UiT The Arctic University of Norway

Faculty of Bioscience, Fisheries and Economics

Department of Arctic and Marine Biology

Seasonal regulation of melanogenesis in ptarmigan

Daniel Curthoys

Master's thesis in biology Bio-3950 August 2023

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Abstract

Ptarmigan are one of the few animals, and only bird species, to undergo seasonal colour change. Every year they moult between a white winter morph and brown summer and autumn morphs and back again. Skin samples were collected from *Lagopus muta* and *Lagopus lagopus* during the autumn moult between brown and white morphs to compare areas with brown feathers and areas that had grown white feathers. The relative gene expression for selected key genes of melanogenesis was then calculated through qPCR. The genes tested were POMC, PC1, PC2, ASIP, MC1R, TYR, TYRP1 & DCT. Most did not show any significant difference between the two sample groups in either species, except TYRP1 which had significantly lower expression in the white samples for *Lagopus muta*. The promoter regions of the above genes and CORIN, CREB1, MITF, OCA2, SLC7A11, SLC45A2 & TBX19 were compared using EMBOSS polydot and CiiiDER workflow. These comparisons looked at the Transcription Factor Binding Sites (TFBS) in seven galliform species: the three Lagopus species (*L. muta*, *L. lagopus* and *L. leucura*) and *L. l. scoticus* as well as *Gallus gallus*, *Coturnix japonica* & *Centrocercus urophasianus.* There were several differences between the promoter regions, the most common was a missing TFBS for Pax2 in several genes in *L. l. scoticus.* None of the TFBS differences have been directly linked to melanogenesis of follicle cycling in other research. The results of these studies do not suggest seasonal differences in the expression of the first eight genes but the differences in the promoter regions do suggest that the several of the genes could have different regulation methods. This comparison of promoter regions creates a starting point for future promoter analysis and further research on seasonal regulation of melanogenesis in ptarmigan.

Acknowledgements

I don't think it can be overstated how grateful I am to the entire department at UiT for creating the Arctic Animal Physiology course. From the cleaners to the technicians to the teachers the whole place has delivered an amazing learning experience and a welcoming and enjoyable environment. I would not have been able to write this thesis without the support of everyone involved whether it was answering questions about ptarmigan and different scientific methods (both stupid and difficult) or just spending a lunch break with.

I also want to thank both David and Daniel specifically for helping me turn ideas and concepts into actual hypotheses and experiments. You have proved invaluable resources, even when I veered outside of your expertise, and I could not have done any of this without you.

Lastly I want to thank my fellow master's students, Magda, Mariel, Sara, Silje & Sona for keeping me focused, helping me de-stress but mostly for being my friends. I've loved the two years we spent together and I wish you all the best for your future endeavours.

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1. Introduction

1.1. Photoperiod and seasons

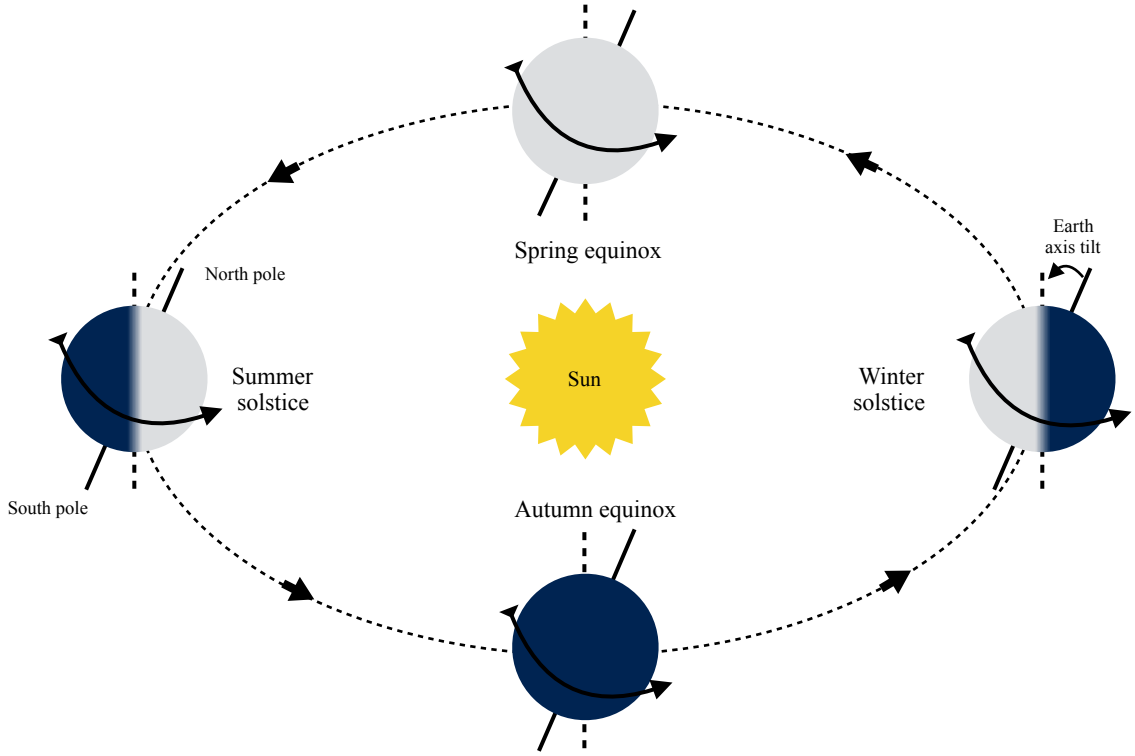

Figure 1. *Model showing the relative position of the Earth and Sun in space for both of the equinoxes and solstices. Arrows on the dotted line show the Earth's annual orbit around the sun, solid arrows show the Earth's daily rotation. The axial tilt of ~23.5° is indicated by the vertical, dotted line and the solid, diagonal line through each model of the Earth.*

As the Earth rotates on its axis, any given place will turn to face the sun and then away again with a twenty-four hour cycle, giving us day and night (figure 1) (Blix, 2005). In the tropics, there is very little annual difference in the duration of day and night, but because the Earth's axis is tilted by about 23.5 degrees, to its orbit around the Sun, the areas in higher latitudes experience annual changes to the duration of day and night, creating the four seasons. These are typically divided by the spring and autumn equinox (where day and night are the same length) and the summer and winter solstice (or the longest and shortest day respectively). At the poles, the axial tilt is experienced at its most extreme, and the sun does not appear above, or below, the horizon for several weeks around the solstices (figure 2a) (Blix, 2005).

Incoming solar radiation heats up the surface of the Earth; meaning that there is a direct, causative, relationship between day length and ambient temperature, which is why summer is warmer than winter at non-tropical latitudes (figure 2). This is then further compounded by the change in the angle of incoming solar radiation across the curvature of the Earth. At the equator, sunlight enters the atmosphere and strikes the ground at about 90º. Increasing latitude increases the amount of atmosphere that the sunlight must penetrate before hitting the ground and consequently the poles receive about 40% of the solar radiation that equatorial regions do. This leads to decreasing, average, ambient temperatures with latitude and explains why the poles are colder than the tropics

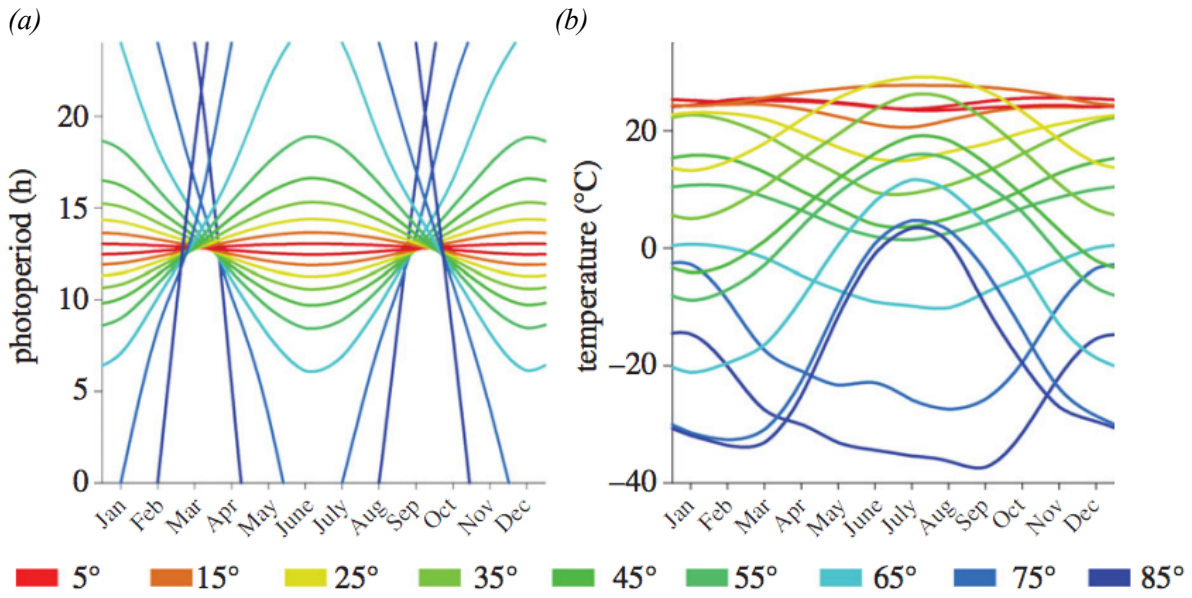

Figure 2. (a) *Variation in annual photoperiod across nine latitudes ranging from equatorial to polar, with each latitude repeated for both hemispheres. Variation increases with latitude; 75° and 85° experience both total darkness and total sunlight.* ***(b)*** *Variation in annual ambient temperature across nine latitudes ranging from equatorial to polar, with each latitude repeated twice for both hemispheres. Variation increases with latitude while mean temperature decreases. Equatorial regions rarely reach below 20°C while polar regions barely reach above 0°C. Modified from Hut et al., 2013.*

and why snow and ice are much more common in temperate and polar regions (figure 2b). The snow and ice covering means that the poles have a high albedo, or reflectance, and consequently they can reflect about 90% of the incoming solar radiation away from the Earth's surface making the region even colder than would otherwise be expected. The regular but highly variable changes in light and therefore temperature have led to similar extreme changes in the availability of freshwater and vegetative growth. As phenological events, like the vegetative growing season, are known to shorten with latitude the Arctic latitudes end up with the shortest growing season on Earth (Hopkins, 1920). These regular changes are a strong evolutionary driver and numerous species have adapted to them; either as migratory species, such as geese and reindeer, or as residents such as Arctic foxes and ptarmigan.

1.2. Seasonal colour change

One of the evolutionary adaptations to regular changes in an environment is the moulting and regrowth of fur or feathers; the winter coat is often thicker or has a different microstructure that reduces heat loss and improves thermoregulation (Russell & Tumlison, 1996). When the two coats have different colours it is known as seasonal colour change (SCC), which can be defined one of two ways. Either, as moulting between a camouflaged, non-breeding morph and a - normally more conspicuous - breeding morph (Mcqueen *et al.,* 2019). Or: as moulting between two distinct, camouflaged, morphs between seasons (Zimova *et al.,* 2018). The first definition, of separate breeding and camouflaged morphs, is only found in birds and is more common in migratory species. This thesis will focus on the second definition, of two separate camouflaged morphs. This is more common in mammals but is also found in birds, such as the three species of ptarmigan.

SCC, here defined as the change between two camouflaged morphs, has evolved separately in several Arctic and sub-Arctic lineages including: leporids (snowshoe hare, *Lepus americanus*)*;* muroids (Siberian hamster, *Phodopus sungorus*)*;* mustelids (stoat, *Mustela erminea*)*;* a canid (Arctic fox, *Vulpes lagopus*)*;* and birds (ptarmigan, *Lagopus spp*.). The variety of species indicates that SCC has evolved in response to environmental pressure, it should also be noted that individuals that do not experience the same selection pressures do not always undergo SCC. For example: Arctic hares (*Lepus arcticus*) only have a brown morph in their most southern ranges, where the snow regularly melts in the summer. Similarly, stoats (*M. erminea*) can be found across Eurasia and individuals in lower latitudes do not have a white morph. The red grouse (*Lagopus lagopus scoticus*) a subspecies of willow ptarmigan (*Lagopus lagopus*) that is endemic to the UK, also lacks a white morph and remains brown year-round, again correlating to annual snow cover (Miranda *et al.,* 2021; Walsh, 2021; Kozma, 2016).

Although the species that undergo SCC have a white morph it is commonly mostly white and patches of dark fur or feathers are retained throughout the winter; for example: the black rectrices (tail feathers) of *Lagopus muta* and *Lagopus lagopus* as well as the black eyestripe of male *L. muta*; the black ear tips of *Lepus spp*. and the black tail tip of *M. erminea*. There are also pigment variations in brown morphs. Most of the mammals that exhibit SCC have dorso-ventral countershading in their summer morphs and have pigmented fur covering their back and sides and white, or lighter, fur covering their throat, thorax and abdomen; a trait they share with other non-seasonally changing mammals (Caro, 2013). Additionally, all three ptarmigan species have white primaries (the larger of the wing feathers) year-round; which, along with their rectrices, are moulted once a year in the summer. This contrasts with the fur of mammals, which is completely moulted and regrown to change between morphs.

Because SCC has evolved in different vertebrate groups it is not necessarily the same mechanism in each species, and it has been less studied in birds. It is clear that the seasonal timing is determined by changes in photoperiod. Decreasing photoperiod in the autumn induces a moult to the white, winter morph and increasing photoperiod in the spring induces a moult to the brown, summer morph (Höst, 1942). This is true for most species that exhibit SCC and keeps them in synch with the changing environment and the subsequent changes in snow cover and temperature (Zimova *et al*., 2018). However SCC species also have an endogenous clock that keeps time in the absence of environmental information. If kept in photoperiod conditions that simulate an artificially long winter *P. sungorus* will start the spring moult into brown fur within 38 weeks despite a lack of increasing photoperiod (Zimova et al., 2018). *L. muta hyperborea* on the other hand start the autumn moult into their white plumage after 20 weeks of an artificially long summer (Aspelund, 2012). Both of these species exhibit photo-refractoriness; becoming insensitive to the usual stimulation of photoperiod after prolonged exposure. *P. sungorus* are short day photo-refractory and *L. m. hyperborea* are long day photo-refractory. Their endogenous clocks are entrained by natural photoperiod cues to maintain an annual moulting rhythm but can function on their own (Dunlap et al., 2004).

While temperature does not affect the timing of the moult, it can affect the speed. In warmer autumns the moult will take longer and colder autumns lead to faster moults. The opposite is true in the spring; warmer springs lead to faster moults and colder springs to slower ones (Zimova *et al*., 2018; Watson, 1973). This plasticity has the obvious benefit of matching colouration and insulative

properties with the environment, although the mechanism through which these changes take place is unclear.

Moulting is under hypothalamic control based on hormonal secretions from the pituitary gland. In birds, thyroid hormone concentration increases before or during moulting and also stimulates gonadotropin-releasing hormone neurons to activate gonadal growth and development in preparation for the breeding season (Nakane & Yoshimura, 2014; Höhn & Braun, 1980). Prolactin concentration increases throughout the breeding season peaking at the time of the post-nuptial moult. Moulting has been prevented through active immuno-neutralisation of both prolactin and vasoactive intestinal polypeptide (the neuropeptide that stimulates the release of prolactin) in both starlings (a passerine) and turkeys (a galliform) (Kuenzel, 2003). Thus the seasonal secretions of thyroid hormones and prolactin are required for the initiation of moulting in birds. However, this research is mostly based on birds that moult once a year after breeding and has not been related to melanin production. Thyroid hormone concentration increases around the time of all three moults in ptarmigan (Zimova *et al*., 2018; Höhn & Braun, 1980). Therefore it seems plausible that pigment production and moulting are controlled by separate factors. The regulation of pigment synthesis and deposition may be controlled locally by the cells of the feather follicles and the surrounding skin. It is therefore important to understand the physical structure of follicles and how feathers are grown and replaced.

1.3. Feather follicles and follicle cycling

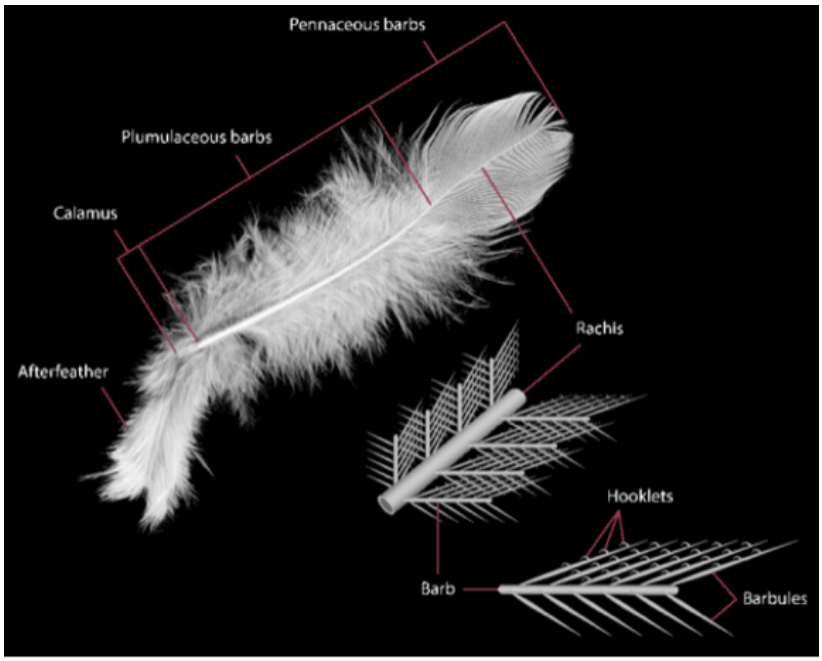

Figure 3. *A photograph of a feather from the back of a Svalbard ptarmigan, Lagopus muta hyperborea, with illustrations depicting the interlocking barbs and barbules. Credits: photograph by Benjamin Judix, illustrations by Vidar Holie. Modified from Nord et al., 2023.*

Feathers are a uniquely avian trait and provide important defensive, thermoregulatory, and locomotive properties through their structure (Stettenheim, 2000). The arrangement of interlocking keratin fibres: reduces mechanical damage to the skin, traps air close to the skin where it can retain body heat, and allows birds to fly (figure 3). This interlocking structure also creates a relatively

large surface area for colouration, at least compared to the analogous keratin structure of fur in mammals. Through the synthesis and deposition of different pigments, such as melanins or carotenoids, birds can use their feathers to camouflage into their environments and reduce predation. They also facilitate communication about sexual availability and fitness to other members of their species, serving to both intimidate rivals and attract mates (Yoshioka & Akiyama, 2021; Chen *et al.,* 2015; Dawson *et al.,* 2001; Ralph, 1969). These functions are not evident in all bird species. Some are famous for sexual dimorphism; with clear differences in the appearance and behaviour of the two sexes, like peacocks, birds of paradise or mallard ducks, where males are more conspicuous and females more camouflaged. Others, like geese, show camouflage but not dimorphism. Others still, like parrots, show neither dimorphism nor camouflage (figure 4).

Figure 4. *Photographs of different bird species displaying sexual dimorphism and/or camouflage.* ***Top left:*** *Anser anarauna.* ***Bottom left:*** *Branta canadensis.* ***Middle:*** *Anadorhynchus hyacinthus.* ***Top right:*** *Anas platyrhynchus (female).* ***Bottom right:*** *Anas platyrhynchus (male). Credits: Linh Moran.*

Because the function of feathers is so closely tied to their structure, if they become damaged they can become non-functional or even detrimental to a bird's survival. Broken flight feathers would impair flight, potentially leading to increased predation risk or reduced hunting success, while a different appearance could reduce mating success. Birds can replace individual feathers as needed but most birds also moult their feathers and grow new ones once a year (Ralph, 1969). Moulting can also be an opportunity to change feather colouration. Most species have a different appearance as chicks and adults and some bird species have separate, often conspicuous, breeding morphs that they moult in and out of for the breeding season (Ralph, 1969, McQueen *et al.,* 2019).

