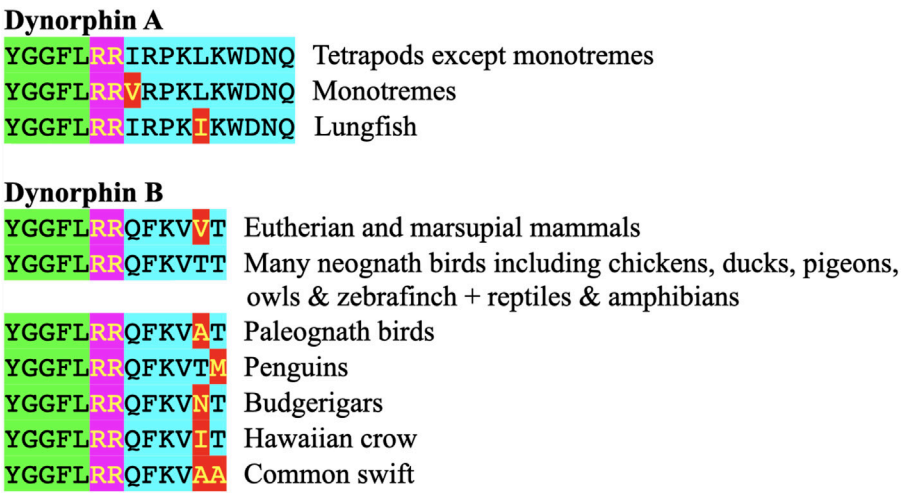

FIGURE 4
Comparison of the structures of dynorphin A and dynorphin B in vertebrates. Key: Green highlight indicates enkephalin motif Pink highlights indicate basic amino acid pair (putative site for proteolysis) Red highlight indicates different amino acid residue from that in chickens, many other birds, reptiles and amphibians; this being the presumptive ancestral form Light blue highlight indicates additional amino acid residues in dynorphin A or B.

Monotremes do not appear to have dynorphin B with the reported sequence exhibiting marked degeneracy compared to dynorphin B in other tetrapods.

- YGASRPRPFKPVT Platypus,
- YGAVRPRPYKLVA Australian echidna.

Moreover, in at least one amphibian species (*Microcaecilia unicolor*), dynorphin B may not be present based on the absence of dibasic cleavage site in prodynorphin (see Supplementary Figure S2).

Despite the degree of identity between dynorphin A and B in their homologues across tetrapods, the regions of prodynorphin that are not part of dynorphin neuropeptides exhibit considerable variation even across the class Aves (see Supplementary Figure S2).

3.3 Converting Enzymes and Prodynorphin

Prodynorphin is subject to proteolysis by convertase(s) generating neoendorphins and dynorphins together with potentially Leu-enkephalin in mammals (Berman et al., 1999; reviewed Ner and Silberring, 2013) and both Met- and Leu-enkephalins in birds. In mammals, there is evidence that prodynorphin is cleaved in a disparate manner in different regions of the brain and pituitary gland (Cone et al., 1983; Seizinger et al., 1994a; Seizinger et al., 1994b).

In mammals, the principal cleavage products of prodynorphin are the following:

- α-Neo-endorphin (YGGFLRKYPK).
- β-Neo-endorphin (YGGFLRKYP).
- Dynorphin A 1–17 (YGGFLRRIRPKLKWDNQ).
- Dynorphin A 1–8 (YGGFLRRIR.
- Dynorphin B 1–29 (YGGFLRRQFKVVTRSQEDPSAYYEEL FDV)

(e.g., Seizinger et al., 1984b). Other putative neuropeptides include dynorphin B 1–13 (YGGFLRRQFKVVT), dynorphin B 1–9 (YGGFLRRQF), 8 and 10 KDa prodynorphin intermediates (see Figure 4) and, potentially, Leu-enkephalin (Day et al., 1998). The predicted structures of avian neuropeptides cleavage products of avian prodynorphin are shown in Figure 3. There are differences between avian and mammalian prodynorphin, comparing prodynorphin products in chickens and cattle. For instance, there are Met-enkephalin motifs in both the putative avian α-/β-neoendophin and dynorphin A neuropeptides instead of Leu-enkephalin motifs in mammals.

3.4 Biological activity of dynorphin

Dynorphin B had a potency of 700 compared to Leu-enkephalin in a guinea pig ileum longitudinal muscle assay; the effects of dynorphin B being blocked by naloxone (Goldstein et al., 1979. The chicken κ opioid receptor is activated by dynorphin A and B (Bu et al., 2020).

3.5 Release of dynorphin

There is limited information on the release of dynorphin in mammals and none in birds. There is release of both immunoreactive dynorphin and α-neoendorphin from the perfused rat duodenum *in vitro* (Corbett et al., 1988). Release of α-neoendorphin and dynorphin were increased in the presence of nicotine in cultured human phaeochromocytoma cells (Yanase et al., 1987); this action presumably acting via nicotinic cholinergic receptors.

3.6 Circulating concentrations of dynorphin

There are limited reports on plasma concentrations of dynorphin or neoendorphin in mammals (humans: Shen and Wang, 1998; Moniaga et al., 2019; Shahkarami et al., 2019) with basal concentrations of 13.1 pmol L^{-1} (Moniaga et al., 2019). The plasma concentrations of dynorphin A were increased in pilots subjected to hypoxia (Shen and Wang, 1998). To the best of our knowledge, there are no reports of plasma concentrations of dynorphin in either poultry or wild birds. Plasma concentrations of IR-dynorphin have been reported in human volunteers as 40.3 ± 6.4 pmol L^{-1} (calculated from Margioris et al., 1990) but markedly lower in control subjects being compared to heart transplant patients [3.3 ± 0.2 pmol L^{-1}] (calculated from Ationu et al., 1993). Plasma concentrations of IR-dynorphin were elevated in human subjects receiving administration of hypertonic saline (Margioris et al., 1990).

Another compartment of blood, leukocytes, have been demonstrated to synthesize dynorphin in mammals. For instance, preprodynorphin expression is reported in peripheral blood cells (Shahkarami et al., 2019). There are no reports of leukocytic expression of preprodynorphin in leukocytes or for that matter in erythrocytes or thrombocytes in birds.

3.7 Stress and circulating concentrations of prodynorphin derived neuropeptides in birds

Plasma concentrations of an immune-reactive α-neoendorphin have been reported in an abstract:

- Adult female chickens 11.5 ± 0.86 pmol L^{-1}.
- Adult male chickens 15.9 ± 0.57 pmol L^{-1} (calculated from Pierzchala and Przewlocki, 1989).

Plasma concentrations of α-neoendorphin in pullets were increased following crowding stress. This effect is blocked by the prior administration of naltrexone (Pierzchała-Koziec et al., 1996).

3.8 Neoendorphin and dynorphin: expression, tissue concentrations and release

There is no information on the expression of the prodynorphin together with tissue distribution and release outside of the brain or on circulating concentrations of either dynorphin A and B in birds. There is some information on these in mammals. High concentrations of IR-dynorphin are detected in the mammalian posterior pituitary gland (reviewed: Margioris et al., 1990) with marked expression in magnocellular neurons in the hypothalamus that project into the posterior pituitary gland (Sherman et al., 1986a). Moreover, expression of pro-dynorphin shifts in a manner similar to that of vasopressin in supraoptic and paraventricular nuclei (Sherman et al., 1986b). Also, there is increased expression of the prodynorphin gene in the hypothalamus of dehydrated or salt loaded rats (Sherman et al., 1986a).

Both dynorphin and α-neoendorphin are released from rat duodenal tissue *in vitro* (Majeed et al., 1987). Moreover, release of IR-dynorphin and IR α-neoendorphin is increased by serotonin (rat: Majeed et al., 1987). There is no information on the distribution in tissues or the control of the release of dynorphin A or B in any avian species.

3.9 Questions

What is not known are the following:

1. The forms of dynorphin A and B together with neoendorphin that are produced by various avian tissues.
2. The biological activities of different prodynorphin derived peptides in birds.
3. Are circulating concentration of prodynorphin derived neuropeptides exerting hormonal effects or do they reflect "spill over" from neural, paracrine or autocrine effects?
4. The physiological control of dynorphin A and B together with neoendorphin release.
5. Whether avian leukocytes or, for that matter, erythrocytes and thrombocytes, produce prodynorphin derived neuropeptides and contribute to their concentrations in the plasma.
6. The extent to which different neuropeptides are released and whether this varies with different tissues.

4 Pronociceptin (PNOC) and the derived neuropeptide nociceptin (orphanin FQ)

4.1 Introduction to nociceptin

The structure of pronociceptin is shown in Figure 5. There is peptide containing an enkephalin motif generated from the prepronociceptin mRNA in mammals, birds and reptiles. In contrast, there are two peptides with enkephalin motifs encoded by prepronociceptin mRNA in boney and cartilaginous fish, amphibians and lungfish. Nociceptin is the ligand for the nociceptin opioid receptor; this being insensitive to naloxone.

4.2 Structure and evolution of nociceptin

Nociceptin is a neuropeptide with 17 amino-acids (see Figure 4; Supplementary Figures S3, S4). There is an identical structure for nociceptin across mammalian species (see Figure 6; Supplementary Figures S3, S4). This differs from other tetrapods with five substitutions of amino acid residues (see Figure 4). The last common ancestor for mammals and reptiles/birds is estimated as living 315 million years ago (during the Carboniferous period) (Irisarri et al., 2017). In contrast, there are identical structures for nociceptin in non-mammalian tetrapods together with non-tetrapod Sarcopterygii (lungfish and coelacanths) (see Figure 6;

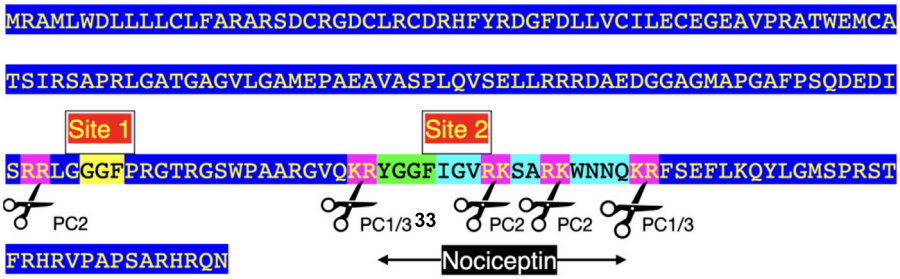

FIGURE 5
Structure of chicken prepronociceptin (Genbank XM_040697232). Key: PC prohormone convertase Green highlight indicates enkephalin motif Pink highlights indicate basic amino acid pair (putative site for proteolysis) Yellow highlight indicates degenerate enkephalin motif e.g., lacking two basic amino-acid residue pairs on C flanking of the partial enkephalin motif. Light blue highlight indicates additional amino acid residues in nociceptin Blue highlight other amino acid residues.