Feathers are produced by, and attach to the skin through, feather follicles; cylindrical invaginations of the epidermis with a keratinised lining surrounded by collagen and elastic fibres (figure 5) (Stettenheim, 2000). The outer dermal tissues, and by extension the follicle itself, have a good supply of blood vessels and sensory fibres. Most are connected by smooth muscle bundles and elastic fibres that hold the calamus, or lower shaft, of the feather in the follicle through isometric contraction (Stettenheim, 2000). The high variability in feather morphology does not translate to the

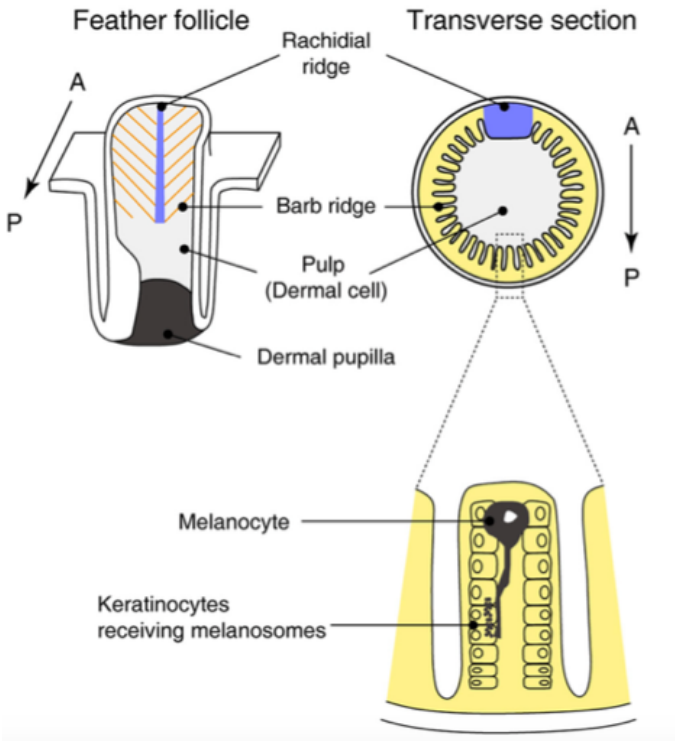

Figure 5. *Schematic of a feather follicle during the growth phase, showing the placement of melanocytes and adjacent keratinocytes in the barb ridges. Modified from Inaba & Chuong, 2020.*

follicles; aside from diameter and surrounding musculature, the follicles are nearly identical morphologically across the bird and even between species (Stettenheim, 2000).

Although feathers are larger and structurally more complex than mammalian hair the basic principle regarding their growth is the same. Follicles produce keratin, which is built into the required shape, they then halt keratin production until the hair or feather is moulted and they grow a new keratin structure. These alternating growth and resting phases can be further broken down into: initiation; growth; rest; and moulting; before repeating (Chen *et al.,* 2015). As mentioned earlier, because different species moult a different number of times, and at different times, throughout the year the length of the resting phase is highly variable across species. Additionally the cycle can be forced into a premature repeat by removal of the feather, either intentionally by the bird or by outside factors, such as predators. The colour and pattern of the feathers is determined by structural proteins and pigments that are synthesised in the skin and built into the developing feathers by the keratinocytes (figure 5) (Galván & Solano, 2016). Once the feather has been produced its appearance is fixed unless changed by an outside factor, such as abrasion or soiling. The most common colouration, particularly for SCC, is brown; this is created by the synthesis and deposition of melanins.

1.4. Melanin structure and properties

Melanin refers to a group of water insoluble, phenol-based pigments most commonly derived from the oxidation of the non-essential amino acid tyrosine; it can also be derived from other phenolic compounds (Wakamatsu & Ito, 2021; Sugumaran & Barek, 2016; Urabe *et al.,* 1994). More specifically it can either be the dark, brown or black eumelanin or the lighter, red or yellow phaeomelanin. There is also a third type, neuromelanin, but it is used in a non-pigmentary capacity

in the brain (Wakamatsu & Ito, 2021; Sugumaran & Barek, 2016; Urabe *et al.,* 1994). Depending on the ratio of eumelanin to phaeomelanin a fairly wide range of colours can be produced, for example, these two pigments give rise to the variety of colour seen in human hair and the different morphs of ptarmigan (figure 9).

1.5. Melanogenesis

Melanogenesis, or melanin synthesis, occurs within the melanosomes of melanocytes (figure 5). Post-production the melanosome will be transferred along the dendritic projections of the melanocyte to the adjacent keratinocytes where it will be built into keratin-based structures such as feathers or fur. Each melanocyte can project to multiple keratinocytes and the normal ratio is 1:30 (Wakamatsu & Ito, 2021).

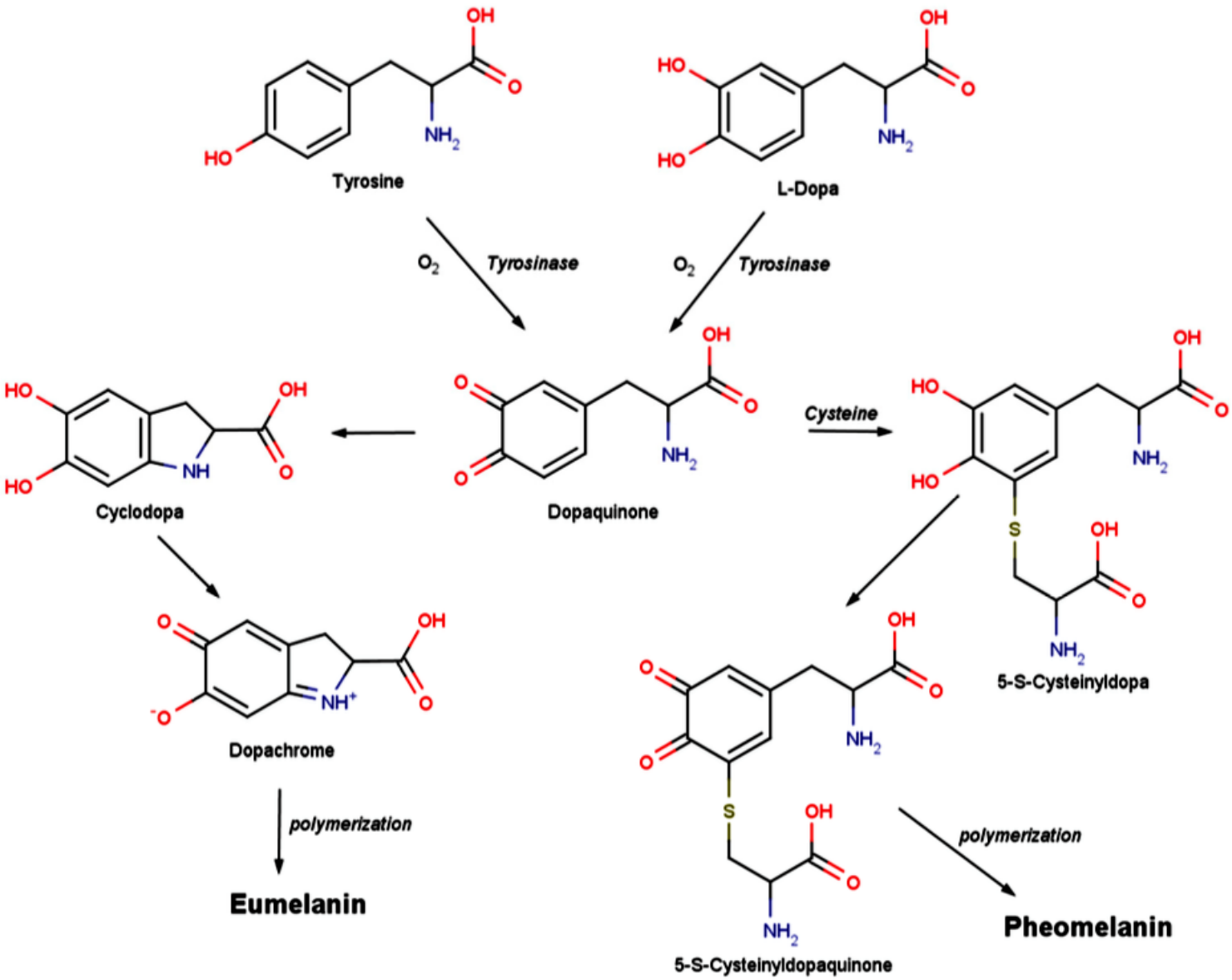

***Figure 6**. Schematic of melanogenesis for both eumelanin and phaeomelanin from tyrosine or L-dopa. Modified from Pralea et al., 2019.*

1.5.1. Regulation within the melanocyte

In the classical melanocortin signalling pathway the first step of eumelanogenesis is the conversion of either tyrosine or L-dopa to dopaquinone, catalysed by the enzyme tyrosinase (figure 6). Dopaquinone spontaneously cyclises to cylcodopa which then oxidises into dopachrome. Dopachrome is then isomerised by the enzyme DCT (dopachrome tautomerase) to produce quinone methide, which is converted to either DHICA (5,6-dihydroxyindole-2-carboxylic acid) or DHI (5,6-dihydroxyindole). The enzyme TYRP1 (tyrosinase related protein 1) has a dual role of oxidising DHICA and stabilising tyrosinase (Murisier & Beermann, 2006). The end result is a combination of DHICA-melanin, DHI-melanin, and mixed-DHICA-DHI-melanin within the melanosome, all categorised as eumelanin (figure 6) (Pralea *et al*., 2019;Sugumaran & Barek, 2016; Urabe *et al.,* 1994).

Eumelanins can also be produced from other phenol-based compounds such as adrenaline, noradrenaline, and dopamine. All three of these compounds can be oxidised by tyrosinase into quinones, which can then be converted into leucochrome in the place of dopaquinone; the rest of the pathway can continue in the same way as from tyrosine (Sugumaran & Barek, 2016; Urabe *et al.,* 1994).

Phaeomelanogenesis can be considered as an alternate, or default, branch of the eumelanogenesis pathway. Once tyrosinase has produced dopaquinone, or a dopaquinone-like compound such as dopamine quinone, spontaneous addition reactions will occur with thiols, such as cysteine, producing thiolated catecholamines (figure 6). These thiolated catecholamines then go through a series of oxidation, cyclic adduction, aromatisation and finally oxidative polymerisation reactions to produce phaeomelanins (Sugumaran & Barek, 2016; Urabe *et al.,* 1994). Whether phaeomelanins or eumelanins are produced requires a switch between these branches, or more accurately, considering the wide variety of shades and patterns available in the natural world, a fine-tuned dial of hormonal secretion that alters the ratio of eumelanins to phaeomelanins (figures 6 & 7).

1.5.2. Regulation outside of the melanocyte

A key part of this dial is the interaction of MC1R (melanocortin receptor 1), ASIP (agouti signalling protein) and α-MSH (α-melanocyte stimulating hormone) in the cell membrane of melanocytes. MC1R is a transmembrane G-protein coupled receptor, located in the outer cell membrane of melanocytes. When stimulated by α-MSH it starts a signal cascade by increasing intracellular levels of cAMP (cyclic adenosine monophosphate) leading to increased tyrosinase activity and the ultimate production of eumelanins (figure 7) (Boswell & Takeuchi, 2005). ASIP also binds to MC1R and acts as the antagonist to α-MSH; decreasing intracellular levels of cAMP, leading to decreased tyrosinase activity and the ultimate production of phaeomelanins (figure 7) (Boswell & Takeuchi, 2005). In snowshoe hares, *Lepus americanus,* there is a significantly higher *Asip* expression in the old, white hair follicles compared to the follicles that had recently moulted and were growing brown fur (Ferreira *et al.,* 2020). This means that ASIP can be responsible for switching to phaeomelanin production or simply switching off melanin production all together.

In the context of SCC most species have a system more akin to a switch than a dial and it is activated twice a year: once in the spring when melanogenesis is turned on and once in the autumn when it is turned off. As ptarmigan have more than two moults in a year, with different morphs requiring different ratios of melanins, they seem to have a more complicated system than other

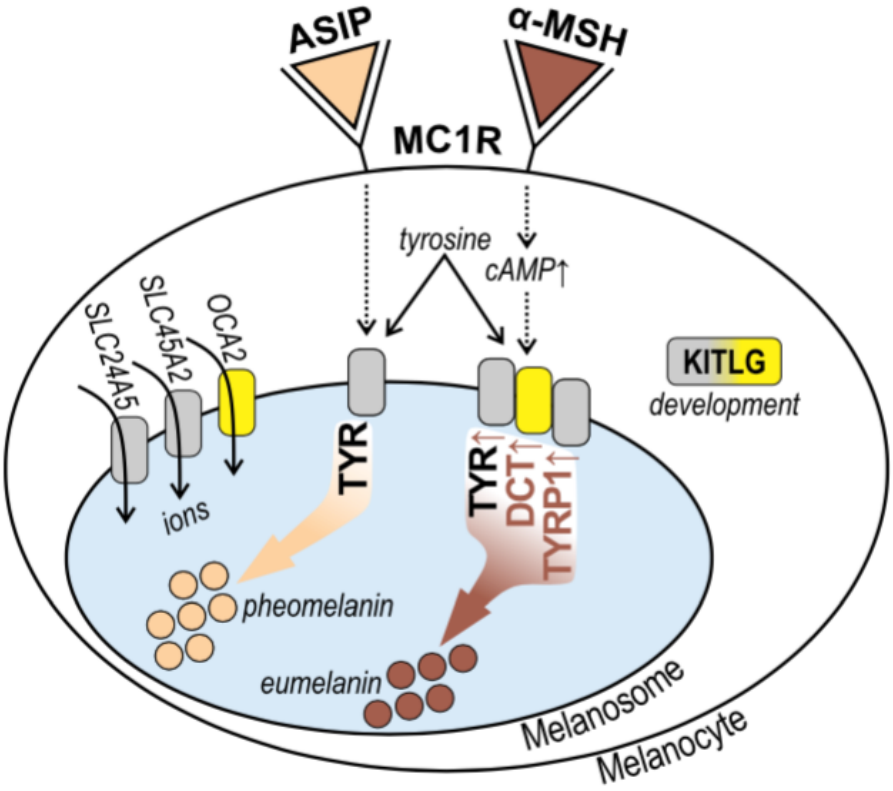

***Figure** 7. Schematic of melanogenesis in a human melanocyte. ASIP and α-MSH bind with MC1R in the melanocyte cell membrane, leading to a decrease or increase in cAMP signalling and tyrosinase activity respectively. ASIP binding, and decreased tyrosinase activity, leads to the production of phaeomelanin. α-MSH binding, and increased tyrosinase activity, leads to the production of eumelanin. Melanogenesis occurs in the melanosome, ion transporter proteins, like OCA2, SLC45A2 & SLC24A5, cause the melanosome to migrate along the dendritic projections of the melanocyte to the keratinocytes (not pictured). Modified from Hudjašov, 2013.*

animals. Melanogenesis is controlled not just by α-MSH and ASIP but also the gonadal hormones testosterone and luteinising hormone (figure 8). It is also possible that the white morph is created not by an absence of hormonal stimuli, as suggested by Höhn and Braun (1980) (figure 8), but is instead created by an increase in ASIP concentration within the skin, as seen in *L. americanus.* Whether this is the default for SCC is unknown as it has not been tested in all species (Zimova *et al*., 2018). Siberian hamsters (*Phodopus sungorus*) have a peak in tyrosinase activity at the time of both the moult in and out of their white winter morph (Logan & Weatherhead, 1980). This suggests that the absence of melanin in their fur is due to the inhibition of a post-tyrosinase step, and not related to ASIP activity. In most SCC species there is also the additional factor of regional regulation; melanogenesis can be localised to only part of the body, such as the tail tip of *M. erminea*. This is compounded in male *L. muta* which produce white primaries alongside pigmented body feathers in the summer and black feathers around the eyes, alongside white body feathers in the spring.

Agouti is a hormone encoded by the *Asip* gene; α-MSH, however, is only one of the possible end products of the post-translational modification of POMC (pro-opiomelanocortin). Once POMC has been translated from RNA to an amino acid chain it has the potential to become different peptides

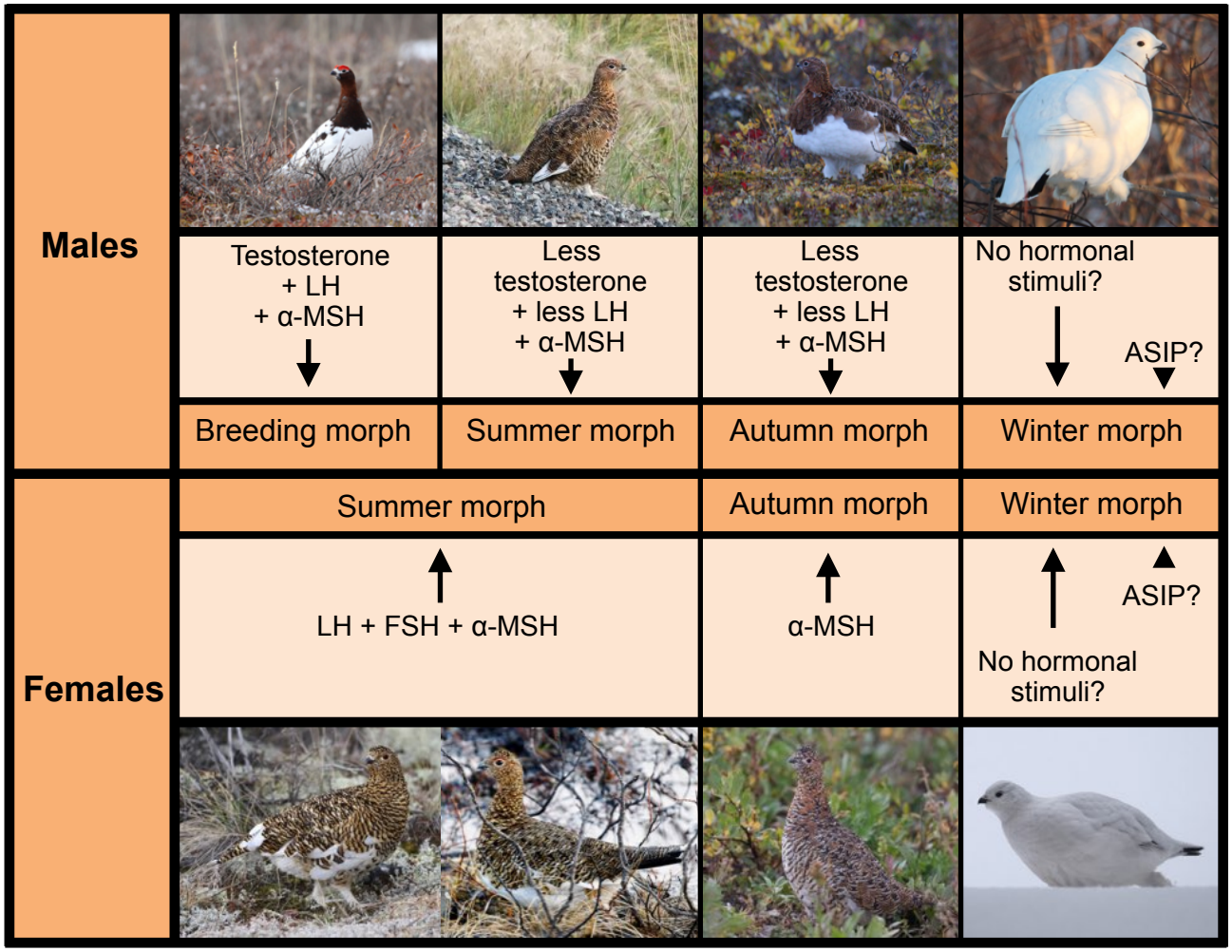

Figure 8. *A suggested model for the hormonal control of plumage colour in Lagopus lagopus. LH = luteinising hormone, FSH = follicle stimulating hormone, MSH = α- melanocyte stimulating hormone, ASIP = agouti signalling protein. Modified from Höhn and Braun, 1980. Photos sourced from macaulaylibrary.org Credits, clockwise from top left: Seth Beadreault, Michael Jacques, Jennyq Fu, Adele Dueck, Alvan Buckley, Blair Dudeck, Matti Rekilä, Alex Lamoreaux.*

depending on exactly how it is modified. These include: ACTH (adrenocorticotropic hormone), joining peptide, CLIP (corticotropin-like intermediate hormone), and α-, β-, and ɣ-MSH (Harno *et al.,* 2018).

POMC is initially cleaved by PC1/3 (pro-protein convertase 1/3), into pro-ACTH and β-LPH (β-lipotropin). PC1/3 then cleaves pro-ACTH into ACTH, pro-ɣ-MSH and joining peptide. ACTH is then cleaved by PC2 (pro-protein convertase 2) into ACTH(1-17) and CLIP. ACTH(1-17) then has the amino acids at its COOH terminal removed by CPE (carboxypedtidase E) to form ACTH(1-13). ACTH(1-13) is then amidated by PAM (peptidyl-glycine α-amidating monooxygenease) to form ACTH(1-13)NH_2. This new NH_2 terminal is then acetylated by N-AT (N-actyltransferase) to form α-MSH (Scanes & Pierzchała-Koziec, 2021; Harno *et al.,* 2018). PC1/3 and PC2 are therefore both necessary in the first steps for the synthesis of α-MSH and their presence indicates that POMC is being processed not just present.

Not only does POMC have the capacity to be modified into different proteins but α-MSH and ASIP can also interact with other receptors. MC1R is one member of a family of melanocortin receptors

(MC1-5R); these receptors are all activated by end products of POMC, with varying levels of binding preference. MC1R is only found in melanocytes and localised to tissues that require pigment, such as the skin or irises. The signalling cascade that produces eumelanin can be initiated by both α-MSH and ACTH binding with MC1R (Harno *et al*., 2018). The other melanocortin receptors (MC2-5R) are expressed in different tissues and are involved in different pathways including: glucocorticoid synthesis, energy homeostasis and appetite regulation, and the stimulation of exocrine glands in the skin. This means that a change in the plasma concentration of circulating POMC could have several effects on different systems of the body and it is unclear how, or if, this is regulated in species that undergo SCC.