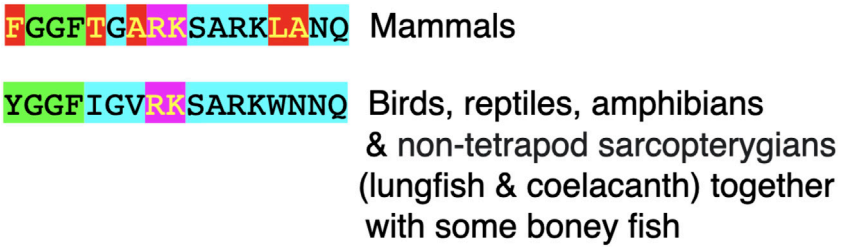

FIGURE 6
Structure of nociceptin in vertebrates. Key Green highlight indicates enkephalin motif (YGGF) or partial motif with flanking pairs of basic amino acids residues both N and C terminals. Pink highlight indicates a pair of basic amino acid residues. Red highlight indicates differences with the sequence of amino acid residues relative to that in birds, reptiles, amphibians and non-tetrapod sarcopterygians Light blue highlights indicate other amino-acid residues.

Supplementary Figures S3, S4). The last common ancestor for tetrapods and non-tetrapod Sarcopterygii is estimated as living 428 million years ago (in the Silurian period) (Irisarri et al., 2017).

What is particularly surprising is the substitution of tyrosine to phenylalanine residues in mammalian nociceptin (Figure 4; Supplementary Figure S3). This is an unique case of such a substitution and is not found with any other neuropeptides that include enkephalin motifs.

4.3 Nomenclature

Nociceptin/orphanin FQ (N/OFQ) is a reasonable name in mammals due to the presence of phenylalanine (F) at the N terminal and a glutamine (Q) at the C terminal. However, in other tetrapods together with lungfish, this is not appropriate. Instead, it is more accurate to refer the avian neuropeptide as nociceptin/orphanin YQ [due to the N terminal being tyrosine (Y) and glutamine being the C terminal] or simply as nociceptin.

4.4 Biological role of nociceptin

Both the chicken nociceptin and κ opioid receptors are activated by nociceptin (Bu et al., 2020). It might be assumed that nociceptin is exerting an analgesic effect in birds. In addition, nociceptin plays a role in embryonic development. Ectodermal expression of nociceptin is increased by somatostatin with nociceptin playing a role in the formation of placode progenitors in chick embryos (Lleras-Forero et al., 2013). However, there are no studies on the effects of avian nociceptin on either avian physiology or pathology. There are, however, reports of the effect of mammalian nocicentin in birds (see below). If nociceptin was not important, it is difficult to envision why an identical structure is found across the tetrapods together with non-tetrapod Sarcopterygians. Thus, the structure was retained in its entirety through at least 428 million years since the last common ancestor of tetrapods and non-tetrapod Sarcopterygians (Irisarri et al., 2017). In addition, an identical structure is found in some boney fish (Figure 6; Supplementary Figures S3, S4).

4.5 Nociceptin and the hypothalamic control of feeding in birds

In young meat line chickens, intracerebroventricular injection of mammalian nociceptin was followed by increased consumption of feed (Abbasnejad et al., 2005; Zendehdel et al., 2013; Zendehdel et al., 2015; Zendehdel et al., 2017; Zendehdel et al., 2019). There is evidence for beta-adrenergic, serotoninergic, dopaminergic and

histaminergic involvement in feeding in chickens induced by mammalian nociceptin. Nociceptin induced feed intake was increased after prior administration of a β_2 adrenergic antagonist (Zendehdel et al., 2017). However, there was no evidence for α- or β_1 or β_3 adrenergic involvement in mammalian nociceptin induced feed consumption (Zendehdel et al., 2017). Nociceptin induced feed consumption was increased by either pharmacological blocking serotonin (5-HT) synthesis or a 5-HT receptor 2 antagonist (Zendehdel et al., 2013). In addition, prior administration of the dopamine precursor, L-DOPA depressed mammalian nociceptin induced feeding while a D_2 dopamine antagonist increase the response to N/OFQ (Zendehdel et al., 2019). Moreover, the effect of mammalian nociceptin was increased by prior administration of an H_1 histamine antagonist but not a H_2 antagonist (Zendehdel et al., 2015). In contrast, the effect of mammalian nociceptin on feed consumption was decreased by prior administration of an H_3 histamine antagonist (Zendehdel et al., 2015). It is cautioned that mammalian nociceptin differs by five amino-acid residues out of a total of 17 compared to that in other tetrapods (Figure 6). Studies employing avian nociceptin are needed. If nociceptin was not important, it is difficult to envision why an identical structure is found across the tetrapods except for mammals (Figure 6; Supplementary Figures S3, S4).

4.6 Circulating concentrations of nociceptin

There are reports of plasma concentrations of nociceptin in humans but not in other mammals or birds. Post operative plasma concentrations of nociceptin have been reported as 39 pmol L^{-1} in individuals with intravenous patient-controlled analgesia (Lee and Jeon, 2013). In contrast, plasma concentrations of nociceptin were 0.55 pmol L^{-1} in patients with sepsis and 1.7 pmol L^{-1} in patients with sepsis who subsequently died (Williams et al., 2008). There is a need for examination of plasma concentrations of nociceptin in birds under both a series of physiological and pathological situations.