1.5.3. Other genes related to melanogenesis

Previous studies (Ferreira *et al*., 2020; Boswell & Takeuchi, 2005; Urabe *et al.,* 1994; Höhn & Braun, 1980), have put a strong emphasis on the agonist/antagonist relationship that the hormones α-MSH and ASIP have with MC1R but there are over 150 genes that have been linked to pigment production and deposition, with varying importance and specificity (Zimova *et al.,* 2018). These include: CORIN, MITF, and SLC45A2; that have functions in the melanogenesis pathway but are not necessarily involved in melanin production itself.

CORIN (serine peptidase) is expressed in the dermal papilla and inhibits ASIP-MC1R binding, as well as being expressed in the heart and helping to regulate blood pressure (Bourgeois *et al.,* 2016). MITF (melanocyte inducing transcription factor) binds to the e-box sequence in the promoter region of different genes, including selected key genes of melanogenesis: TYR, TYRP1, DCT & MC1R. But also for genes unrelated to melanogenesis: hypoxia inducible factor 1α, cyclin-dependent kinase inhibitors, and T-box 2 protein, among others (Vachtenheim & Borovanský, 2010). MITF expression can be induced by a pathway set off by MC1R, involving cAMP and CREB signalling, and creates a positive feedback loop increasing the production of melanin within a stimulated melanocyte (Aoki & Moro, 2002). SLC45A2, is expressed in the melanosome organelle membrane and is one of a number of cation exchange channels that are involved in the transport of the melanosome to the keratinocytes (Bourgeois *et al.,* 2016).

The other non-classical pathways for melanogenesis include the endothelin signalling pathway and WNT/β-catenin signalling pathway. EDNRB (endothelin receptor type B) is a G-protein coupled receptor, like MC1R, and stimulates the proliferation of melanoblasts, the precursors to melanocytes, through the binding of EDN3 (endothelin-3). This multi-step pathway includes the activation of: PKC (protein kinase C); MAPK (mitogen-activated proteinase cascade) and MITF (Kulikova, 2021). Members of the WNT family of lipoglycoproteins bind to their corresponding Frizzled receptor, another G-protein coupled receptor, which induces the expression of MITF and the differentiation of melanoblasts into melanocytes (Kulikova, 2021).

Ptarmigan have been shown to produce new pigmented feathers in response to injections of posterior pituitary extract and α-MSH, after the previous white feathers had been partially plucked (Höhn & Braun, 1980). While this does prove that the melanocortin pathway is active in ptarmigan, and indeed active during the winter, it does not give any indication on the seasonal regulation of this, or any other pathway. In humans POMC expression can be induced by the transcription factor *p53* in response to UV irradiation, and the resulting synthesis of melanin helps prevent further damage to the cell nucleus from UV light. This is not the only pathway for melanogenesis, but

seems to predominate in human skin; other animals, such as ptarmigan, do not have exposed skin and the expression of POMC in the skin is not completely understood (Vachtenheim & Borovanský, 2010). Because SCC only occurs in a select number of species, all within one environment, it is not as widely studied as colour variation or polymorphism in non-SCC species that remain in one morph for most of their lives.

1.6. Genetically-based melanin variation

Mutations in the genes involved in melanogenesis can create individuals with a different appearance. When a group, or groups, within a population have a different appearance to the species standard this becomes known as polymorphism (McLean & Stuart-Fox, 2014).

Much of our understanding of the genetic pathway of melanogenesis in birds comes from "loss of -" and occasionally "gain of - function" mutations in domestic chickens, *Gallus gallus domesticus.* They have been bred to have a wide variety of polymorphs from their non-domestic ancestor the red jungle fowl, *Gallus gallus*. This breed variety, general availability, lack of or low natural selection, and fully sequenced genome has made chickens an ideal study animal for the genetics of melanogenesis in birds (Akiyama & Kinoshita, 2021).

Because MC1R codes for a switch between eu- and phaeo- melanogenesis, mutations in this receptor can cause polymorphism in numerous species. MC1R mutants can still synthesise and deposit melanin but the amount and resulting colour differs to their non-mutant counterparts (Akiyama & Kinoshita, 2021; Mundy, 2005). Mutations that cause over-expression lead to darker individuals, such as the black meat chicken, the lesser snow goose, (*Anser c. caerulescens*) which has numerous discrete blue morphs, and the Arctic fox, which has a blue morph in place of its usual white winter coat (Mundy, 2005; Våge *et al*., 2005). Mutations that cause dysfunctional MC1Rs generally lead to paler morphs than non-mutants, such as the brown-red morph of grey partridge, *Perdix perdix* (van Grouw, 2017).

Barn owls, *Tyto alba,* normally have light brown feathers on their backs and white feathers on their ventral side, including the legs and under the wings, their feathers also feature small eumelanistic spots (San-Jose *et al.,* 2016). A substitution mutation in their MC1R gene leads to the production of phaeomelanin in their ventral feathers and they appear a light brown-red colour with small black spots, known as a rufous morph (San-Jose *et al.,* 2016). The standard white morphs have lower expressions of MC1R, TYR, TYRP1, OCA2, SLC45A2, KIT and DCT and a higher expression of ASIP than the rufous individuals, as would be expected based on their colouration (San-Jose *et al.,* 2016). The expression of PC2 had a positive correlation with phaeomelanin content, but only in the white morphs, i.e. the non-mutant individuals. This indicates that MC1R is able to influence the expression of genes both down- and up-stream of itself in the melanogenesis pathway (San-Jose *et al.,* 2016).

Great tits, *Parus major*, have a eumelanin-based variation in phenotype. The black stripe running down the middle of their breast varies in width and this impacts breeding success and survival. There is no corresponding variation in the amino acid sequence of MC1R, however, and it is likely caused by other genes (Riyahi *et al*., 2015). This shows that there is not always an obvious relationship between phenotypic and genetic differences. The same polymorph could be caused by differences in expression of a number of different genes (van Grouw, 2017).

There are also morphs that are not normally found in the wild because they lead to negative selection pressures that mean the mutations are unlikely to be passed along to offspring. These can be relatively benign, such as albinism or leucism; where lack of pigment makes the animal more conspicuous. Or the mutations can lead to changes in morphology, physiology and behaviour alongside a different morph. These changes can be more malignant; for example: artificial selection of a recessive, lighter morph of mallard ducks led to a higher mortality rate of embryos and ducklings suggesting an associated semi-lethal weakness (Lee & Keeler, 1951). There is also the 'lethal-yellow' phenotype in mice, characterised by light yellow fur, late onset-obesity and hyperphagia, type 2 diabetes, and an increased likelihood of developing tumours (Dinulescu & Cone, 2000). This phenotype is caused by a mutation in *Asip*, where it becomes attached to the promoter and first non-coding intron of the unrelated *Raly* gene. Because ASIP is now expressed when RALY should be, it is removed from any of its usual regulation and becomes over-expressed in multiple tissues, interacting with any of the other melanocortin receptors to induce body wide signalling cascades (Dinulescu & Cone, 2000).

Although these studies have improved our understanding of melanogenesis and the genes involved, they do not necessarily help with our understanding of seasonal regulation. Particularly of SCC which has evolved convergently in unrelated species due to shared selection pressures, and particularly of ptarmigan species which have multiple morphs all with different expressions of eu- and phaeo-melanin. *L. lagopus* and *L. l. scoticus* show very little difference in the genes associated with melanogenesis, like MC1R, TYR and DCT, despite having clear differences in melanin-based phenotype (Skoglung & Höglund, 2010). It seems more likely that seasonal variation in colour deposition and overall phenotype is due to the regulation of pigmentary genes than of differences in the genes themselves (Bourgeois *et al.,* 2016).

1.7. Ptarmigan as a study animal

The ptarmigan genus, Lagopus, consists of three species, the willow ptarmigan, *Lagopus lagopus,* the rock ptarmigan, *Lagopus muta* and the white-tailed ptarmigan, *Lagopus leucura*, in the order galliform, or the grouse family of birds. All three of these species live in the northern hemisphere, ranging from temperate to high Arctic latitudes and the Svalbard ptarmigan, *Lagopus muta hyperborea*, is the northernmost resident bird staying in the Svalbard archipelago year-round, at about 78º latitude. While the lagopus genus as a whole cannot boast the uniqueness of the Svalbard ptarmigan it is worth noting that 80% of bird species live and breed in the tropics (Dawson *et al.,* 2001). This means that ptarmigan have adapted to a more seasonally variable environment than most other birds. Hence, they display pronounced seasonal rhythms, including: activity, fat deposition, reproduction, and moulting (Melum, 2018; Kozma, 2016).

The collective interest in ptarmigan species has lead to the full genome sequencing of all three species and the subspecies *L. l. scoticus* (*L. muta* - Bioproject: PRJNA853367, Accession: JAMCCT000000000.1.; *L. leucura* - Bioproject: PRJNA752366, Accession: JAHKMA000000000.1.; *L. lagopus* & *L. l. scoticus* - Skoglund & Höglund, 2010). This means that the species can be compared at both a phenotypic and genetic level both to each other and to themselves at different times of the year and at different stages in annual cycles. These different adaptive cycles have been well studied, although not all are fully understood. This thesis will focus

on the cycle of feather moulting and the resulting morphs that are common to the three ptarmigan species, but not *L. l. scoticus*.

1.8. Moulting and morphs of ptarmigan

Most birds have one morph and moult once a year, after the breeding season, normally in spring/ summer so that the energetically costly feather growth coincides with readily available food (Dawson *et al.,* 2001). Ptarmigan moult more than once a year, with several distinct morphs that vary between species (figure 9). Like most other species there is a full body moult in the summer

Figure 9. *Photographs of different morphs of male ptarmigan species in different seasons. Photos sourced from macaulaylibrary.org with original photographers credited.*

where ptarmigan will replace their primaries and rectrices (outer wing and tail feathers respectively), but they also have partial moults throughout the rest of the year, where only some or most of the feathers are replaced (Zimova *et al.,* 2018; Pyle, 2007; Höst, 1942).

Male *L. lagopus* first moult in the spring towards the end of March. New pigmented feathers are grown on their head, neck, upper breast and back in a dark red/brown colour, the rest of the feathers remain unshed and unchanged from the previous white winter morph. The naked wattles, or combs, above the eyes become enlarged and develop a bright red colour (figure 9) (Zimova *et al.,* 2018; Höst, 1942). The next moult occurs in early summer, after the breeding season, and all of the feathers are shed and regrown with an overall less red and more brown colouration and the combs regress in size and lose their red colouring. This summer morph is then quickly replaced with a third moult into a lighter brown and red autumn morph, these morphs can appear in such quick succession that the autumn moult can start before the summer moult has completely finished (Zimova *et al.,* 2018; Höst, 1942). The final moult occurs around October and new un-pigmented feathers are grown across the entire body starting from the flanks and abdomen and finishing on the head, except for the rectrices, which remain black with white tips (figure 9). They also grow thicker, white, feathers on their legs and feet which not only aid in thermoregulation but also mobility, combining with longer claws to act as snow shoes, allowing them to walk on the surface of the snow layer rather than through it (Zimova *et al.,* 2018; Höhn, 1977; Höst, 1942). Female *L. lagopus* do not have breeding plumage; their first moult also starts towards the end of March but they moult into their summer morph which they will keep until the end of the brooding season, a few weeks after their eggs have hatched, at which point they will moult into their autumn morph and then winter morph in the same pattern as the males (Zimova *et al.,* 2018; Höst, 1942).

As mentioned earlier there is a subspecies of *L. lagopus* in the UK, the red grouse, *L. l. scoticus,* that has been geographically isolated, and since genetically isolated, from *L. lagopus* (Kozma, 2016). Likely due to the inconsistent annual snow cover in the UK, *L. l. scoticus* have lost their white morph and instead remain brown year-round, with the males growing red combs in the breeding season (figure 9) (Walsh, 2021).

L. muta are very similar to *L. lagopus* and distinguishing the two species can be difficult. The males lack a distinct breeding plumage and their fitness is conveyed by the whiteness of their plumage, which becomes increasingly conspicuous as the snow begins to melt (figure 10) (Piersma & Dent, 2003). Like *L. lagopus*, the males also grow red combs above their eyes during the spring breeding season (figure 9) (Zimova *et al*., 2018). Both sexes moult from their white winter morphs into their pigmented summer morphs in the spring, starting with the feathers on the crown, then the breast and back and lastly the feathers on the abdomen. The males typically start moulting later than the females but both seem to come to the end of their moults at the same time (Zimova *et al.,* 2018; Hewson, 1973). Most sources describe the summer morph as a dark brown but *L. m. hyperborea* have been described by Steen & Unander (1985) as having distinct sexual dimorphism, with the females summer morph being a lighter golden brown, more similar to *L. lagopus*, than the greyish brown of the males (figure 9). This discrepancy could be due to the quick succession of summer, autumn, and winter moults as individuals can be constantly moulting during the summer and autumn months. Regardless of the exact colouration the summer morph is still moulted into the autumn morph, which is then moulted into the winter morph around October (figure 10). The winter morph of *L. muta* is similar to *L. lagopus* in that it is mostly white with black rectrices but in

addition to this the males also have a prominent black eyestripe that they maintain throughout the winter until they moult again in spring (figure 9) (Zimova *et al.,* 2018; Hewson, 1973).

Lagopus leucura are also similar to *L. lagopus* and also have a partial moult into their breeding plumage in the spring followed by a pigmented summer morph, a separate pigmented autumn morph and an un-pigmented winter morph (figure 9). The difference between the species is that, as the name white-tailed ptarmigan implies, *L. leucura* do not have pigmented rectrices and they are completely white in the winter, the males also have white feathers on their lower breast and abdomen year-round (figure 9) (Zimova *et al.,* 2018).

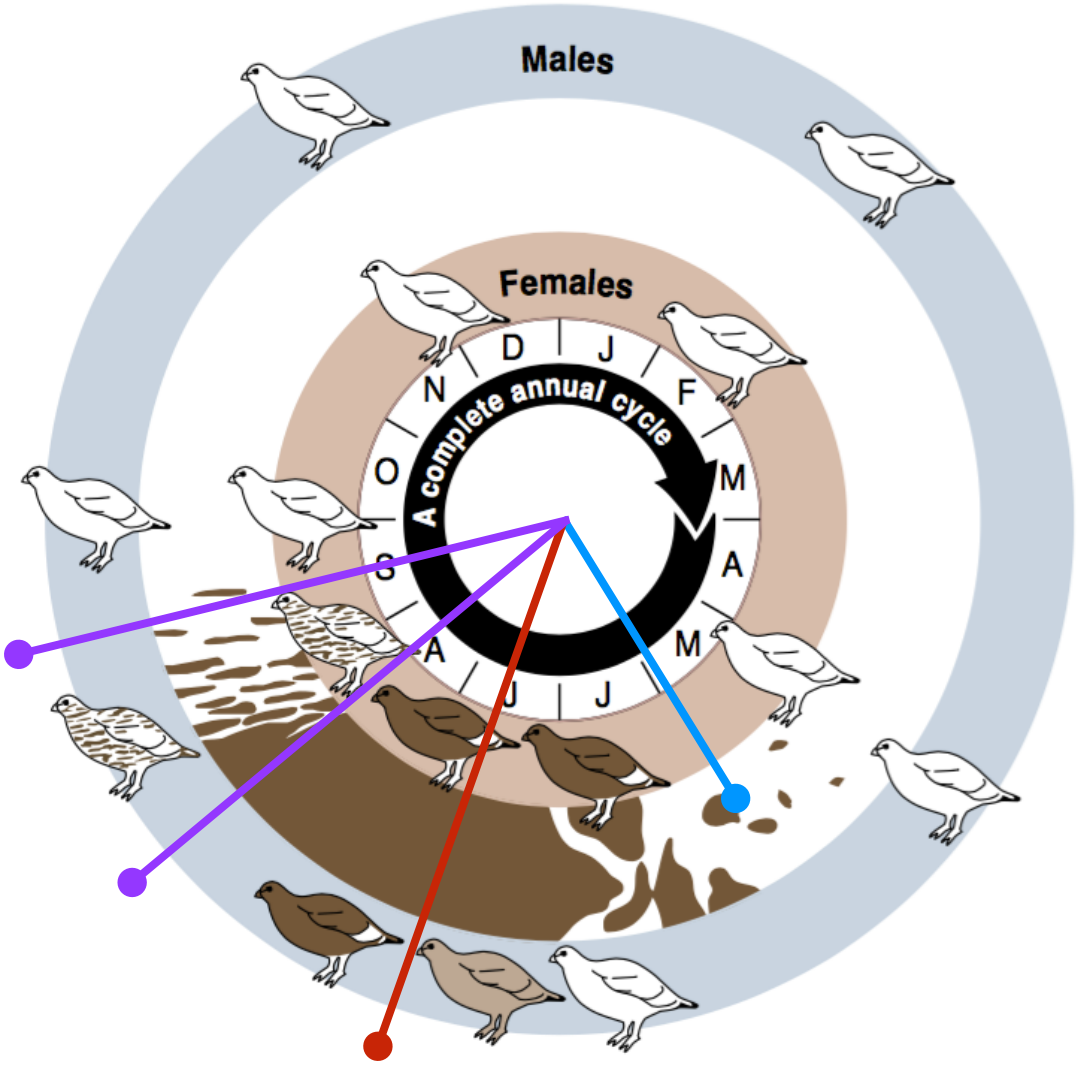

Figure 10. *Annual cycle of the appearance of Lagopus muta in the Canadian Arctic in relation to seasonal changes in snow cover (indicated by the appearance of the second circle). The changes in appearance are caused by moulting of feathers, aside from the males in early summer which soil their white plumage after breeding. The blue and red lines indicate roughly where melanogenesis starts, for females and males respectively. The purple lines indicate the start and end of the moult into the winter morph where melanogenesis stops. Modified from Piersma & Dent, 2003.*

As noted earlier the duration of the autumn and spring moults (in and out of the winter morph respectively) is variable and related to temperature, while the start date is fixed. For female ptarmigan the start of the summer moult (into their autumn morph) is also variable and can change

based on their reproductive timing. The summer moult starts at the end of the brooding season, which itself is dependent on the breeding season. Females that mate later in the year, or have had their nests destroyed and mated twice, finish brooding later and consequently moult into their autumn morph later than individuals that mated and finished brooding earlier in the year (Stokkan *et al.,* 1988; Hewson, 1973). This temporal discrepancy is likely due to the prolonged peak of blood plasma prolactin concentration. Prolactin is responsible for many parental behaviours and physiological processes, such as the production of milk in female mammals, crop milk in columbiform birds, and brooding behaviour in many bird species (Dawson *et al.,* 2001; Riddle *et al.,* 1933). This contrasts with many other bird species that moult in response to an increase in the concentration of blood plasma prolactin and is thought to be a result of photo-refractoriness (Kuenzel, 2003; Stokkan *et al*., 1988).

Although this plasticity only occurs in the females it is similar to the plasticity found in the duration of the white morph. All species studied, that display SCC, show the same relationship between temperature and moulting speed, but ptarmigan are unique in that they can produce incomplete morphs (Zimova *et al*., 2018; Hewson, 1973). Dark brown feathers that do not match their autumn morph can be grown alongside new white feathers during the autumn moult; and white bars or spots can appear on otherwise brown feathers during the spring moult (Zimova *et al*., 2018; Hewson, 1973). The downside of these variable speeds is that faster feather growth leads to the production of low quality feathers, so although they will not be as conspicuous as if they took longer to moult, individual ptarmigan may experience deleterious effects such as impaired thermoregulation or flight (Dawson *et al.,* 2001).

These multiple moults mean that ptarmigan can fit into both definitions of SCC; with a conspicuous breeding plumage, at least for *L. lagopus* and *L. leucura*, and two camouflaged morphs. As stated earlier the focus of this thesis is the second definition and the moulting between pigmented and unpigmented morphs, not between two pigmented morphs. These two moults suggest that, alongside hormonal signals to initiate feather growth, there are separate signals that stimulate and inhibit melanogenesis at different times of the year (figures 8 & 10). These signals could be the up- and down-regulation of genes involved in melanogenesis such as POMC, ASIP, MC1R & TYR and could occur in the feather follicles under local, not hypothalamic, control.

1.9. Aims and hypotheses

Ptarmigan pose an intriguing challenge for studying SCC. As it occurs in different lineages SCC seems to be a convergent evolutionary strategy as the result of environmental selection pressure. Therefore similarity between species may not extend beyond phenotype and could be caused by different regulatory mechanisms in different species. As seen, for example, in the autumn moult in hares and hamsters with the white morph being induced through an up-regulation in *Asip* in hares and an undefined post-tyrosinase down-regulation in melanin synthesis or deposition in hamsters. Ptarmigan are the only bird species to undergo SCC and have more morphs than any of the mammalian species, making them distinct among the SCC group. There are commonalities between the species though: melanogenesis only takes place in the growth phase of follicle cycling and the timing of moulting is determined by photoperiod and can be modified by hormones and temperature (Stokkan, 1987; Hewson, 1973). Greater specificity highlights the differences between species, not the similarities.

Despite a clear seasonal rhythm, and experiments proving changing photoperiod to be the underlying cause, there is little evidence to connect melanogenesis to photoperiod. Light cues induce the follicles to re-enter the growth phase and produce new feathers, during this phase melanocytes will produce melanin (if appropriate) and transport it to the keratinocytes. If the feather is lost the follicle will re-enter the growth phase and an appropriately patterned new feather will be produced although the photoperiod has now changed. Different photoperiods have not been directly linked to melanogenesis in either POMC or ASIP concentration and the timing of moulting and melanogenesis seems almost coincidental. It seems entirely possible that there are two congruent annual cycles within ptarmigan; one controlling the timing of moulting and feather growth and the other controlling the synthesis and/or deposition of melanin, both possibly cued by photoperiod. A similar model has been theorised in *P. sungorus*; unlike the other mammals that exhibit SCC they do not have a clearly defined moulting period, instead they moult continuously in patches. Consequently they can have different areas of skin in different stages of follicle cycling while maintaining two distinct morphs (Zimova *et al*., 2018). To prove or disprove this 'two cycle model' the link between melanogenesis and annual changes in photoperiod needs to be elucidated, whether it is part of the moulting cycle or not.

Melanogenesis is localised to the melanosome organelles of melanocytes and the most common pathway is through the stimulation of the transmembrane protein MC1R. This receptor can bind with either the hormone α-MSH, derived from POMC, or the hormone ASIP. If stimulated by α-MSH, MC1R will start a signalling cascade that produces eumelanins through the oxidation of tyrosine, catalysed by the enzymes TYR, TYRP1 & DCT among others. If MC1R instead binds to ASIP then phaeomelanins will be produced, a high presence of ASIP can also lead to the total inhibition of melanogenesis and white colouration, at least in one species (Ferreira *et al*., 2020). The different morphs that ptarmigan moult between suggest sophisticated regulation of this MC1R melanogenesis pathway on an annual basis, with the possible exception of the autumn moult into the white morph.

Polymorphism in animals is not uncommon but the difference between seasonal and developmental regulation is unclear. It is unlikely that the genetic mutations that cause polymorphism also cause seasonal morphs and it is more likely to be the regulation of these genes. Sequencing has shown high similarity between the genes involved in melanogenesis for *L. lagopus* and the non SCC *L. l. scoticus* (Skoglug & Höglund, 2010). The region for α-MSH in POMC is highly conserved across archosaurs (birds and crocodilians) and suggests that its structure, and therefore function, is highly important (Scanes & Pierzchała-Koziec, 2021). This suggests that seasonal regulation of melanogenesis is not a feature of the genes themselves, however the promoter regions for the genes have not been compared.

Table 1 displays the genes that will be the focus of this thesis and their function in melanogenesis. The first eight, POMC-DCT, will be used for comparing relative gene expression between two groups of samples and for comparing the promoter regions among different species. The last seven, CREB1-TBX19, will only be used for comparing the promoter regions.

POMC, whether secreted from the pituitary gland or by cells in the skin or follicle, stimulates melanogenesis through its derivative α-MSH. POMC will be used to compare gene expression of α-MSH between groups, but, because POMC has low potency and is only bioactive in high

concentrations, PC1/3 & PC2 will also be used as proxies and (particularly for PC2) to indicate that α-MSH is being produced not another POMC derivative (Harno *et al*., 2018).

Comparing relative expression of ASIP & CORIN will determine if ptarmigan have a similar or opposite autumnal regulation pattern to hares. Another possibility is that melanogenesis is down-regulated through the removal of the MC1R protein from the melanocyte cell membrane and that

Table 1. Summary of selected genes involved in melanogenesis that were annotated for all three Lagopus spp. and *L. l. scoticus*

Gene code	Gene name	Function	qPCR	Promoter
POMC	Pro-opiomelanocortin	Signalling protein and precursor to α-MSH among other proteins	Yes	Yes
PC1/3	Pro-hormone convertase 1/3	Part of the modification of POMC to α-MSH	Yes	Yes
PC2	Pro-hormone convertase 2	Part of the modification of POMC to α-MSH	Yes	Yes
ASIP	Agouti signalling protein	Competes with α-MSH to bind to MC1R and induce the production of phaeomelanin	Yes	Yes
MC1R	Melanocortin receptor 1	Transmembrane receptor that initiates the production of melanins within a melanocyte	Yes	Yes
TYR	Tyrosinase	Rate-limiting enzyme of melanogenesis, converts tyrosine and/or dopa to dopaquinone	Yes	Yes
TYRP1	Tyrosinase related protein 1	Involved in eumelanogenesis, stabilises tyrosinase and oxidises DHICA	Yes	Yes
DCT	Dopachrome tatomerase	Involved in eumelanogenesis, converts dopachrome to quinone methide	Yes	Yes
CREB1	cAMP responsive element binding protein 1	Induces gene transcription in response to the stimulation of the cAMP pathway, which includes melanogenesis	No	Yes
CORIN	Corin serine peptidase	Inhibits ASIP binding to MC1R	No	Yes
OCA2	OCA2 melanosomal transmembrane protein	Codes for P protein, believed to transport small molecules in melanocytes and/or regulate post-translational processing of tyrosinase	No	Yes
MITF	Melanocyte inducing transcription factor	Creates a positive feedback loop up regulating MC1R, TYR, TYRP1 & DCT.	No	Yes
SLC45A2	Solute carrier family 45 member 2	Cation exchange channel that is involved in the transport of the melanosome	No	Yes
SLC7A11	Solute carrier family 7 member 11	Cation exchange channel that is involved in the transport of the melanosome.	No	Yes
TBX19 (TPIT)	T-box transcription factor 19	Induces expression of POMC in the pituitary gland	No	Yes

more are present during the summer than the winter. TYR, TYRP1, DCT, CREB1, OCA2 & MITF are all involved in melanogenesis and act within the melanocyte.

SLC45A2 & SLC7A11 are also active in the melanocyte and are involved in the transport of the melanosome, not melanogenesis itself. TBX19, also known as TPIT, is a cell restricted transcription factor that induces POMC expression in the pituitary gland of mammals, with knockout mice being unable to produce melanin (Boswell & Takeuchi, 2005). Unlike mice humans do not have a defined intermediate pituitary lobe, which makes them more similar to birds. In humans a-MSH is produced locally in the skin in a glucocorticoid based stress response, but it is unclear where it is produced in bird species and in response to what (Boswell & Takeuchi, 2005). Differing promoter regions between SCC and non-SCC species could indicate that melanogenesis is not seasonally regulated but melanin transport and deposition within keratinocytes is.

The aim of this thesis is to investigate SCC in ptarmigan and expand our knowledge of how this is regulated.

The hypotheses regarding this aim are as follows:

1. Melanogenesis is regulated through the expression of key genes of melanogenesis: POMC, PC1, PC2, MC1R, ASIP, TYR, TYRP1 & DCT.
2. These genes will be up-regulated during the production of brown feathers and down-regulated during the production of white feathers.
3. This difference in gene expression will be detectable in the skin and feather follicles of ptarmigan during their moult between brown and white plumage.
4. The promoter regions of the genes involved in melanogenesis (table 1) will differ between *L. l. scoticus,* which does not undergo SCC, and the ptarmigan species that do undergo SCC: *L. muta, L. lagopus*, and *L. leucura*.

2. Materials and methods

Skin samples were taken from *L. lagopus* and *L. muta hyperborea* during the autumn moult. The *L. m. hyperborea* samples came from one individual (ID: Yellow 7) housed at the ptarmigan facility at the University of Tromsø that was euthanised due to illness on the 28th of September, 2022. These samples were used to test different RNA extraction methods and labelled YB1-13X, where 'X' denotes brown or white feathers. The *L. lagopus* samples came from multiple individuals killed during a hunting trip in the Tromsø area in the first week of October and labelled LB1-12X, with X again denoting brown or white feathers.

2.1. RNA extraction - method comparison

Samples were taken from a moulting *L. muta hyperborea* (ID: Yellow 7). Skin was taken from the leg, wing, breast and neck and snap-frozen in a -80ºC freezer. Giving a range of samples from skin that had previously produced brown feathers and skin that had recently moulted to produce white feathers. Different methods of RNA extraction were evaluated based on their NanoDrop 2000c Spectrophotometer (Thermo scientific) scores, the results are displayed in table 3. The RIN^e (RNA Integrity Number) values from the 4200 Tapestation (Agilent technologies) were used to verify the NanoDrop scores but not repeated for every sample.

Attempt 1 (11/1)
Samples YB1W & YB2W from 'Yellow 7' bird, leg:white
RNA extraction using QIAGEN RNeasy Plus Mini Kit (Cat. no. 74134) and corresponding protocol

1. Tissue sample was placed in 600µl Buffer RLT Plus solution with ball bearing before being disrupted for 3 minutes using TissueLyzer (Qiagen).
2. Ball bearing was removed and lysate was centrifuged for 3 minutes at maximum speed.
3. Lysate was pipetted out and placed in a gDNA eliminator spin column (supplied) inside a 2ml collection tube (supplied) before being centrifuged for 30 seconds at 8000xg
4. The spin column was discarded, 600µl of 70% ethanol was added to the flow-through and mixed by pipetting.
5. 700µl of this mixture was transferred to a RNeasy spin column (supplied) and centrifuged for 15 seconds at 8000xg, the flow-through was discarded.
6. 700µl of Buffer RW1 was added to the spin column and centrifuged for 15 seconds at 8000xg, the flow-through was discarded.
7. 500µl of Buffer RPE was added to the spin column and centrifuged for 15 seconds at 8000xg, the flow-through was discarded.
8. 500µl of Buffer RPE was added to the spin column and centrifuged for 15 seconds at 8000xg, the flow-through was discarded.
9. The spin column was placed in a new 2ml collection tube (supplied) and centrifuged for 1 minute at maximum speed.
10. The spin column was placed in a 1.5ml collection tube (supplied), 30µl of RNase-free water was pipetted directly onto the spin column membrane, this was then centrifuged for 1 minute at 8000xg.

Attempt 2. (12/1)
Samples YB1W & YB2W
Same procedure as attempt 1.

Attempt 3. (12/1)
Samples YB3B & YB4B
Sample dipped in liquid Nitrogen before being placed in pestle and mortar and hand crushed before being divided in two and placed in microtubes with ball bearing and buffer solution; following QIAGEN procedure as above for attempt 1.

Attempt 4. (13/1)
Samples YB5W & YB6W
Sample dipped in liquid Nitrogen and mortar and pestle cooled in -80ºC freezer before use before being divided in two and placed in microtubes with ball bearing and lysed for 1 minute then placed in a QIAGEN shredder spin column and centrifuged at maximum speed for 2 minutes. QIAGEN procedure for attempt 1. was then followed.

Attempt 5. (13/1)
Samples YB7W & YB8W
Sample placed in glass tube with buffer solution and lysed with tissueruptor then divided in two, placed in microtubes with ball bearing and continued with QIAGEN protocol as for attempt 1.

Attempt 6.
Samples had RNAlater added to microtubes and placed back into -80ºC freezer. Metal grinding jars were placed in a -80ºC freezer for 15 minutes. Samples were placed into grinding jars and lysed for 1 minute at 20Hz, then judged by eye to be in the same condition as samples: YB5-8W.

Attempt 7. (19/1)
Samples YB9B, YB10B & YB11B
Sample had RNAlater added to microtube and placed back into -80ºC freezer. Mortar and pestle were placed in -80ºC freezer for 1 hour. Liquid nitrogen and sample were placed into mortar and ground into a powder. Powder and nitrogen were poured into 1.5ml microtubes and nitrogen was allowed to evaporate off. 600µl buffer was added to each microtube and homogenised with a needle and syringe. QIAGEN protocol was followed as above.

Attempt 8. (30/1)
Samples YB12B & YB13W
Due to the hazardous nature of the QIAzol lysis reagent (Cat. no. 79306) and chloroform this procedure was performed inside a fume cupboard.

1. Samples were cut to an appropriate size on ice and placed in a 2ml microtube containing 2 metal beads, 900µl of QIAzol was added and they were homogenised for 3 minutes at 20Hz, before standing at room temperature for 5 minutes.
2. 100µl of gDNA eliminator solution and 180µl of chloroform was added to each tube, vortexed for 15 seconds and left to stand at room temperature for 2 minutes.
3. The samples were then centrifuged at 4ºC for 15 minutes to separate the solution into phases, 550µl of the upper clear aqueous phase was carefully pipetted out into a new microtube.
4. 550µl of 70% ethanol solution was also added to the phase in the new microtube and the solution was mixed by pipetting.
5. 700µl of this solution was transferred to an RNeasy spin column in a collection tube and centrifuged for 8000xg for 15 seconds, the flow-through was discarded.

6. 700µl of Buffer RWT solution was added to the spin column and centrifuged at 8000xg for 00:15, the flow-through was discarded.
7. 500µl of Buffer RPE solution was added to the spin column and centrifuged at 8000xg for 00:15, the flow-through was discarded.
8. 500µl of Buffer RPE solution was added to the spin column and centrifuged at 8000xg for 2 minutes, the flow-through was discarded
9. The spin column was placed in a new collection tube and centrifuged at maximum xg for 1 minute to dry out the membrane.
10. 30µl of RNase-free water was pipetted directly onto the membrane and centrifuged at 8000xg for 1 minute, the flow-through was pipetted back onto the membrane and again centrifuged at 8000xg for 1 minute, the spin column was discarded.

The resulting elutions were evaluated with NanoDrop with the aim of a high RNA concentration and a 260/280 value of 2.00±10%; which would indicate that the sample contained pure RNA. The results of these different methods are summarised in table 2.

Table 2: Results from NanoDrop and Tapestation, where applicable, for methods of RNA extraction from skin samples of *L. muta* (Yellow 7). N(l) = liquid nitrogen, CO_2(s) = dry ice.

Samples 'Yellow 7'	Method	Attempt	Colour	Conc. (ng/µl)	260/ 280	260/ 230	RIN^e	RIN^e Conc. (ng/µl)
YB1W (11/1)	**QIAGEN std.**	**1**	**White**	57.4	2.11	1.63	6.7	59
YB2W (11/1)	**QIAGEN std.**	**1**	**White**	30.5	2.08	1.69	N/A	N/A
YB1W (12/1)	**QIAGEN std.**	**2**	**White**	140.9	2.09	0.96	6.4	144
YB2W (12/1)	**QIAGEN std.**	**2**	**White**	14.3	2.15	0.75	6.6	44
YB3B (12/1)	**QIAGEN +N(l)**	**3**	**Brown**	115.1	2.09	1.63	6.8	60
YB4B (12/1)	**QIAGEN +N(l)**	**3**	**Brown**	116.5	2.09	1.63	6.9	140
YB5W (13/1)	**QIAGEN Shredder +N(l)**	**4**	**White**	53.0	2.12	1.65	N/A	N/A
YB6W (13/1)	**QIAGEN Shredder +N(l)**	**4**	**White**	56.9	2.14	0.65	N/A	N/A
YB7W (13/1)	**QIAGEN +Tissueruptor**	**5**	**White**	13.3	1.99	0.13	N/A	N/A
YB8W (13/1)	**QIAGEN +Tissueruptor**	**5**	**White**	3.3	2.08	0.05	N/A	N/A
YB9B (19/1)	**QIAGEN +N(l) +CO_2(s)**	**7**	**Brown**	173.2	2.07	1.52	8.3	34
YB10B (19/1)	**QIAGEN +N(l) +CO_2(s)**	**7**	**Brown**	141.9	2.09	0.78	8.2	22
YB11B (19/1)	**QIAGEN +N(l) +CO_2(s)**	**7**	**Brown**	140.6	2.09	0.55	8.2	18
YB12B (30/1)	**QIAzol**	**8**	**Brown**	975.9	2.04	1.99	N/A	N/A
YB13W (30/1)	**QIAzol**	**8**	**White**	225.7	2.03	1.59	N/A	N/A

2.2. RNA extraction - samples

To test the first three hypotheses, skin samples were collected from multiple *L. lagopus* individuals. As with Yellow 7 the *L. lagopus* samples were taken from areas of skin that had moulted into white feathers and areas of skin that still had brown feathers from freshly caught birds. The samples were placed in microtubes containing 1.5ml RNAlater in the field and then brought to the lab and placed in a -20ºC freezer. As attempt 8, the Qiazol method, produced the highest concentrations of RNA, (table 2), this method was used for the *L. lagopus* samples (table 3).

Table 3: Samples for qPCR reactions, with results from NanoDrop and calculations for concentrations to make equal volumes of cDNA. 'YBXX' samples are from the *L. muta* individual 'Yellow 7'; 'LBXX' samples are from multiple *L. lagopus* individuals.

Sample ID	Conc. (ng/µl)	260/280	260/230	Volume H_2O added to cDNA reaction
YB1W (12/1)	140.9	2.09	0.96	6.14
YB9B (19/1)	173.2	2.07	1.52	5.00
YB10B (19/1)	141.9	2.09	0.78	6.10
YB11B (19/1)	140.6	2.09	0.55	6.16
YB12B (30/1)	975.9	2.04	1.99	0.89
YB13W (30/1)	225.7	2.03	1.59	3.84
LB5B	432.1	2.01	1.93	2.00
LB5W	432.3	2.02	1.74	2.00
LB7B	143.0	2.03	1.79	6.05
LB7W	100.2	2.02	1.49	8.64
LB8B	451.5	2.05	2.09	1.92
LB8W	127.5	2.04	2.09	6.79
LB9B	135.2	2.06	2.05	6.40
LB9W	96.2	1.98	0.24	9.00
LB10B	483.2	2.04	1.95	1.79
LB10W	207.1	2.05	1.98	4.18

2.3. Primer design

Using the NCBI genome database for *L. muta* (https://www.ncbi.nlm.nih.gov/datasets/genome/?taxon=64668) (Squires *et al.,* 2023) the sequences for selected key genes involved in melanogenesis were isolated; namely: POMC, PC1, PC2, ASIP, MC1R, TYR, TYRP1 & DCT. The sequence for ASIP was not found and was blasted from the equivalent sequence in *L. leucura* (White tailed ptarmigan). MC1R was also not found initially but after searching for the nucleotide sequence was found to be annotated as TUBB3, the downstream neighbour of MC1R (Dalziel *et al*., 2011).

These sequences were then analysed using the online tool Primer3 (https://primer3.ut.ee/), which gave a selection of possible primers. Using the online tool NCBI primer blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi), primers were then narrowed down to the two best options based on their individual self-complementary scores and similarity to other sequences in the genomes for *L. muta, L. leucura* and the domestic chicken, *Gallus gallus*. Because of the close phylogenetic relationship between *L. muta* and *L. lagopus* it is assumed that the same primers will work for both species (Kozma, 2016).

Primers (Sigma-Aldrich) were diluted to a concentration of 100µmol based on the technical data sheet and vortexed to mix. 20µl of this primer dilution was then added to a separate microtube with 180µl DEPC water and vortexed again, to make dilutions of 10%, these solutions were then used in PCR reactions to determine primer efficiencies.

Table 4: Summary of primer sequences used in qPCR.

Gene	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
POMC	AGGAAGACGGAGAAGGGTTG	CTTCCTCCTCCTGCTCTTCC
PC1	AGTATCTAGGAAGTCAAGGC	CTCCATCACATTCTTCTTCC
PC2	CCAAAGGTTATATGGTGCG	GCATTTGTATTTGGCAAGG
ASIP	TACTGTTAAACCCAAAGTGC	AAGCTCTCTACTTTGATTGC
MC1R	TTCATCACCTACTACCGC	CATGTGAATGTAGAGCACC
TYR	CAGAGGCAATTTCATGGG	TAGGTTAAGGTAGGCAAGG
TYRP1	TGCAATGGTAATTTCTCTGG	TTGATTTGCTGGTTACACG
DCT	CAAGTTTGGTTGGACTGG	CTCTGTTGTCAAGGAATGG
PPIB	CTGACGAGAACTTCAAGC	TGGTGATGAAGAACTGGG

2.4. Primer efficiency

Samples 1(12/1) & 3(12/1) were used to make cDNA using the High Capacity RNA-to-cDNA Kit from Applied Biosystems (Cat. no. 4387406) with: 10µl of 2x RT Buffer mix, 1µl of 20x RT Enzyme mix and 9µl of the RNA sample. This solution was incubated at 37ºC for 1 hour and 95ºC for 5 minutes and then stored at -80ºC for later use.

The cDNA was then made into a serial dilution for use in qPCR. Master mix for each primer pair was made using Promega - GoTaq (table 6). 19µl of the master mix and 1µl of the serial dilution were pipetted into a hard shell PCR plate (Bio-Rad) and run through a qPCR protocol on a CFX connect Real-time system (Bio-Rad). The primers were run through different primer concentrations and qPCR protocols to determine their efficiencies. The concentration and protocol that yielded efficiencies between 85% and 115% were then selected for further use (table 5).