4.7 Nociceptin tissue concentrations and release

There is no information on the distribution in tissues or the control of the release of nociceptin in any avian species.

4.8 A putative avian nocistatin

In mammals, pronociceptin also encodes a second biologically active peptide, nocistatin (Okuda-Ashitaka et al., 1998; reviewed; Fricker et al., 2022). This is generated by proteolytic cleavage at basic amino-acid residue pairs (Okuda-Ashitaka et al., 1998; reviewed; Fricker et al., 2022) (see Figure 5). The length of nocistatin exhibiting marked variability (bovine: 17; human: 31 amino-acid residues). The C–terminal for nocistatin in humans and cattle consists of the following hexapeptide: EQKQLQ (Meunier et al., 1995; Nothacker et al., 1996; Okuda-Ashitaka and Ito, 2000). Based on a PubMed search, there are no reports of nocistatin in birds. However, there are predicted sequences of a putative avian nocistatin in a series of birds (see Figures 5, 6 also see Supplementary Figure S5). These are separated by basic amino-acid residue pairs. In birds, these are characterized by having an identical or very similar C terminal hexapeptide, AARGVQ; this being found in, for instance, in Okarito brown kiwi (XM_026067016), chicken (XM_040697232) and Hawaiian crow (XM_048298403) together with similar hexapeptides as AAKGVQ (common canary—XM_050971908) and TARGVQ (California condor—XM_050895080).

4.9 A putative avian non-opioid neuropeptide derived from pronociceptin

Fricker et al. (2022) included non-opioid peptide(s) derived from pronociceptin when nocicentin was cleaved (Figures 5, 6). This viewed these likely to be biologically active (Fricker et al., 2022). This was presumably a neuropeptide as are all the peptides derived from proenkephalin, prodynorphin, and pronociceptin (Fricker et al., 2022).

It was questioned whether such a putative biologically active peptide might exist in birds and, also, in reptiles. The deduced sequence for nociceptin has a pair of basic amino-acid residues (lysine-arginine) at both its N and C terminal in birds and reptiles (Figure 5; Supplementary Figure S3) together with mammals (Fricker et al., 2022). Proteolytic cleavage would be expected to occur at these sites generating nociceptin and a second peptide(s) again in birds, reptiles and mammals (Fricker et al., 2022).

There is strong similarity between the N terminal of the putative peptide with identical residues at positions 1, 2, 3, 4, 7, 8, and 9 in mammals (e.g., human and Tasmanian devil) compared to birds (e.g., chicken and kiwi) (Supplementary Figure S6). This would have 30 or 31 amino-acid residues (also see Supplementary Figure S6). This putative C terminal neuropeptide would be considered as non-opioid as it lacks the YGGF motif (Figure 6).

Comparison of the putative peptide from deduced structures of pronociceptin in birds suggested that there are two structures of the nociceptin C terminal non-opioid peptide in birds:

- FSEFLKQYLGMSPRSTFRHRVPAPSARHRQN in chickens (with the V replaced by an I in some species).
- FSEFLKQYLGMSPRSSEYDIAGGISEHNEI (Supplementary Figure S6).

They share a 15 amino-acid residue peptide (FSEFLKQYLGMSPRS). The C terminal of each in different species have multiple cases of identical amino-acid residues (Supplementary Figure S6).

What was unexpected that both forms were found in avian species in the Infra-order Paleognathae and Neognathae, in the Clade Neoaves, in the Clade Australaves (e.g., members of the orders Passeriformes, and Falconiformes), in the Clade Afroaves (Strigiformes and Accipitriformes) and within both the orders Piciformes and Passeriformes (avian classification and evolution based on Brusatte et al., 2015). This is not consistent with a simplistic evolutionary interpretation. The explanation for this is not readily apparent.

4.10 Questions

What is not known includes the following:

1. Which forms of nociceptin and other putative neuropeptides derived from pronociceptin are produced by various avian tissues.
2. What are the biological activities of peptides derived from pronociceptin are produced by various avian tissues.
3. Are circulating concentration of nociceptin exerting hormonal effects or do they reflect "spill over" from neural, paracrine or autocrine roles?
4. The physiological control of the release of nociceptin and other peptides derived from pronociceptin.
5. Whether avian leukocytes or, for that matter, erythrocytes and thrombocytes, produce nociceptin and other peptides derived from pronociceptin contribute to plasma concentrations of nociceptin.

5 Conclusion

What is almost completely missing in avian species is information on cleavage pattern of proenkephalin, prodynophin and pronociceptin in different tissues and the relative activities of the multiple endogenous opioids/peptides via the δ-opioid, κ-opioid and μ-opioid receptors in chickens or other birds. Moreover, given the multiplicity of opioid peptides and their roles, it is questioned whether at least some are released in response to the welfare challenges such as stress or injury. It is assumed that the biologically active peptides derived from proenkephalin are Met-enkephalin and Leu-enkephalin. However, other peptides are derived from proenkephalin and, based on mammalian studies, they have markedly different biological activities. The sequence of amino-acid residues in these peptides is identical in birds and mammals arguing for their importance. Avian prodynorphin is likely to be subject to proteolytic cleavage generating dynorphin A and B and, probably also an avian neoendorphin with the first two exhibiting marked similarity in sequence. Avian pronociceptin is cleaved to produce nociceptin, nocistatin and a non-opioid C terminal peptide with the first and third having close homology with their mammalian counterparts. (Pierzchala and Van Loon, 1990; Chen et al., 2007; Thompson et al., 2014; Avenali et al., 2017).

Author contributions

Authors declare equal contribution in writing; CS-conceptualization. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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References

Abbasnejad, M., Jonaidi, H., and Pourrahimi, A. M. (2005). Feeding and locomotion responses to centrally injected nociceptin/orphanin FQ in chicks. *Physiol. Behav.* 85, 383–386. doi:10.1016/j.physbeh.2005.03.016

Ationu, A., Sorensen, K., Whitehead, B., Singer, D., and Carter, N. (1993). Ventricular expression and circulating levels of immunoreactive dynorphin in heart transplant recipients. *Clin. Sci. (Lond.)* 85, 1–4. doi:10.1042/cs0850001

Avenali, L., Fulas, O. A., Sondermann, J., Narayanan, P., Gomez-Varela, D., and Schmidt, M. (2017). Nocistatin sensitizes TRPA1 channels in peripheral sensory neurons. *Channels (Austin)* 11, 11–19. doi:10.1080/19336950.2016.1207025

Barron, B. A., Pierzchala, K., and Loon, G. R. (1990). Source of stress-induced increase in plasma met-enkephalin in rats: Contribution of adrenal medulla and/or sympathetic nerves. *J. Neuroendocrinol.* 2, 381–388. doi:10.1111/j.1365-2826.1990.tb00422.x

Berman, Y., Juliano, L., and Devi, L. A. (1999). Specificity of the dynorphin-processing endoprotease: Comparison with prohormone convertases. *J. Neurochem.* 72, 2120–2126. doi:10.1046/j.1471-4159.1999.0722120.x

Blebea, J., Mazo, J. E., Kihara, T. K., Vu, H.-H., McLaughlin, P. J., Atnip, R. G., et al. (2000). Opioid growth factor modulates angiogenesis. *J. Vasc. Surg.* 32, 364–373. doi:10.1067/mva.2000.107763b

Brusatte, S. L., O'Connor, J. K., and Jarvis, E. D. (2015). The origin and diversification of birds. *Curr. Biol.* 25, R888–R898. doi:10.1016/j.cub.2015.08.003

Bu, G., Cui, L., Lv, C., Lin, D., Huang, L., Li, Z., et al. (2020). Opioid peptides and their receptors in chickens: Structure, functionality, and tissue distribution. *Peptides* 128, 170307. doi:10.1016/j.peptides.2020.170307

Chen, J. X., Zhao, X., Yue, G. X., and Wang, Z. F. (2007). Influence of acute and chronic treadmill exercise on rat plasma lactate and brain NPY, L-ENK, DYN A1-13. *Cell. Mol. Neurobiol.* 27, 1–10. doi:10.1007/s10571-006-9110-4

Cone, Ri., Weber, E., Barchas, Jd., and Goldstein, A. (1983). Regional distribution of dynorphin and neo-endorphin peptides in rat brain, spinal cord, and pituitary. *J. Neurosci.* 3, 2146–2152. doi:10.1523/JNEUROSCI.03-11-02146.1983

Corbett, A. D., McKnight, A. T., and Kosterlitz, H. W. (1988). Tissue content of opioid peptides in the myenteric plexus-longitudinal muscle of Guinea-pig small intestine. *J. Neurochem.* 51, 32–37. doi:10.1111/j.1471-4159.1988.tb04831.x

Coulter, C. L., Giraud, A. S., Hooper, S. B., Parker, L., and McMillen, I. C. (1990). Fetal asphyxia stimulates an increase in fetal plasma catecholamines and [Met]-enkephalin-arg6-phe7 in the late-gestation sheep fetus. *J. Dev. Physiol.* 14, 267–272.

Cullen, J. M., and Cascella, M. (2022). Physiology, enkephalin. https://www.ncbi.nlm.nih.gov/books/NBK557764/.

Day, R., Lazure, C., Basak, A., Boudreault, A., Limperis, P., Dong, W., et al. (1998). Prodynorphin processing by proprotein convertase 2. Cleavage at single basic residues and enhanced processing in the presence of carboxypeptidase activity. *J. Biol. Chem.* 273, 829–836. doi:10.1074/jbc.273.2.829

Dhaliwal, A., and Gupta, M. (2022). *Physiology, opioid receptor*. Tampa, FL, USA: StatPearls.

Fricker, L. D., Margolis, E. B., Gomes, I., and Devi, L. A. (2022). Five decades of research on opioid peptides: Current knowledge and unanswered questions. *Mol. Pharmacol.* 98, 96–108. doi:10.1124/mol.120.119388

Goldstein, A., Tachibana, S., Lowney, L. I., Hunkapiller, M., and Hood, L. (1979). Dynorphin-(1-13), an extraordinarily potent opioid peptide. *Proc. Natl. Acad. Sci. U. S. A.* 76, 6666–6670. doi:10.1073/pnas.76.12.6666

Hucklebridge, F. H., Hudspith, B. N., Muhamed, J., Lydyard, P. M., and Brostoff, J. (1989). Methionine-enkephalin stimulates *in vitro* proliferation of human peripheral lymphocytes via δ-opioid receptors. *Brain, Behav. Immun.* 3, 183–189. doi:10.1016/0889-1591(89)90019-6