Table 5: Summary of genes, with protocols, primer concentrations and efficiencies, melt curves started at 65ºC for 5s before heating up to 95ºC in 0.5ºC intervals. ‖: indicates repetition of steps.

Gene	Protocol						Primer	Efficiency
	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6		
POMC	95ºC, 2:00	95ºC, 00:15	63ºC, 00:15	66ºC, 1:00	‖: 2-4 x39	Melt Curve	x1	0.8887
PC1	95ºC, 2:00	95ºC, 00:15	57ºC, 00:15	60ºC, 1:00	‖: 2-4 x39	Melt Curve	x1	0.9810
PC2	95ºC, 2:00	95ºC, 00:15	57ºC, 00:15	60ºC, 1:00	‖: 2-4 x39	Melt Curve	x2	0.9166
ASIP	95ºC, 2:00	95ºC, 00:15	57ºC, 00:15	60ºC, 1:00	‖: 2-4 x39	Melt Curve	x2	1.0518
MC1R	95ºC, 2:00	95ºC, 00:15	57ºC, 00:15	60ºC, 1:00	‖: 2-4 x39	Melt Curve	x1	0.9360
TYR	95ºC, 2:00	95ºC, 00:15	57ºC, 00:15	60ºC, 1:00	‖: 2-4 x39	Melt Curve	x2	1.0263
TYRP1	95ºC, 2:00	95ºC, 00:15	57ºC, 00:15	60ºC, 1:00	‖: 2-4 x39	Melt Curve	x2	0.9477
DCT	95ºC, 2:00	95ºC, 00:15	57ºC, 00:15	60ºC, 1:00	‖: 2-4 x39	Melt Curve	x1	0.9315
PPIB	95ºC, 2:00	95ºC, 00:15	57ºC, 00:15	60ºC, 1:00	‖: 2-4 x39	Melt Curve	x1	0.9074

2.5. qPCR

RNA samples of high concentration (≧95ng/µl) were converted into cDNA as described above. All cDNA reactions contained a total of 865.8ng RNA. This amount is based on the lowest concentrated RNA sample (96.2 ng/µl) and the maximum volume of the RNA sample which can be added to the cDNA reaction (9µl). See table 3 for the appropriate RNA sample volumes for each cDNA reaction. The qPCR plate was then pipetted based on the usable samples and appropriate master mixes and protocols for each gene and most efficient primer pair (table 6).

Table 6: Guide for ‘Master mix’ of DNA primers. Replicated for 1 well, 1 line (13 wells), 21 samples repeated twice (45 wells) and for doubling the amount of primers in each master mix, gene dependent.

Reagent	1 Well (µl)	13 Wells (µl)	1 Well 2x primer (µl)	13 Well 2x primer (µl)	45 Wells (µl)	45 Wells 2x primer (µl)
DEPC H_2O	6.9	89.7	4.9	63.7	310.5	220.5
GoTaq	10	130	10	130	450	450
Forward	1	13	2	26	45	90
Reverse	1	13	2	26	45	90
CxR	0.1	1.3	0.1	1.3	4.5	4.5
Total	19	247	19	247	855	855

2.6. Analysis and statistics

Relative gene expression was calculated from the Cq values produced by the CFX connect Real-time system; the Cq values (quantification cycles) represent the cycle number at which the fluorescence of the dye is higher than the threshold value and, in qPCR, acts as a proxy for DNA concentration. Using the housekeeping gene PPIB (Peptidylprolyl Isomerase B) (GenBank: JAMCCT010000010.1) as a reference point for both *Lagopus spp*.

Each sample was run twice and the average was used in calculations, the values for white samples was used as a zero point average and the IMLOG2 function to create values for relative gene expression as a log2 fold change. These values were then analysed on RStudio using a t-test to determine if there was a significant difference between the two groups, 'B' and 'W'. Because the LB samples were from multiple individuals they were run through a paired t-test to account for individual variation (Log base 2), YB samples were run through an unpaired t-test as they were all from the same individual (tables 7 & 8). Graphs were also created on RStudio using the libraries: 'ggplot2', 'magrittr', 'tidyverse'& 'ggtext'.

2.7. Sequencing

PPIB's sequences were previously confirmed and were therefore excluded from the analysis outlined here. Gel electrophoresis was performed to further evaluate the efficiencies of the primers. First the eight genes were run through the same qPCR protocols as before but without melt curves using the cDNA sample LB4•1, which had a concentration of 1470.5ng/µl. The samples were then pipetted out of the PCR plate into individual tubes and frozen at -20ºC for later. The gel was made of 100ml 1xTAE and 1g agarose powder, dissolved and heated, then 2µl of EtBr, ethidium bromide, was added and the solution was poured into the mould to set; the set gel was then submerged in buffer solution.

4µl of purple loading dye was added to each of the 20µl primer sample tubes (1:6) and mixed, 12.5µl of both ladders (1kb and 100bp) were pipetted into the middle two wells, 20µl of the primer sample was pipetted into each well, four on each side of the ladders. The electrodes were attached and the system was run at 90v for 50 minutes, the gel was then removed and placed into a ChemiDoc MP imaging system (BioRad). The UV irradiation then causes the dye to fluoresce where the DNA is present; if multiple genes have been amplified by the primers then two bands will appear under UV light. None of the primers tested produced multiple bands, suggesting that they each amplified only the intended target gene.

The two amplicon samples that had not been used in gel electrophoresis were run through the NanoDrop machine to test their concentration and the highest of these was used for bacterial cloning and sequencing. The amplicons were purified with the QIAquick PCR purification kit (QIAGEN, Cat. no. 28104) and the corresponding protocol.

1. 95µl of Buffer PB were added to each 19µl DNA sample, vortexed to mix, and pipetted into a 2ml collection tube.
2. The sample was centrifuged at 13,000xg for 30s and the flow-through was discarded.
3. 750µl of Buffer PE was added to the column, and again centrifuged at 13,000xg for 30s, discarding the flow-through.

4. The column was centrifuged at 13,000xg for 1 minute and placed in a 1.5ml micro centrifuge tube.
5. The DNA was eluted with 50µl of Buffer EB and centrifuged at 13,000xg for 1 minute

The samples were then run through NanoDrop again to determine the concentration of DNA used in ligation of the plasmid. Biomath calculator (no.promega.com) was used to calculate the molar ratio of the DNA sample needed based on the number of base pairs in each amplicon and the concentration of the DNA sample from the NanoDrop, this was then added to a 5ml microtube with: 1µl of salt, 1µl of PCR-II-Blunt-TOPO (ThermoFisher Scientific, Cat. no. K280002) and the appropriate volume of H_2O to make a 5µl total volume, before being vortexed.

LB broth was made from 500ml H_2O and 12.5g LB broth powder, growth medium was made from 500ml H_2O and 20g LB broth powder with agar; these were then autoclaved and 0.5ml of the antibiotic kanamycin was added to each. 15ml of the growth medium was pipetted onto 8 petri dishes and left to set before being covered with parafilm to make them airtight.

Each transfection and aliquot of transfection-competent cells (*Escherichia coli*) was thawed on ice and 25µl were pipetted into eight 5ml microtubes, 2µl of the DNA ligate for each gene was added to the microtube and they were placed in ice for 30 minutes before being heat shocked in a 42ºC water bath for 30 seconds and returned to ice for another 5 minutes. 475µl of SOC media (Super Optimal broth with Catabolite repression) was pipetted into each microtube and they were shaken at 250rpm for 1 hour at 30ºC before being pipetted onto the petri dishes of growth medium, left to dry for 30 minutes and then turned upside down and left at 37ºC to culture overnight. Sixteen 15ml falcon tubes were filled with 5ml of LB broth, a single colony from each petri dish was transferred to each tube with a pipette tip. The tube was loosely closed and taped and left in 120rpm shaker at 37ºC overnight.

The DNA was then purified using the QIAprep Spin Miniprep Kit; the tubes were centrifuged to pellet the bacteria, which was then resuspended in 250µl of Buffer P1 and transferred to a microtube. 250µl of Buffer P2 were added and inverted 4-6 times until the solution turned blue, 350µl of Buffer N3 were added within 5 minutes, inverted 4-6 times until the solution turned colourless and centrifuged for 10 minutes at 13,000rpm. 800µl of the supernatant was pipetted into a QIAprep 2.0 spin column and centrifuged for 30-60 seconds, discarding the flow-through. 500µl of Buffer PB were added to the column and centrifuged for 30-60 seconds, discarding the flow-through. 750µl of Buffer PE were added to the column and centrifuged for 30-60 seconds, discarding the flow-through. The spin column was transferred to a new collection tube and centrifuged for 1 minute before being transferred to a 1.5ml microcentrifuge tube, the membrane was eluted with 50µl of Buffer EB, left to stand for 1 minute and centrifuged for 1 minute.

The resulting elutions were run through NanoDrop to determine DNA concentration and this was used to calculate the same concentration of DNA for all samples to be used in the BigDye program. The reagents required are 0.5µl of M13F primer, 0.5µl of BigDye, 3µl of 5x sequence Buffer, and a minimum DNA sample of 100ng and enough DEPC H_2O to make 16µl. This was then run through the BigDye program on the thermocycler and the end product was sent for Sanger sequencing by the Genomics Support Centre Tromsø (https://uit.no/prosjekter/prosjekt?p_document_id=468291)

in the Department of Health Sciences and compared to the *L. muta* genome on NCBI using the alignment feature on Benchling (benchling.com).

Four of the sequences, POMC, ASIP, MC1R & DCT, gave inconclusive results; they did not match the genome for *L. muta* but did not indicate the presence of any other genes. This suggested that although the amplicons were correct there were errors in the methodology, such as a failure of the TOPO vector to incorporate DNA fragments from PCR. To remedy this these genes were resequenced using the QIAquick gel extraction method which uses band extracts that are enriched in the specific amplicon. New amplicons were made from LB4•2 and run through the same gel electrophoresis protocol as before but for 40 minutes. The band for each gene was then excised and placed in a 2ml microtube, 270µl of Buffer QG was added to each tube and they were incubated at 50ºC for 10 minutes, vortexing every 2-3 minutes.

Once the agar gel had been dissolved 270µl of isopropanol was added to each tube and mixed by pipetting. 750µl of this solution was added to a QIAquick spin column in a 2ml collection tube and centrifuged at 13,000rpm for 1 minute, the flow-through was discarded. 75µl of Buffer PE was pipetted into the column and left to stand at room temperature for 2 minutes before being centrifuged at 13,000rpm for 1 minute, the flow-through was discarded and the column was again centrifuged at 13,000rpm for 1 minute. The column was transferred to a 1.5ml micro centrifuge tube and the membrane was eluted with 30µl of Buffer EB, left to stand for 1 minute and then centrifuged at 13,000rpm for 1 minute.

1µl of the DNA solution was added to a 25µl aliquot of stable competent *E. coli* cells and mixed carefully before being left on ice for 30 minutes. The solutions were then heat-shocked at 42ºC for 30 seconds and returned to ice for 5 minutes. 975µl of Stable Outgrowth Medium was pipetted into each solution and shaken at 250rpm for 1 hour at 30ºC. The solution was then pipetted onto prepared petri dishes and spread around in 100µl and 500µl quantities, these plates were then incubated at 37ºC overnight. Individual colonies were isolated as before and run through the same growth, DNA extraction and BigDye protocols as before, then sequenced and compared to the *L. muta* genome.

2.8. Bioinformatics and promoter analysis

The promoter region was defined as the sequence of 5,000 nucleotides upstream of the start of the first intron for each gene. The genomes for *L. muta* and *L. leucura* are available on the National Centre for Biotechnology Information (ncbi.nlm.nih.gov) under the bioproject codes 'PRJNA853367' and 'PRJNA752366' respectively. The genomes for *L. lagopus* and *L. l. scoticus* came from eight individuals sequenced by J. Höglund at Uppsala university (Kozma *et al*., 2019) that were made into a consensus sequence using integrative genomics viewer (IGV_2.16.0).

The sequences were also compared to the promoter regions in other galliform birds: *Gallus gallus* (Bioproject: PRJNA660757, GenBank: GCA_016699485.1), *Centrocercus urophasianus* (Bioproject: PRJNA734947, GenBank: GCA_019232065.1) and *Coturnix japonica* (Bioproject: 292031 GenBank: GCA_001577835.2); which were also available on NCBI.

The sequences were run through the EMBOSS Polydot program (ebi.ac.uk) (Madeira *et al*., 2022). The polydots produced are an 'all-against-all' comparison of the promoter regions for each gene,

with each species repeated on both the x and y axes, the word size was increased from the default ‘6’ to ’10’ to improve clarity. Sequences that match each other will produce a diagonal line from the xy intercept with little to no ‘noise’ on either side e.g. where sequences are compared to themselves (figure 13). Sequences that do not match at all will not produce any diagonal line while partial matches will produce diagonal lines that do not line up with the corners. These partial matches can be caused by: mutations that cause the sequences to no longer align, such as frameshift or addition mutations; differing sequences because they were copied from different points in their code which otherwise would align; or a difference between the introns of the species, before the transcriptional start site, that changes when the promoter region starts. The sequences were compared before they were copied to ensure that the promoter regions started from as close to the same place as possible.

The sequences were also analysed with CiiiDER workflow (ciiider.com) to produce a scan of potential transcription factor binding sites (TFBSs) which were then compared to find sites that were common to *L. muta*, *L. leucura* and *L. lagopus* but that were not found in *L. l. socticus.* The sequences were compared to the ‘JASPAR2020_CORE_vertebrates.txt’ Position Frequency Matrix (PFM) and the TFBSs shown were determined by CiiiDER’s MATCH algorithm that displays any site with a MATCH score above 0.85 (from a maximum score of 1) (Gearing et al., 2019). The CiiiDER outputs were then gone through manually to compare the number and position of the potential TFBSs.

3. Results

3.1. Relative gene expression

qPCR was used to determine the relative gene expression between skin samples that had grown brown feathers and skin samples that had recently moulted and grown white feathers. The samples for *L. lagopus* came from five individuals that had been killed and sampled in the field in mid October and were analysed with a paired t-test. The *L. muta* samples came from one individual housed at UiT and euthanised in late September, these samples were analysed with an unpaired t-test.

None of the LB samples from five *L. lagopus* individuals showed any significant difference ($p<0.05$) in gene expression between brown and white samples (table 7 & figure 11). However, there is consistently higher expression of MC1R in brown samples than white ones for all individuals tested. Similarly PC2 and DCT generally has higher expression in brown samples than white ones (figure 11).

For the YB samples from one individual *L. muta*, only TYRP1 shows a significant difference ($p<0.05$), with higher gene expression in brown samples than white ones (table 8 & figure 12) despite not being significant there does seem to be a similar trend in PC2 and TYR, where gene expression is higher in brown samples than white ones (figure 12). POMC shows the opposite trend, although again not significantly, of higher expression in white samples than brown ones (figure 12).

Table 7: Results of a paired t-test for *Lagopus lagopus* samples (LBXX)

Gene	POMC	PC1	PC2	ASIP	MC1R	TYR	TYRP1	DCT
t value	-0.551	0.087	2.087	-0.147	2.671	0.260	0.131	0.072
df	4	4	4	4	4	4	4	4
p value	0.611	0.935	0.105	0.891	0.056	0.808	0.902	0.946
Mean difference	-0.444	-0.076	1.040	-0.161	1.031	0.361	0.164	-0.090
No. samples	10	10	10	10	10	10	10	10

Table 8: Results of an unpaired t-test for *Lagopus muta* samples (YBXX)

Gene	POMC	PC1	PC2	ASIP	MC1R	TYR	TYRP1	DCT
t value	-0.513	0.092	1.612	-2.301	1.327	2.275	2.770	1.408
df	5.525	7.992	5.756	3.566	1.797	5.382	6.880	4.782
p value	0.628	0.929	0.160	0.091	0.328	0.068	0.028	0.221
x mean	0.444	-0.076	1.040	-5.160	1.041	3.300	3.580	1.280
y mean	0.000	-0.000	0.000	0.000	0.000	-0.000	0.000	-0.000
No. samples	6	6	6	6	6	6	6	6

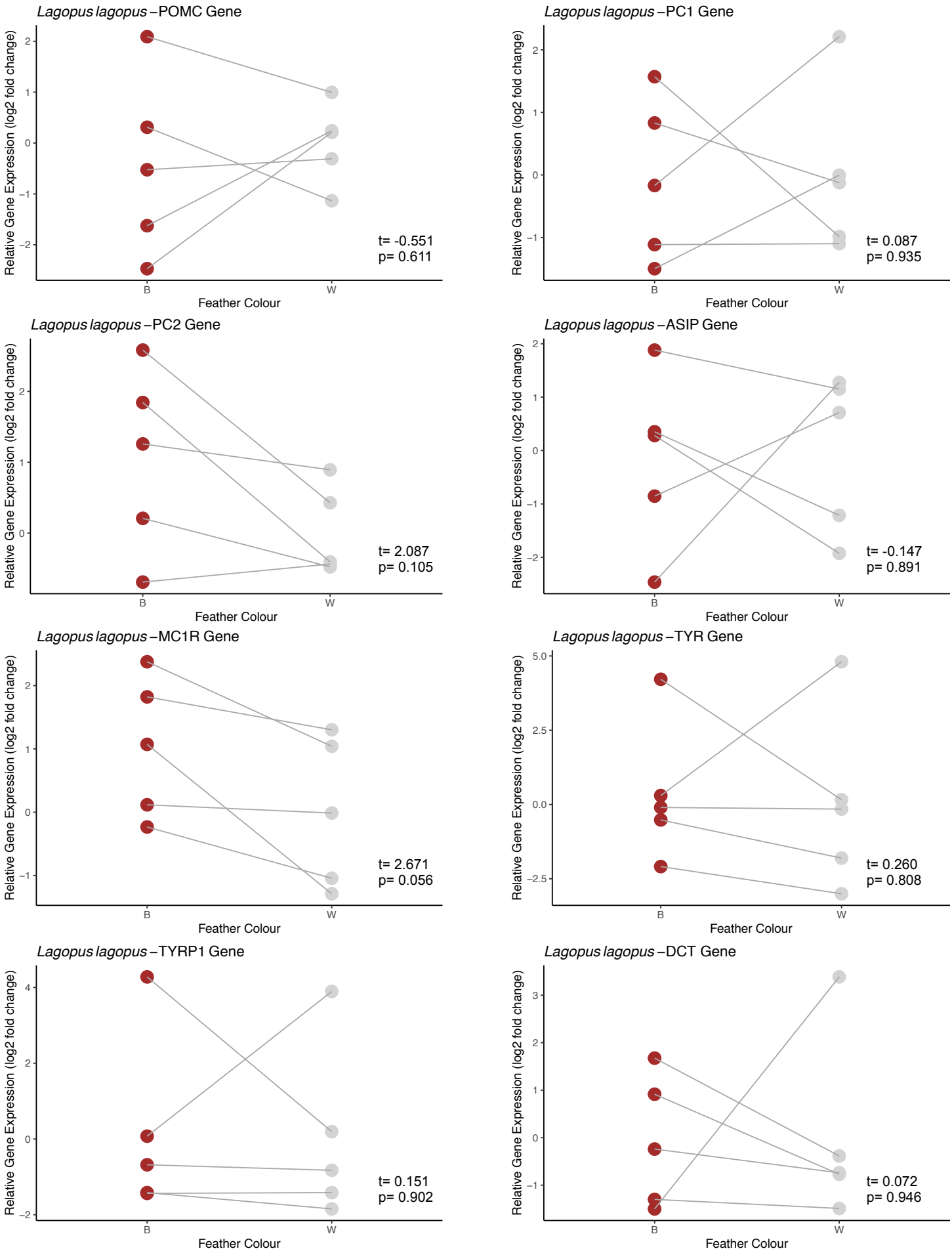

Figure 11. *Graphs showing relative gene expression between skin samples that had grown brown feathers (B) and skin samples that had grown white feathers (W). 10 samples were taken from 5 L. lagopus birds and lines between data points denote individuals, t-test values are shown in the bottom right corner and in table 7.*