Irisarri, I., Baurain, D., Brinkmann, H., Delsuc, F., Sire, J.-Y., Kupfer, A., et al. (2017). Phylotranscriptomic consolidation of the jawed vertebrate timetree. *Nat. Ecol. Evol.* 1, 1370–1378. doi:10.1038/s41559-017-0240-5

Jhamandas, K., and Sutak, M. (1980). Action of enkephalin analogues and morphine on brain acetylcholine release: Differential reversal by naloxone and an opiate pentapeptide. *Br. J. Pharmac.* 71, 201–210. doi:10.1111/j.1476-5381.1980.tb10927.x

Kamphuis, S., Eriksson, F., Kavelaars, A., Zijlstra, J., van de Pol, M., Kuis, W., et al. (1998). Role of endogenous pro-enkephalin A-derived peptides in human T cell proliferation and monocyte IL-6 production. *J. Neuroimmunol.* 84, 53–60. doi:10.1016/s0165-5728(97)00240-3

Kilpatrick, D. L., Taniguchi, T., N Jones, B. N., Stern, A. S., Shively, J. E., Hullihan, J., et al. (1981). A highly potent 3200-dalton adrenal opioid peptide that contains both a [Met]- and [Leu]enkephalin sequence. *Proc. Natl. Acad. Sci. U. S. A.* 78, 3265–3268. doi:10.1073/pnas.78.5.3265

Kolta, M. G., Pierzchala, K., Houdi, A. A., and Van Loon, G. R. (1992). Effect of diabetes on the levels of two forms of Met-enkephalin in plasma and peripheral tissues of the rat. *Neuropeptides* 2 1, 55–63. doi:10.1016/0143-4179(92)90152-m

Kraemer, W. J., Fragala, M. S., Beijersbergen van Henegouwen, W. R. H., Gordon, S. E., Bush, J. A., Volek, J. S., et al. (2013). Responses of proenkephalin Peptide F to aerobic exercise stress in the plasma and white blood cell biocompartments. *Peptides* 42, 118–124. doi:10.1016/j.peptides.2013.01.013

Lee, H., and Jeon, H. (2013). Plasma concentrations of nociceptin/orphanin FQ: Comparison of levels after general and neuraxial anesthesia for arthroscopic knee surgery. *Korean J. Anesthesiol.* 65, 327–330. doi:10.4097/kjae.2013.65.4.327

Lleras-Forero, L., Tambalo, M., Christophorou, N., Chambers, D., Houart, C., and Streit, A. (2013). Neuropeptides: Developmental signals in placode progenitor formation. *Dev. Cell* 26, 195–203. doi:10.1016/j.devcel.2013.07.001

Majeed, N. H., Silberring, J., and Przewłocki, R. (1987). The *in vitro* release of immunoreactive dynorphin and alpha-neoendorphin from the perfused rat duodenum. *Life Sci.* 41, 497–504. doi:10.1016/0024-3205(87)90227-x

Malendowicz, L. K., Rebuffat, P., Tortorella, C., Nussdorfer, G. G., Ziolkowska, A., and Hochol, A. (2005). Effects of met-enkephalin on cell proliferation in different models of adrenocortical-cell growth. *Int. J. Mol. Med.* 15, 841–845. doi:10.3892/ijmm.15.5.841

Margioris, A. N., Brockmann, G., Kalogeras, K. T., Fjellestad-Paulsen, A., Stratakis, C. A., Vamvakopoulos, N., et al. (1990). Effect of hypertonic saline infusion on the level of immunoreactive dynorphin in extracted human plasma. *J. Clin. Endocrinol. Metab.* 71, 298–304. doi:10.1210/jcem-71-2-298

Mason, D. F., Medbak, S., and Rees, L. H. (1987). Circulating [Met]enkephalin and catecholamine responses to acute hypotension and hypertension in anaesthetized greyhounds. *Br. J. Pharmacol.* 91, 103–111. doi:10.1111/j.1476-5381.1987.tb08988.x

Mateo, Z., Napier, L., Gaugl, J. F., Barron, B. A., and Caffrey, J. L. (1995). Hemorrhage alters plasma and cardiac enkephalins and catecholamines in anesthetized dogs. *Am. J. Physiol.* 269, H2082–H2089. doi:10.1152/ajpheart.1995.269.6.H2082

Matus-Ortega, M. E., Gelman, P. L., Calva-Nieves, J. C., Flores-Zamora, A., Salazar-Juárez, A., Torner-Aguila, C.-A., et al. (2017). Mexneurin is a novel precursor of peptides in the central nervous system of rodents. *FEBS Lett.* 591, 1627–1636. doi:10.1002/1873-3468.12679

Meunier, J.-C., Mollereau, C., Toll, L., Suaudeau, C., Moisand, C., Alvinerie, P., et al. (1995). Isolation and structure of the endogenous agonist of opioid receptor-like ORL_1 receptor. *Nature* 377, 532–535. doi:10.1038/377532a0

Mizuno, K., Minamino, N., Kangawa, K., and Matsuo, H. (1980b). A new endogenous opioid peptide from bovine adrenal medulla: Isolation and amino acid sequence of a dodecapeptide (BAM-12P). *Biochem. Biophys. Res. Commun.* 95, 1482–1488. doi:10.1016/S0006-291X(80)80064-7