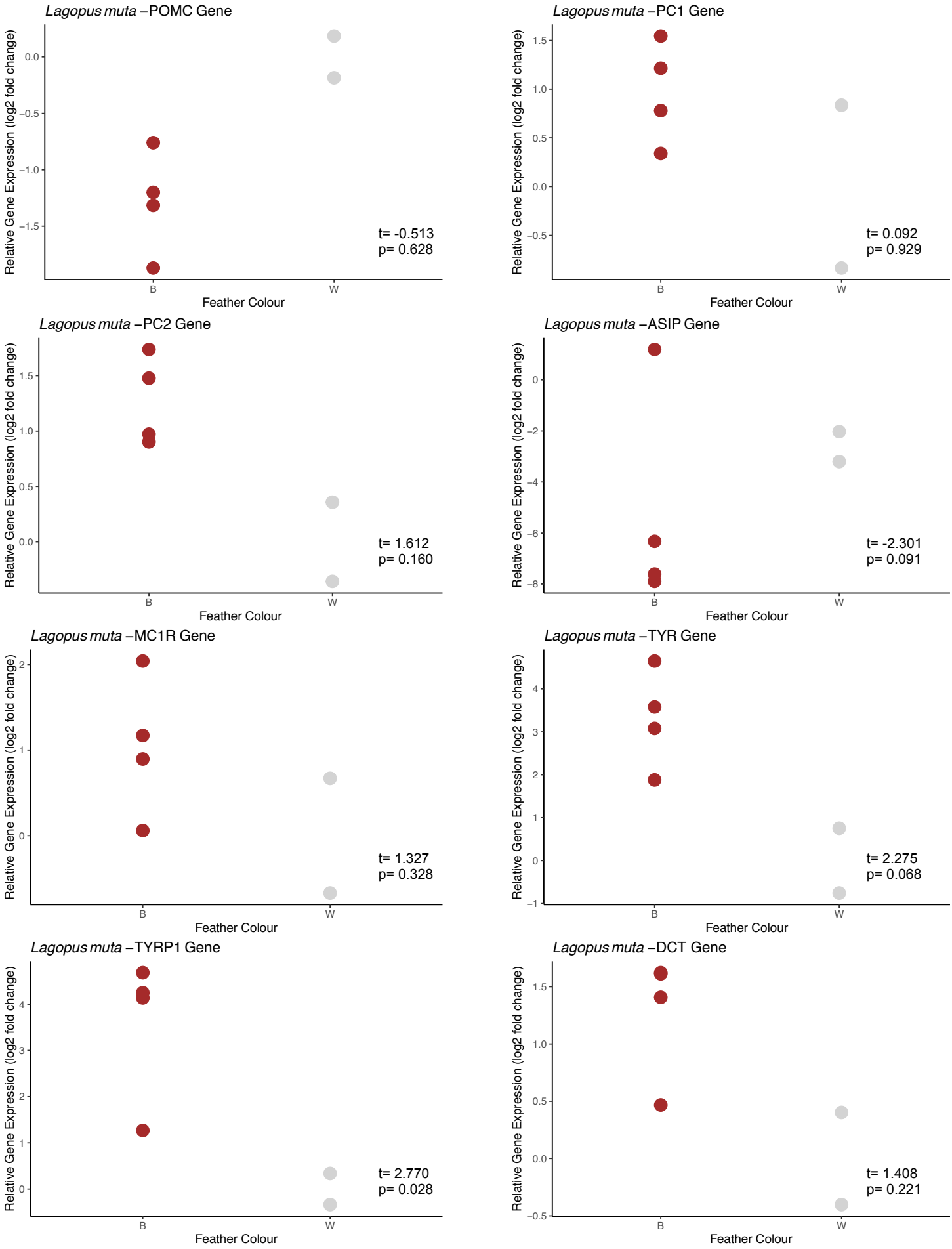

Figure 12. *Graphs showing relative gene expression between skin samples that had grown brown feathers (B) and skin samples that had grown white feathers (W). 6 samples were taken from one L. muta individual, t-test values are shown in the bottom right corner and in table 8.*

3.3. Gene promoter comparison

The promoter regions were defined as the 5,000 base pair sequences upstream from the first intron for each gene. They were either sourced from NCBI (*L. muta, L. leucura, G. gallus, C. japonica* & *C. urophasianus*) or from eight individuals sequenced at Uppsala university and compiled into a consensus sequence using IGV (*L. lagopus* & *L. l. scoticus*). These sequences were then run through the EMBOSS Polydot programme and CiiiDER workflow to compare the sequences for each species. The CiiiDER workflows were then manually marked to highlight similarities and differences between *L. l. scoticus* and the other species.

POMC

The sequences for all Lagopus species show a clear match in the Polydot graphs, the other galliform birds show partial matches with either frame shifts and/or addition or subtraction mutations (figure 13). *G. gallus* seems more similar to Lagopus species than either *C. japonica* or *C. urophasianus*. From the CiiiDER output (figure 14) there is not a lot of difference between *L. l. scoticus* and the other Lagopus species; there is an extra TFBS for RHOXF1 at -3950. *Rhoxf1* (Rox homeobox family member 1) is a member of a subfamily of homeobox genes and highly expressed in the testis of mice with low expression in other organs (Song *et al.,* 2013). The function is unlikely to be tied to melanin production, at least in mammals, and more likely related to reproduction. It should also be noted that *L. l. scoticus* differ from other ptarmigan in more than just their seasonal colouring and POMC can affect numerous parts of a ptarmigan's life including behaviour and appetite alongside melanogenesis (Walsh, 2021).

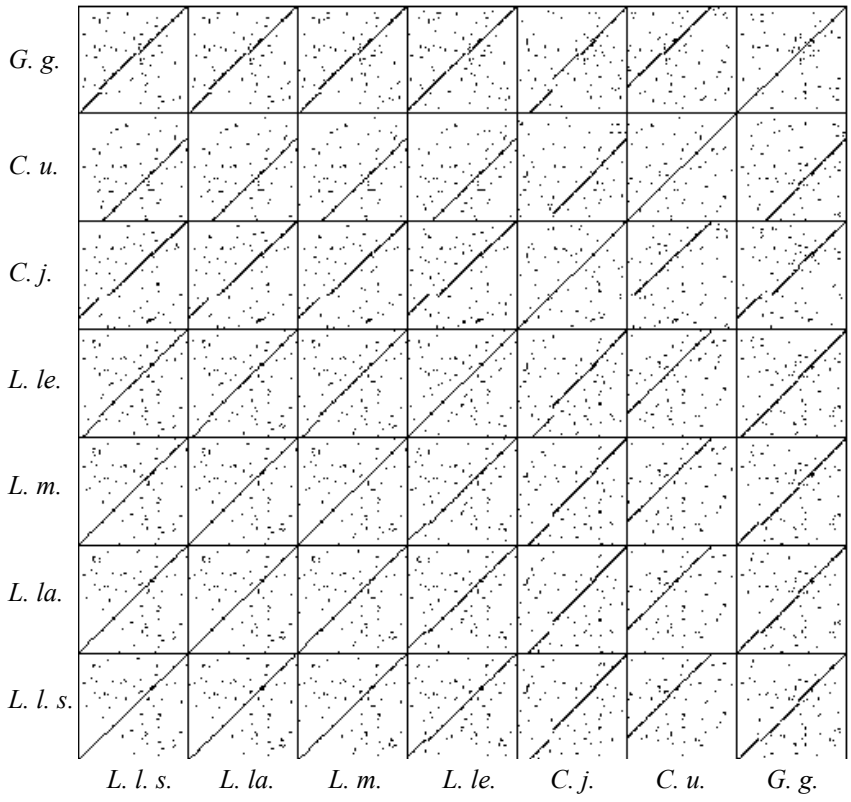

Figure 13 *Promoter polydot for POMC in galliform birds, G. g.=Gallus gallus, C. u.=Centrocercus urophasianus, C. j,=Coturnix japonica, L. le.=Lagopus leucura, L. m.=Lagopus muta, L. la.=Lagopus lagopus, L. l. s.=Lagopus lagopus scoticus.*

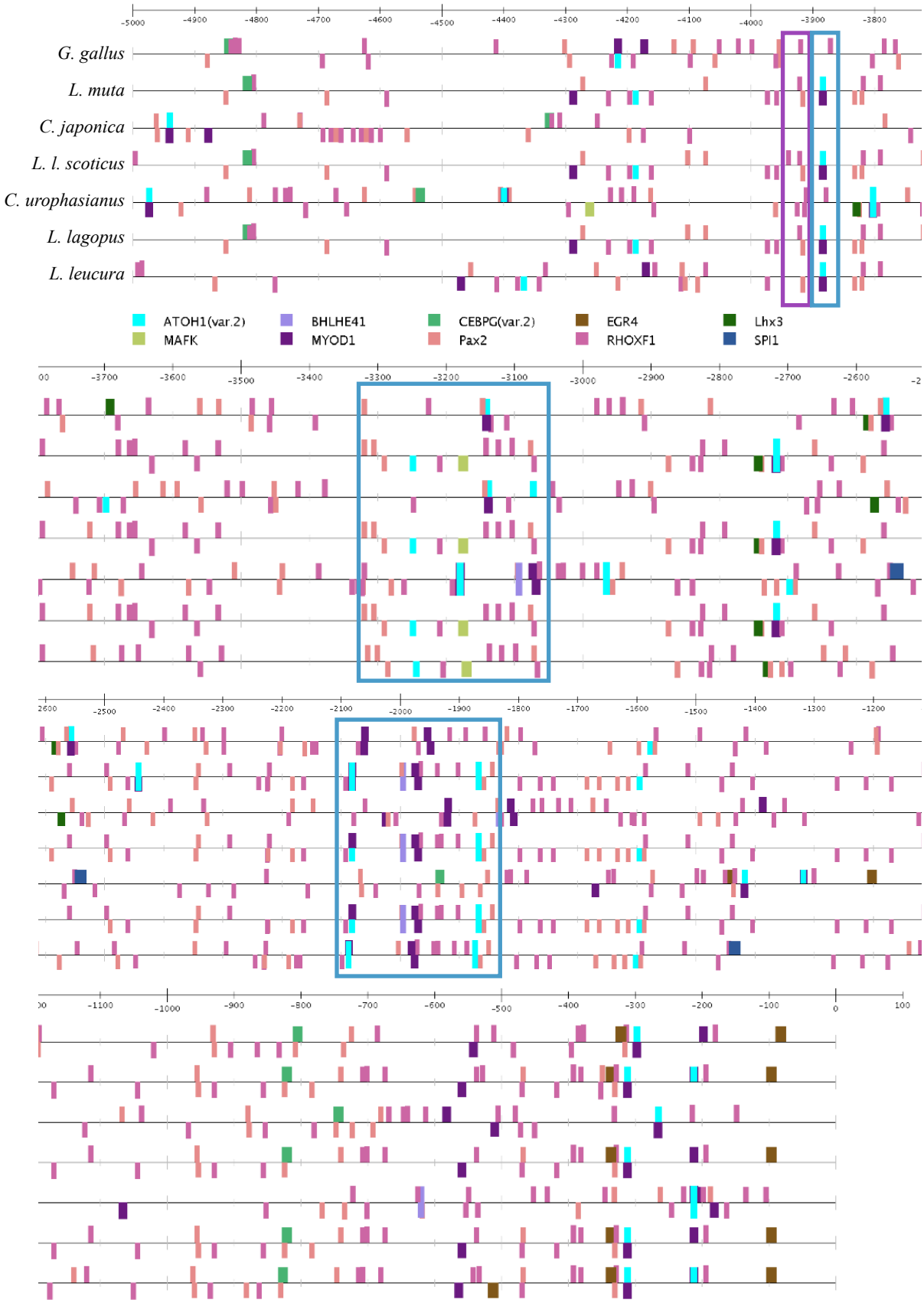

Figure 14. *Promoter regions of POMC for G. gallus, L. muta, C. japonica, L. l. scoticus, C. urophasianus, L. lagopus & L. leucura. Blue boxes indicate TFBS common to Lagopus spp. but not other galliforms. Purple boxes indicate TFBS that show differences between L. l. scoticus and other Lagopus spp. but not similarity between L. l. scoticus and non SCC galliforms.*

PC1

C. urophasianus did not have a promoter sequence for PC1 available on NCBI and so can't be compared with the other species. All species show matching sequences, with *C. japonica* and *G. gallus* being more similar to each other than to Lagopus species and *L. leucura* being the least similar to other Lagopus species (figure 15). *L. l. scoticus* and *G. gallus* lack a TFBS for PAX2 at -1500 that is present in all other species (figure 16). The other Lagopus species have two sites for PAX2 in close proximity, while *L. l. scoticus*, *C. japonica* and *G. gallus* only have one. *L. l. scoticus* and *G. gallus* lack the first site with *G. gallus* having an SNAI3 site in its place, *C. japonica* lacks the second site so the difference here seems minimal. Although *Pax2* (Paired box gene 2) does have a role in melanogenesis it seems to be during development and localised to the eye so it is unclear if these differences are significant to SCC (Creel, 2022).

L. l. scoticus also has extra TFBSs for MEIS1 and TBX3 at -600 while the other lagopus species have only one site for MEIS1 and *G. gallus* and *C. japonica* have none. *L. lagopus* and *L. leucura* only have one site for TBX3 but *G. gallus, C. japonica* and *L. muta* all lack a site, it is possible that for *L. muta* the site has shifted upstream as there is an extra site for TBX3 at -500. *Meis1* (Meis homeobox 1) is another homeobox gene and is involved in cell proliferation and differentiation, crucially it is localised to nuclei in the dermal papilla during anagen and loss of function mutations lead to decreased hair growth and proliferation of hair matrix cells (Namekata *et al*., 2019). *Meis1* also acts as a transcriptional regulator of *Pax6*, a gene related to *Pax2* that is also involved in eye development (Zhang *et al*., 2002). *Pax6* has been directly linked to the regulation of melanogenesis through its interactions with *Mitf* this suggests that *Meis1* may have a dual role of stimulating and

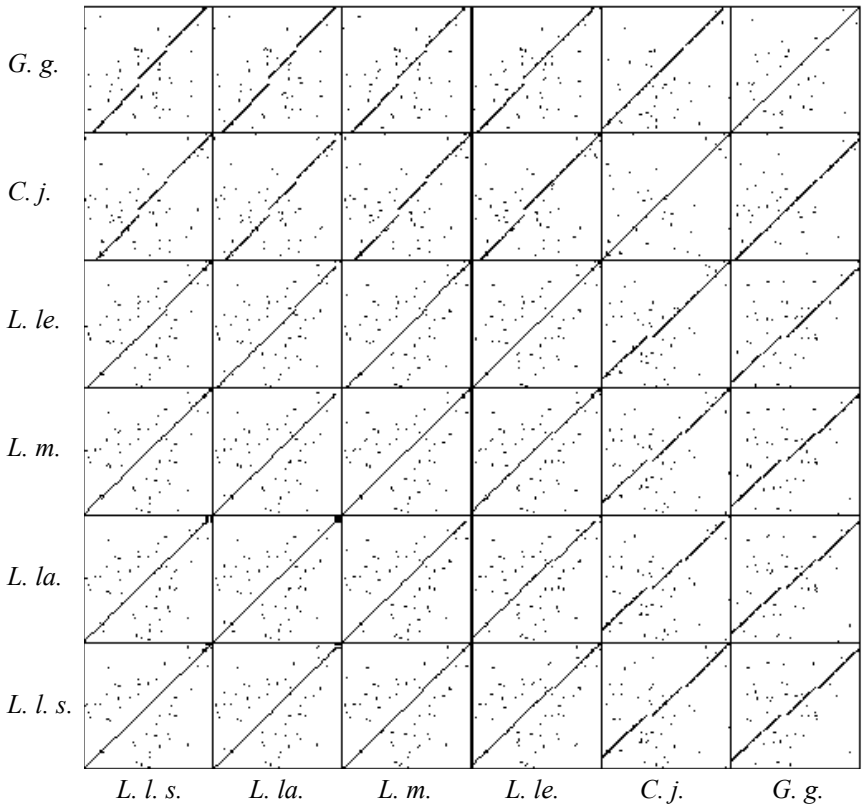

Figure 15 *Promoter polydot for PC1 in galliform birds, G. g.=Gallus gallus, C. u.=Centrocercus urophasianus, C. j,=Coturnix japonica, L. le.=Lagopus leucura, L. m.=Lagopus muta, L. la.=Lagopus lagopus, L. l. s.=Lagopus lagopus scoticus.*

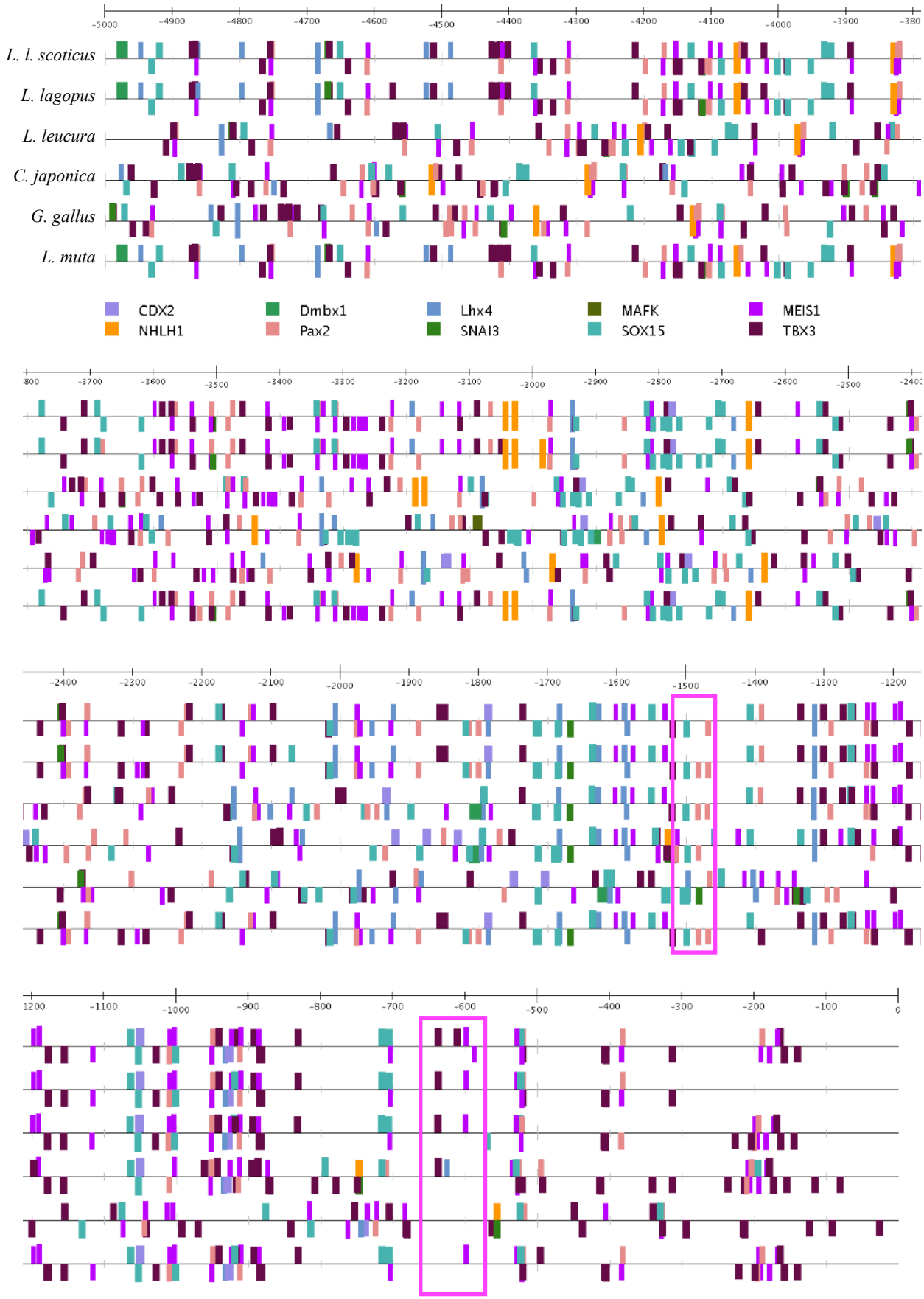

Figure 16. *Promoter regions of PC1 for L. l. scoticus, L. lagopus, L. leucura, C. japonica, G. gallus & L. muta. Purple boxes indicate TFBS that show differences between L. l. scoticus and other Lagopus spp. but not similarity between L. l. scoticus and non SCC galliforms.*

regulating melanogenesis at two different point in the pathway (Raviv *et al*., 2014). *Tbx3* (T-box transcription factor 3) has a role in development and mutations can affect the growth of limbs, teeth and hair in mammals. There is a significant difference in expression between scale- and feather-footed pigeons but no corresponding relationship between chicken breeds (Domyan *et al*., 2016). This suggests a role in feather development but not melanogenesis, however in horses the localised expression of the *Tbx3* gene has been shown to create the 'dun' colouring due to radially asymmetric deposition of melanins (Imsland *et al*., 2016). The presence of these TFBS in PC1 might explain how melanogenesis is localised to the anagen stage of feather growth but in itself doesn't explain the seasonal regulation of melanogenesis that seems to occur in SCC.

PC2

All species have produced diagonal lines on the polydot which suggest matching sequences. *L. leucura, C. japonica* and *C. urophasianus* but not *G. gallus* show a frameshift with the other Lagopus species, which can also be seen in the CiiiDER output (figures 17 and 18). The species that do not undergo SCC all lack a TFBS for SOX4 between -3700 and -3400 (figure 18). *Sox4* (SRY-box transcription factor) is a member of the *Sox* family of genes that encode transcription factors with multiple developmental roles (Jiang *et al*., 2019). *Sox4* specifically is involved in the regulation of epidermal development and the components of the extracellular matrix. It has been shown to have a vital role in the transition of cells from epithelial to mesenchymal phenotypes and is reactivated after wounding and during repair (Miao et al., 2019; Kalluri & Weinberg, 2009). It is therefore unlikely to be involved in melanogenesis, but possibly involved in feather growth.

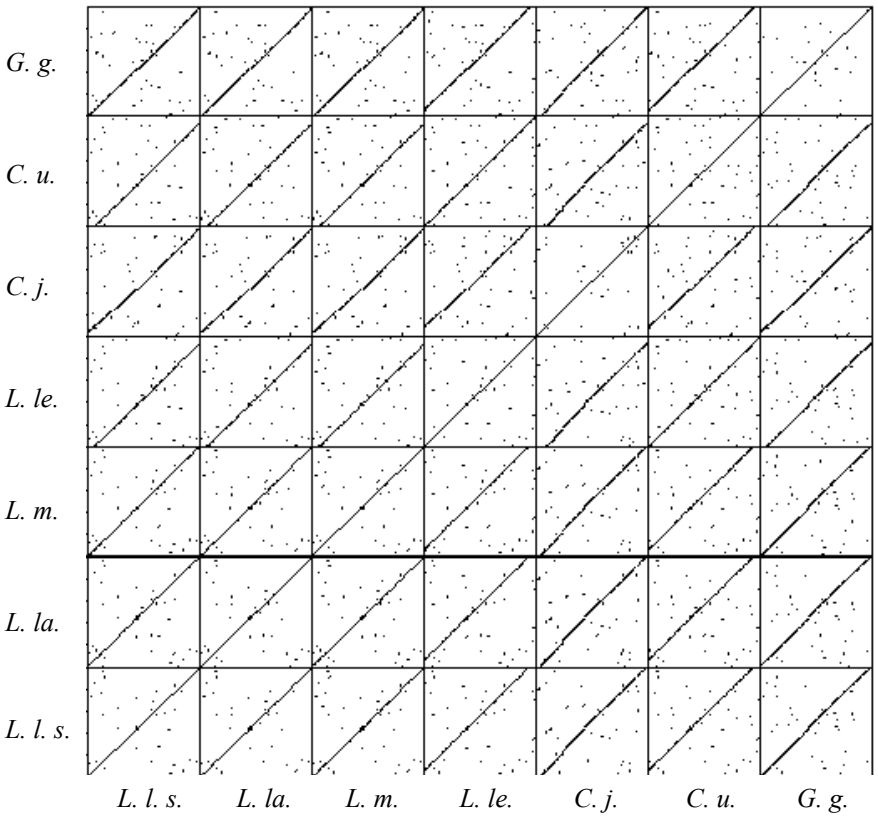

Figure 17 *Promoter polydot for PC2 in galliform birds, G. g.=Gallus gallus, C. u.=Centrocercus urophasianus, C. j,=Coturnix japonica, L. le.=Lagopus leucura, L. m.=Lagopus muta, L. la.=Lagopus lagopus, L. l. s.=Lagopus lagopus scoticus.*

Figure 18. *Promoter regions of PC2 for L. l. scoticus, L. lagopus, G. gallus, L. leucura, C. urophasianus, L. muta & C. japonica. Red boxes indicate TFBS common to SCC species only.*

ASIP

Most species do not match each other: *L. l. scoticus* & *L. lagopus* match and *L. muta* & *L. leucura* match with a partial match with both for *C. urophasianus*. *G. gallus* & *C. japonica* do not match any other species and the two Lagopus groups do not match each other either (figure 19). Based on the CiiiDER output (figure 20) there is no difference between *L. l. scoticus* and *L. lagopus* and little to no similarity with the other species.

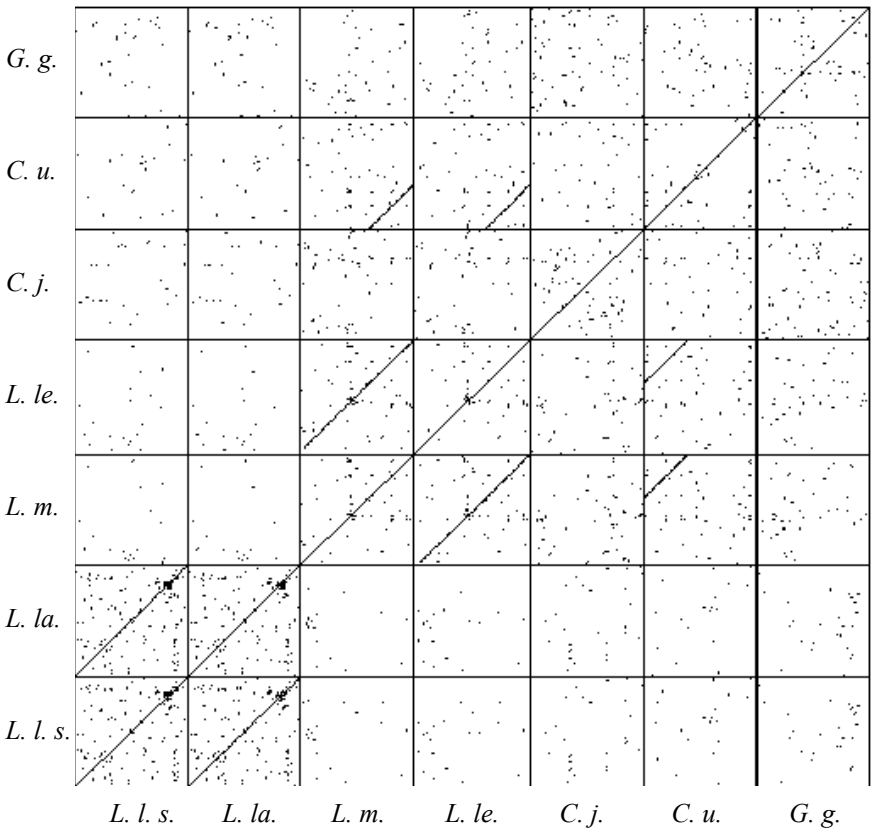

Figure 19. *Promoter polydot for ASIP in galliform birds, G. g.=Gallus gallus, C. u.=Centrocercus urophasianus, C. j,=Coturnix japonica, L. le.=Lagopus leucura, L. m.=Lagopus muta, L. la.=Lagopus lagopus, L. l. s.=Lagopus lagopus scoticus.*

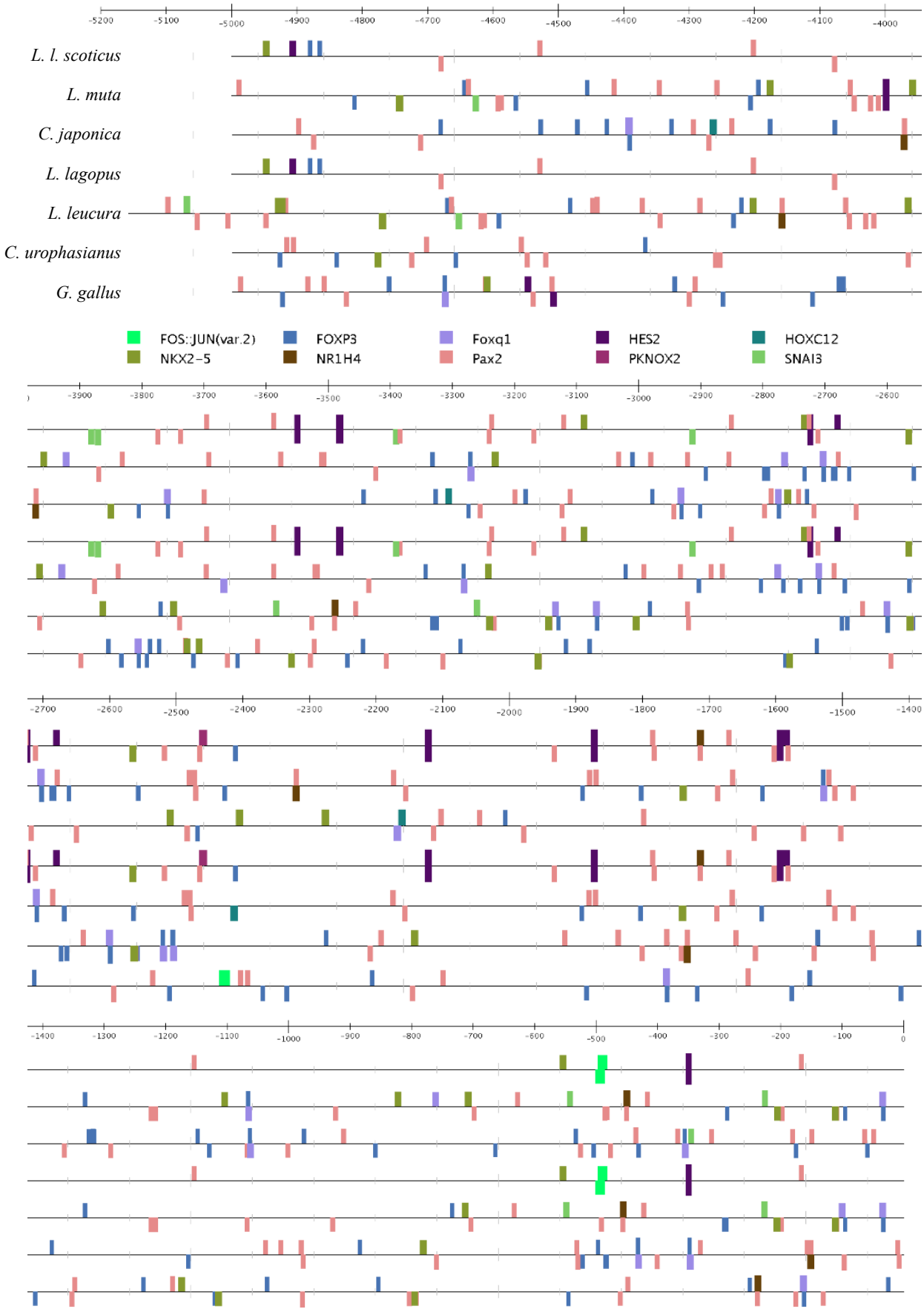

Figure 20. *Promoter regions of ASIP for L. l. scoticus, L. muta, C. japonica, L. lagopus, L. leucura, C. urophasianus & G. gallus.*

MC1R

There are some shifts, where the diagonal lines do not start and end in the corners but all species have matching sequences (figure 21). This is reflected in the CiiiDER outputs which show strong similarity between species (figure 22). However at -3000 *L. l. scoticus* lacks a TFBS for PAX2 that can be found in all other species.

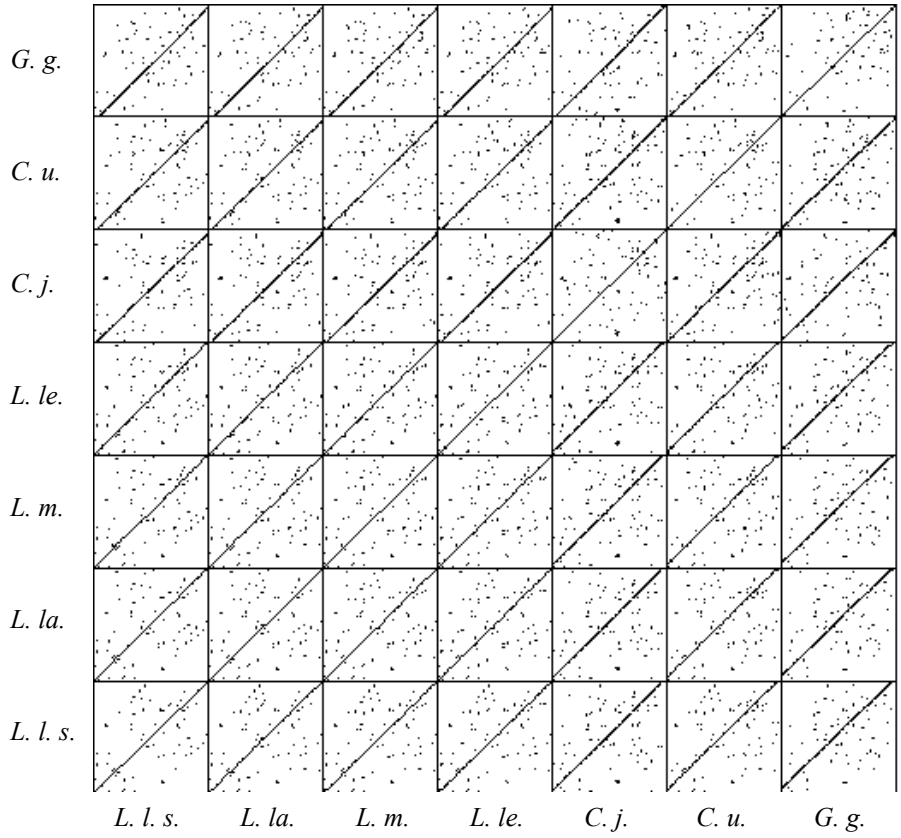

Figure 21. *Promoter polydot for MC1R in galliform birds, G. g.=Gallus gallus, C. u.=Centrocercus urophasianus, C. j,=Coturnix japonica, L. le.=Lagopus leucura, L. m.=Lagopus muta, L. la.=Lagopus lagopus, L. l. s.=Lagopus lagopus scoticus.*

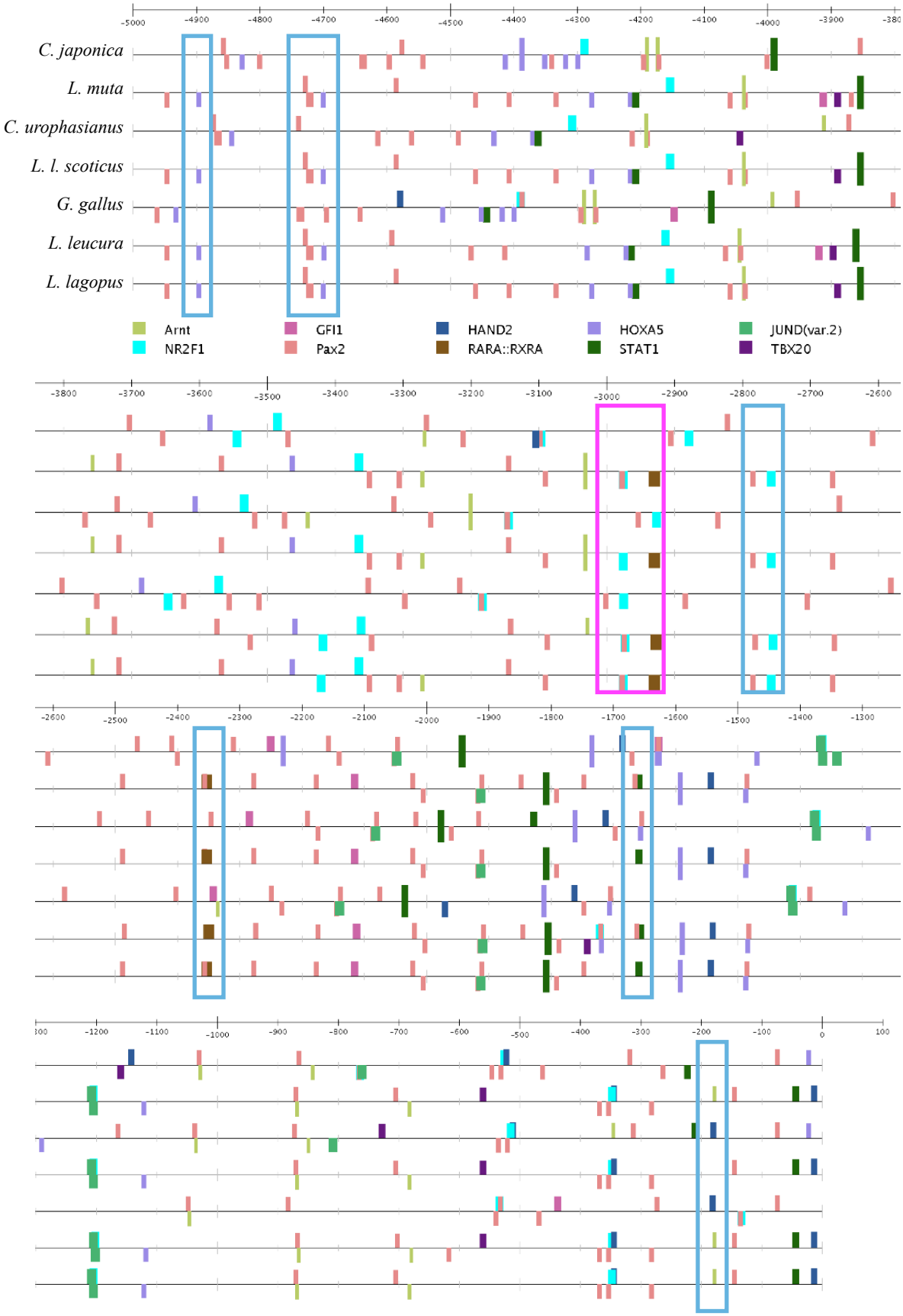

Figure 22. *Promoter regions of MC1R for C. japonica, L. muta, C. urophasianus, L. l. scoticus, G. gallus, L. leucura & L. lagopus. Blue boxes indicate TFBS common to Lagopus spp. but not other galliforms. Purple boxes indicate TFBS that show differences between L. l. scoticus and other Lagopus spp. but not similarity between L. l. scoticus and non SCC galliforms.*

TYR

Most species have matching sequences, except for *L. leucura* which has no matches and *G. gallus* which has a frame shift with the other five species (figure 23). In the CiiiDER output (figure 24) *L. l. scoticus* lacks another PAX2 site at -2800 and and site for MEOX2 at -1100.

Meox2 (mesenchyme homeobox 2) is expressed in the epithelial somites and the sclerotome in the late stages of development and, based on knockout mice, is involved in the development of skeletal muscles in the limbs (Rodrigo *et al*., 2004). There is nothing to suggest that it is involved in melanogenesis and TYRP1 and DCT were used as controls to test the specificity of MEOX2 and BAPX1 in mice (Rodrigo *et al*., 2004).

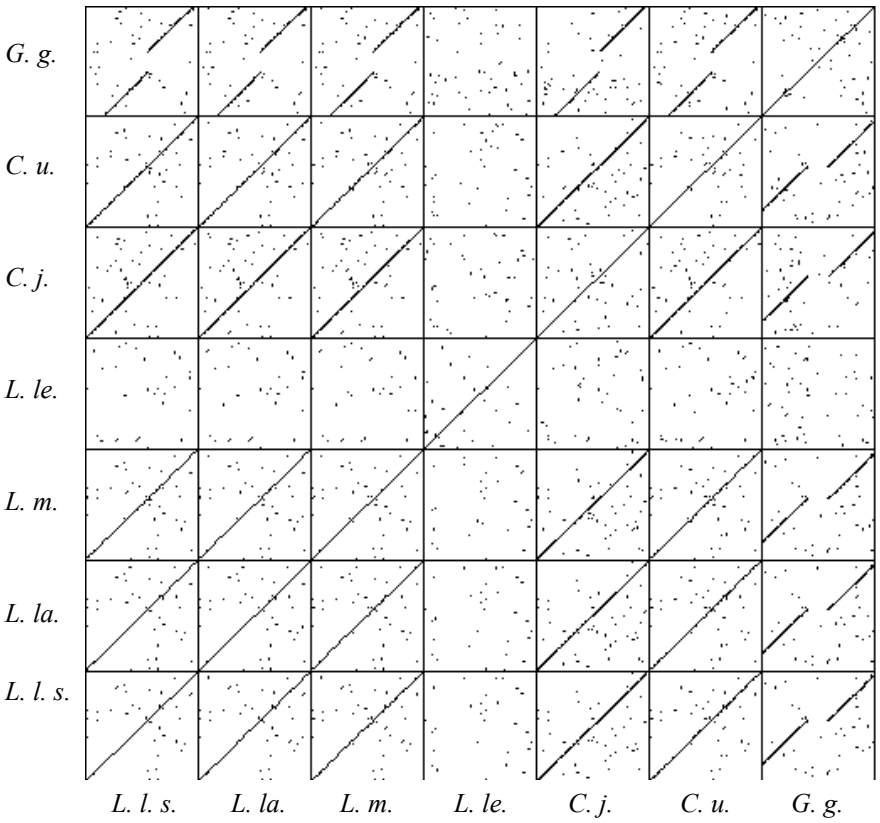

Figure 23. *Promoter polydot for TYR in galliform birds, G. g.=Gallus gallus, C. u.=Centrocercus urophasianus, C. j,=Coturnix japonica, L. le.=Lagopus leucura, L. m.=Lagopus muta, L. la.=Lagopus lagopus, L. l. s.=Lagopus lagopus scoticus.*