Mizuno, K., Minamino, N., Kangawa, K., and Matsuo, H. (1980a). A new family of endogenous "big" met-enkephalins from bovine adrenal medulla: Purification and structure of docosa- (BAM-22P) and eicosapeptide (BAM-20P) with very potent opiate activity. *Biochem. Biophys. Res. Commun.* 97, 1283–1290. doi:10.1016/S0006-291X(80)80005-2

Moniaga, C. S., Iwamoto, S., Kitamura, T., Fujishiro, M., Takahashi, N., Kina, K., et al. (2019). Plasma dynorphin A concentration reflects the degree of pruritus in chronic liver disease: A preliminary report. *Acta Derm. Venereol.* 99, 442–443. doi:10.2340/00015555-3139

Ner, J., and Silberring, J. (2013). Dynorphin convertases and their functions in CNS. *Curr. Pharm. Des.* 19, 1043–1051. doi:10.2174/1381612811319060006

Nothacker, H. P., Reinscheid, R. K., Mansour, A., Henningsen, R. A., Ardati, A., F J Monsma, F. J., et al. (1996). Primary structure and tissue distribution of the orphanin FQ precursor. *Proc. Natl. Acad. Sci. U. S. A.* 93, 8677–8682. doi:10.1073/pnas.93.16.8677

Okuda-Ashitaka, E., and Ito, S. (2000). Nocistatin: A novel neuropeptide encoded by the gene for the nociceptin/orphanin FQ precursor. *Peptides* 21, 1101–1109. doi:10.1016/s0196-9781(00)00247-3

Okuda-Ashitaka, E., Minami, T., Tachibana, S., Yoshihara, Y., Nishiuchi, Y., Kimura, T., et al. (1998). Nocistatin, a peptide that blocks nociceptin action in pain transmission. *Nature* 392, 286–289. doi:10.1038/32660

Owens, P. C., Chan, E. C., Lovelock, M., Falconer, J., and Smith, R. (1988). Immunoreactive methionine-enkephalin in cerebrospinal fluid and blood plasma during acute stress in conscious sheep. *Endocrinology* 122, 311–318. doi:10.1210/endo-122-1-311

Pierzchala, K., and Przewlocki, R. (1989). "Plasma levels of α-neoendorphin in hens and cockerels," in Proceedings of the IIIrd Polish-Czechoslovakian Symposium on Avian Physiology, Krakow, Poland, September 1989.

Pierzchala, K., and Van Loon, G. R. (1990). Plasma native and peptidase-derivable Met-enkephalin responses to restraint stress in rats. Adaptation to repeated restraint. *J. Clin. Invest.* 85, 861–873. doi:10.1172/JCI114513

Pierzchała-Koziec, K., and Mazurkiewicz-Karasińska, D. 2016. Involvement of opioids and glucocorticois in the regulationof food intake in growing chicken. Proceedings of the XXV International Symposium Molecular and Physiological Aspects of Regulatory Processes in the Organism, Cracow, Poland, October 2016, 55–56.

Pierzchała-Koziec, K., Niezgoda, J., Bobek, S., and Przewłocki, R. (1996). Endogenous opioids may modulate the activity of the hypothalamus-pituitary-adrenocortical axis in domestic fowl. *Zentralbl. Veterinarmed A.* 43, 139–146. doi:10.1111/j.1439-0442.1996.tb00438.x

Scanes, C. G., and Pierzchała -Koziec, K. (2021). Functioning of the proopiomelanocortin (POMC) derived hormones: Melanocyte stimulating hormones and adrenocorticotropic hormone in birds. *Folia Biol. (Kraków)* 69, 121–134. doi:10.3409/fb_69-3.14

Scanes, C. G., and Pierzchala-Koziec, K. (2018). Perspectives on endogenous opioids in birds. *Front. Physiol.* 9, 1842. doi:10.3389/fphys.2018.01842

Seizinger, B. R., Grimm, C., Höllt, V., and Herz, A. (1984b). Evidence for a selective processing of proenkephalin B into different opioid peptide forms in particular regions of rat brain and pituitary. *J. Neurochem.* 42, 447–457. doi:10.1111/j.1471-4159.1984.tb02698.x

Seizinger, B. R., Höllt, V., and Herz, A. (1984a). Proenkephalin B (prodynorphin)-derived opioid peptides: Evidence for a differential processing in lobes of the pituitary. *Endocrinology* 115, 662–671. doi:10.1210/endo-115-2-662

Shahkarami, K., Vousooghi, N., Golab, F., Mohsenzadeh, A., Baharvand, P., Sadat-Shirazi, M.-S., et al. (2019). Evaluation of dynorphin and kappa-opioid receptor level in the human blood lymphocytes and plasma: Possible role as a biomarker in severe opioid use disorder. *Drug Alcohol Depend.* 205, 107638. doi:10.1016/j.drugalcdep.2019.107638

Shen, D., and Wang, Y. (1998). Changes of plasma level of neurotensin, somatostatin, and dynorphin A in pilots under acute hypoxia. *Mil. Med.* 163, 120–121. doi:10.1093/milmed/163.2.120

Sherman, T. G., Civelli, O., Douglass, J., Herbert, E., Burke, S., and Watson, S. J. (1986a). Hypothalamic dynorphin and vasopressin mRNA expression in normal and Brattleboro rats. *Fed. Proc.* 45, 2323–2327.

Sherman, T. G., Civelli, O., Douglass, J., Herbert, E., and Watson, S. J. (1986b). Coordinate expression of hypothalamic pro-dynorphin and pro-vasopressin mRNAs with osmotic stimulation. *Neuroendocrinology* 44, 222–228. doi:10.1159/000124649

Simonetta, G., Young, I. R., Coulter, C. L., Hey, N. J., and McMillen, I. C. (1993). Fetal adrenalectomy does not affect circulating enkephalins in the sheep fetus during late gestation. *Neuroendocrinology* 57, 408–415. doi:10.1159/000126386

Simonetta, G., Young, I. R., and McMillen, I. C. (1996). Plasma catecholamine and met-enkephalin-Arg6-Phe7 responses to hypoxaemia after adrenalectomy in the fetal sheep. *J. Auton. Nerv. Syst.* 60, 108–114. doi:10.1016/0165-1838(96)00026-4

Sundström, G., Dreborg, S., and Larhammar, D. (2010). Concomitant duplications of opioid peptide and receptor genes before the origin of jawed vertebrates. *PLoS One* 5, e10512. doi:10.1371/journal.pone.0010512

Thompson, G. L., Canals, M., and Poole, D. P. (2014). Biological redundancy of endogenous GPCR ligands in the gut and the potential for endogenous functional selectivity. *Front. Pharmacol.* 5, 262. doi:10.3389/fphar.2014.00262

Williams, J. P., Thompson, J. P., Young, S. P., Gold, S. J., McDonald, J., Rowbotham, D. J., et al. (2008). Nociceptin and urotensin-II concentrations in critically ill patients with sepsis. *Br. J. Anaesth.* 100, P810–P814. doi:10.1093/bja/aen093

Yanase, T., Nawata, H., Kato, K., and Ibayashi, H. (1987). Preproenkephalin B-derived opioid peptides in human phaeochromocytomas. *Acta Endocrinol. (Copenh.)* 114, 446–451. doi:10.1530/acta.0.1140446

Yasothornsrikul, S., Greenbaum, D., Medzihradszky, K. F., Toneff, T., Bundey, R., Miller, R., et al. (2003). Cathepsin L in secretory vesicles functions as a prohormone-processing enzyme for production of the enkephalin peptide neurotransmitter. *Proc. Natl. Acad. Sci. U. S. A.* 100, 9590–9595. doi:10.1073/pnas.1531542100

Zadina, J. E., Hackler, L., Ge, L.-J., and Kastin, A. J. (1997). A potent and selective endogenous agonist for the mu-opiate receptor. *Nature* 386, 499–502. doi:10.1038/386499a0

Zendehdel, M., Ebrahimi-Yeganeh, A., Hassanpour, S., and Koohi, M. K. (2019). Interaction of the dopaminergic and nociceptin/orphanin FQ on central feed intake regulation in chicken. *Br. Poult. Sci.* 60, 317–322. doi:10.1080/00071668.2019.1596225

Zendehdel, M., Farshid Hamidi, F., and Hassanpour, S. (2015). The effect of histaminergic system on nociceptin/orphanin FQ induced food intake in chicken. *Int. J. Pept. Res. Ther.* 21, 179–186. doi:10.1007/s10989-014-9445-5

Zendehdel, M., Mokhtarpouriani, K., Babapour, V., Baghbanzadeh, A., Pourrahimi, M., and Shahin Hassanpour, S. (2013). The effect of serotonergic system on nociceptin/orphanin FQ induced food intake in chicken. *J. Physiol. Sci.* 63, 271–277. doi:10.1007/s12576-013-0263-x

Zendehdel, M., Parvizi, Z., Hassanpour, S., Baghbanzadeh, A., and Hamidi, F. (2017). Interaction between nociceptin/orphanin FQ and adrenergic system on food intake in neonatal chicken. *Int. J. Pept. Res. Ther.* 23, 155–161. doi:10.1007/s10989-016-9548-2

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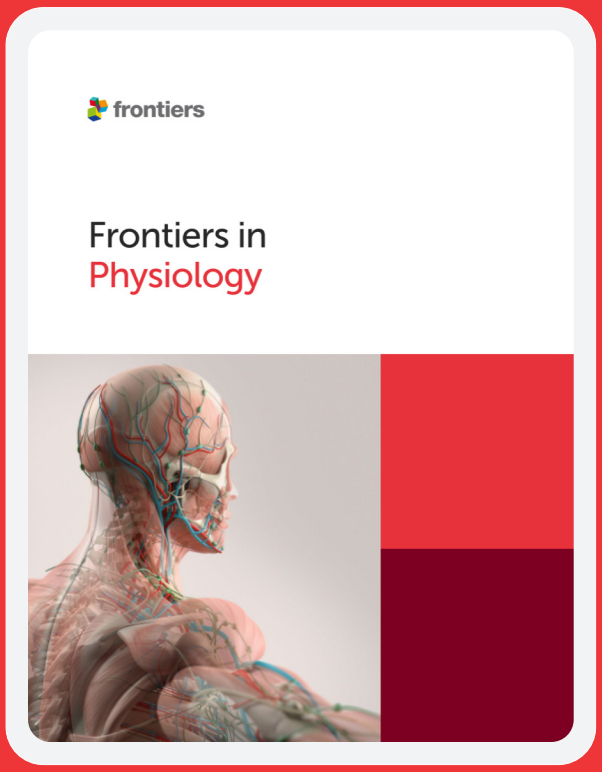

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