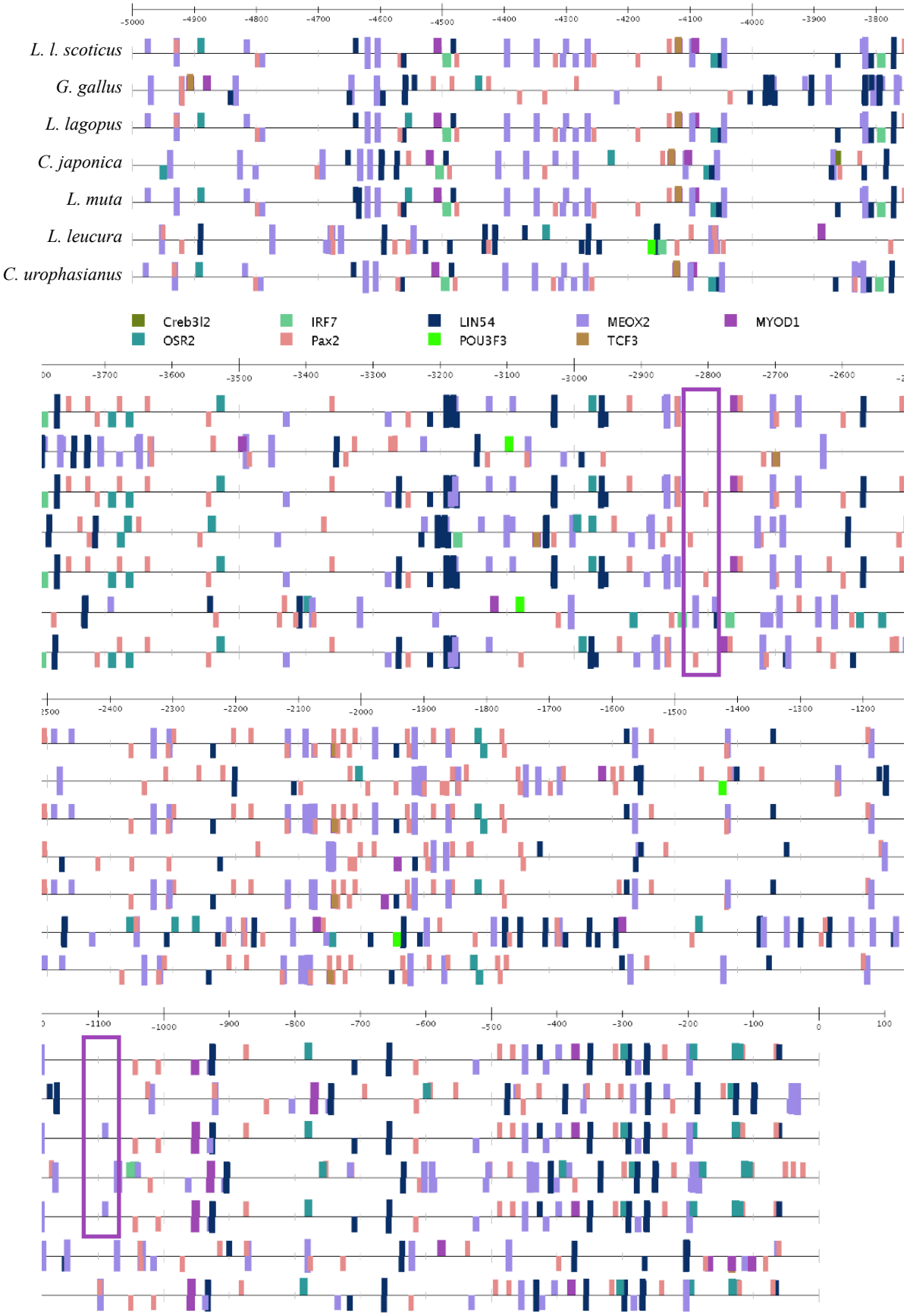

Figure 24. *Promoter regions of TYR for L. l. scoticus, G. gallus, L. lagopus, C. japonica, L. muta, L. leucura & C. urophasianus. Purple boxes indicate TFBS that show differences between L. l. scoticus and other Lagopus spp. but not similarity between L. l. scoticus and non SCC galliforms.*

TYRP1

All Lagopus species have matching promoter sequences, *C. urophasianus* and *G. gallus* have notable frame shifts while *C. japonica* matches no other species (figure 25). There is an extra TFBS for NOBOX at -4500 in *L. l. scoticus* that is not found in other Lagopus species but does match a site in G. gallus at the same location (figure 26). *Nobox* (newborn ovary homeobox gene) plays an important role in the assembly of primordial follicles during ovarian development (Guo *et al*., 2019). Reproductive hormones play an important role in the different morphs of ptarmigan but mostly around the breeding season and so any role that NOBOX may have in SCC is difficult to determine.

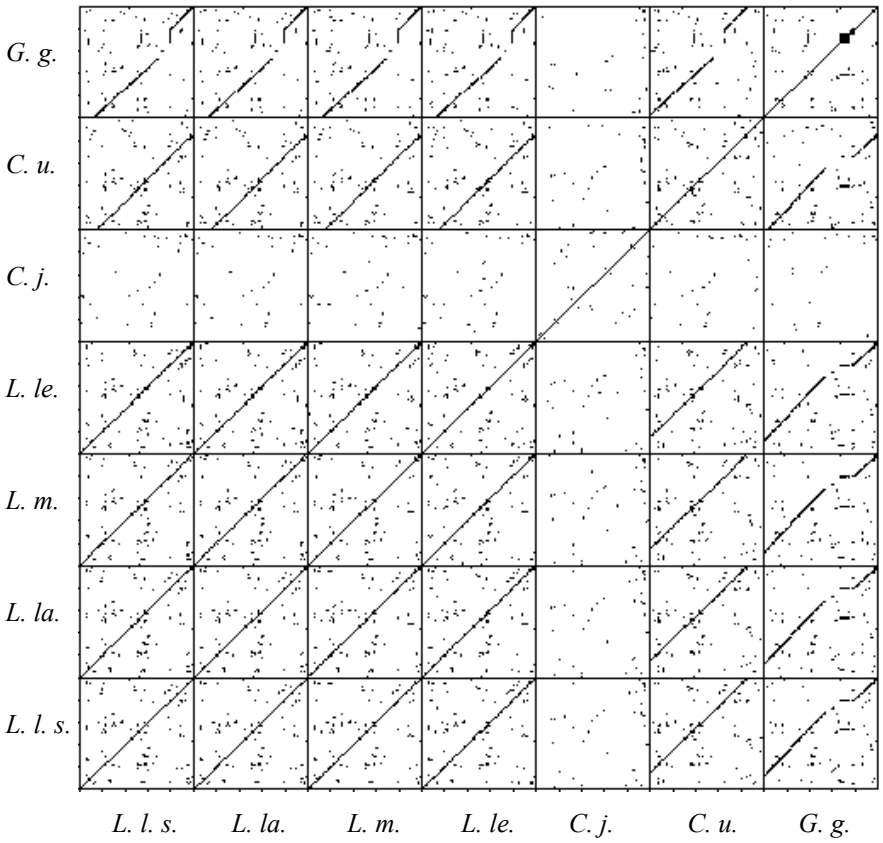

Figure 25. *Promoter polydot for TYRP1 in galliform birds, G. g.=Gallus gallus, C. u.=Centrocercus urophasianus, C. j,=Coturnix japonica, L. le.=Lagopus leucura, L. m.=Lagopus muta, L. la.=Lagopus lagopus, L. l. s.=Lagopus lagopus scoticus.*

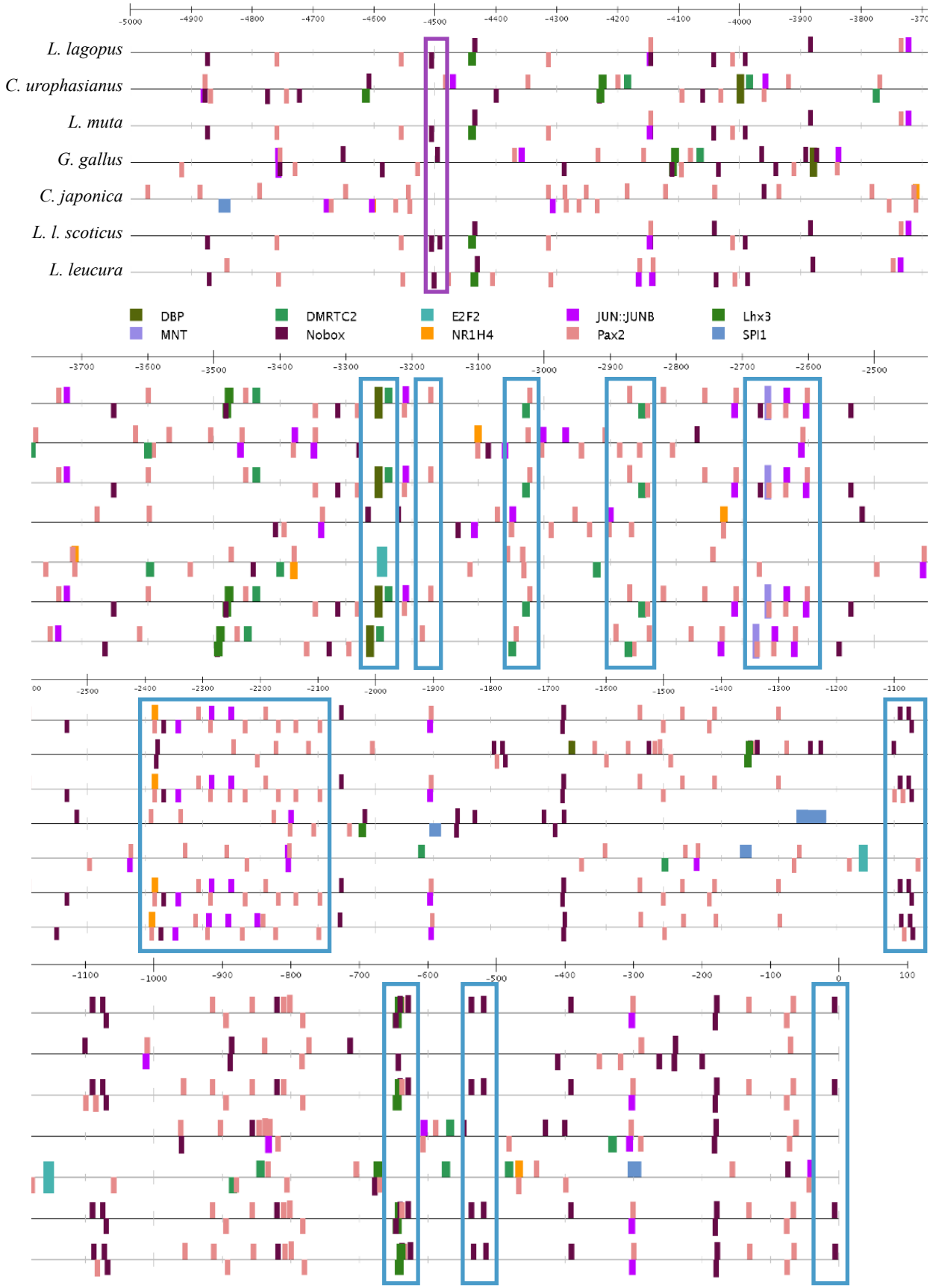

Figure 26. *Promoter regions of TYRP1 for L. lagopus, C. urophasianus, L. muta, G. gallus, C. japonica, L. l. scoticus & L. leucura. Blue boxes indicate TFBS common to Lagopus spp. but not other galliforms. Purple boxes indicate TFBS that show differences between L. l. scoticus and other Lagopus spp. but not similarity between L. l. scoticus and non SCC galliforms.*

DCT

G. gallus matches with no other species and both *C. urophasianus* and *C. japonica* display frame shifts, the Lagopus all match (figure 27). There is no notable difference between the sequences of *L. l. scoticus* and the other Lagopus species or similarity to the other galliforms excluding *G. gallus* (figure 28).

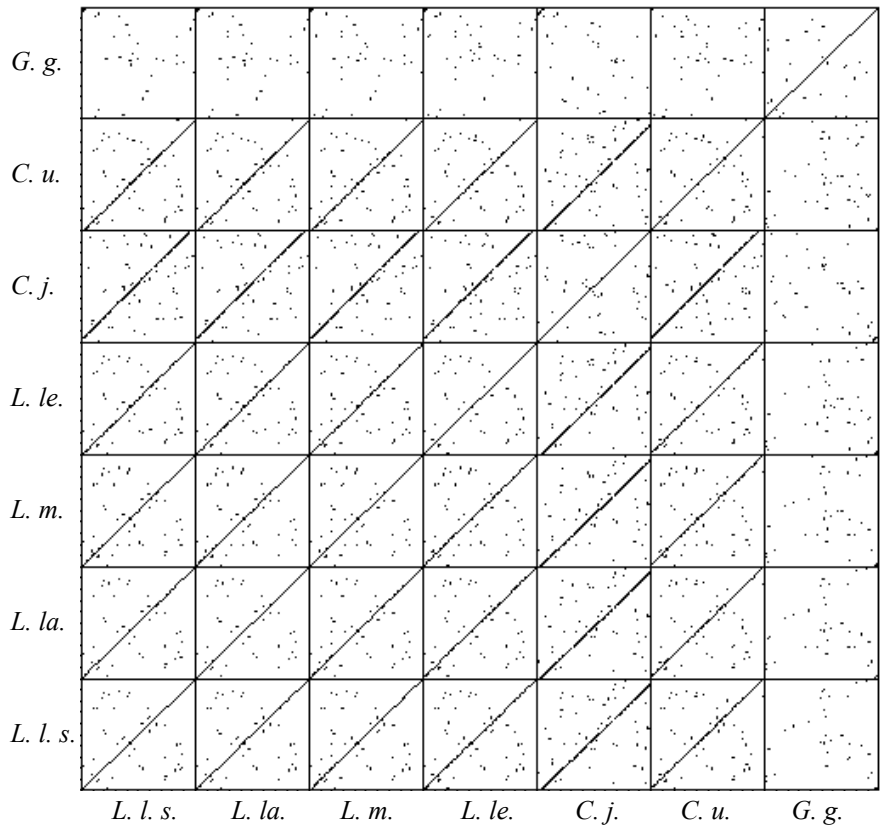

Figure 27. *Promoter polydot for DCT in galliform birds, G. g.=Gallus gallus, C. u.=Centrocercus urophasianus, C. j,=Coturnix japonica, L. le.=Lagopus leucura, L. m.=Lagopus muta, L. la.=Lagopus lagopus, L. l. s.=Lagopus lagopus scoticus.*

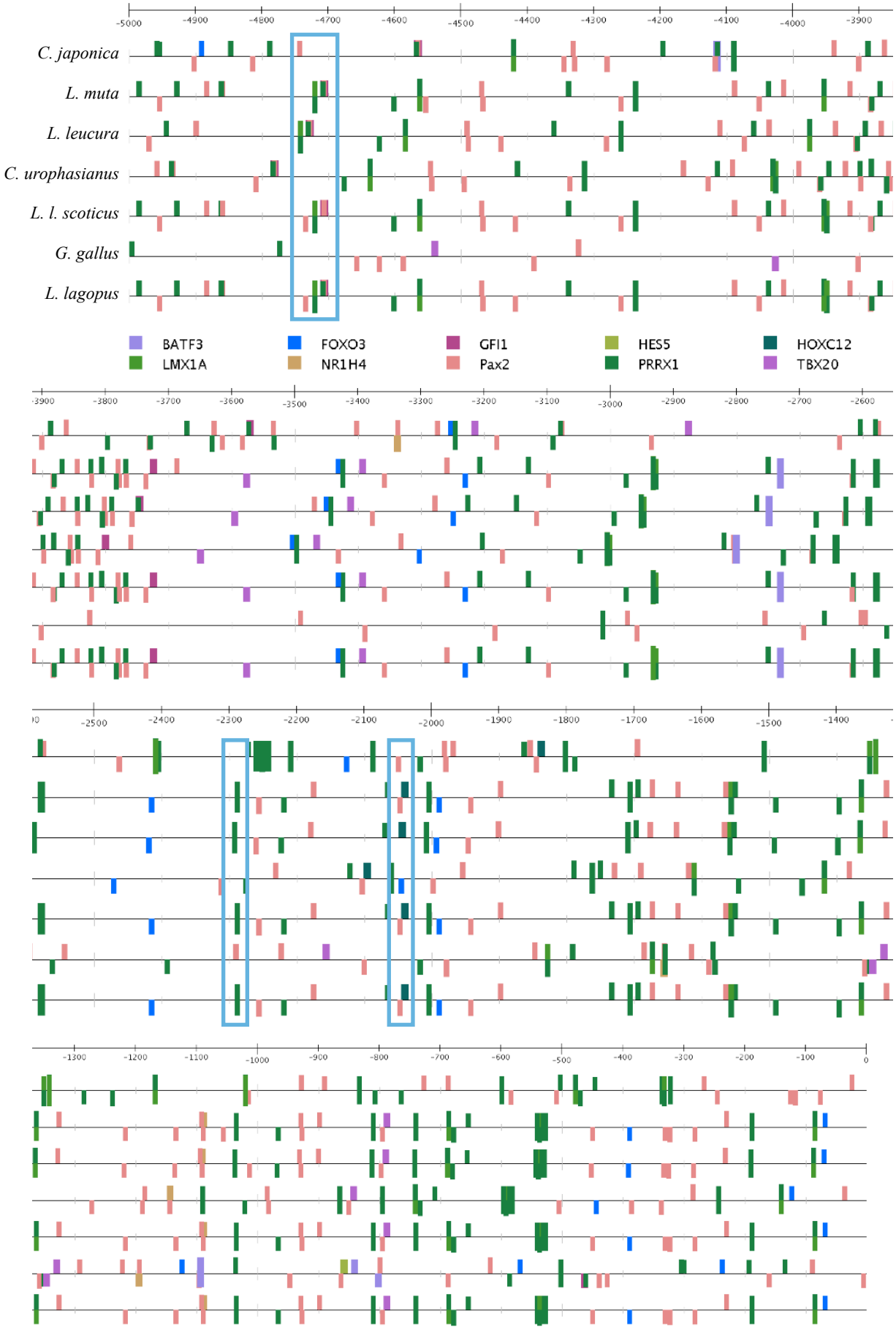

Figure 28. *Promoter regions of DCT for C. japonica, L. muta, L. leucura, C. urophasianus, L. l. scoticus, G. gallus & L. lagopus. Blue boxes indicate TFBS common to Lagopus spp. but not other galliforms. Red boxes indicate TFBS common to SCC species only.*

CORIN

Lagopus species match completely, the other galliform birds also match each other and have a frame shift with the Lagopus species (figure 29). All species that do not undergo SCC, including *L. l. scoticus*, lack a TFBS for PAX2 between -2600 and -2400 (figure 30).

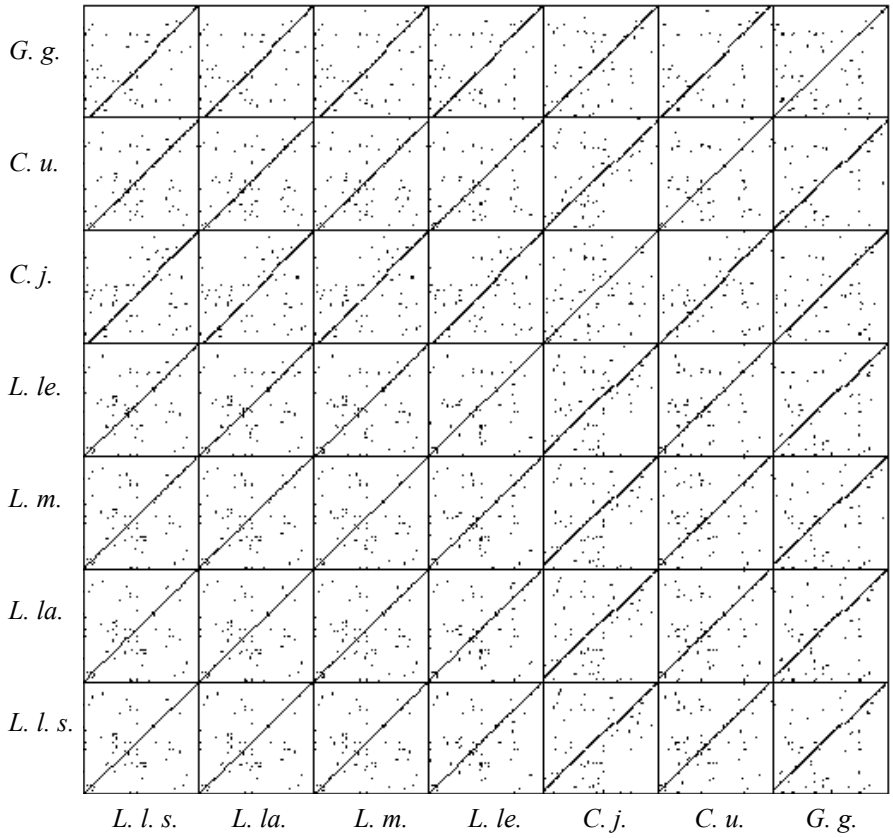

Figure 29. *Promoter polydot for CORIN in galliform birds, G. g.=Gallus gallus, C. u.=Centrocercus urophasianus, C. j,=Coturnix japonica, L. le.=Lagopus leucura, L. m.=Lagopus muta, L. la.=Lagopus lagopus, L. l. s.=Lagopus lagopus scoticus.*

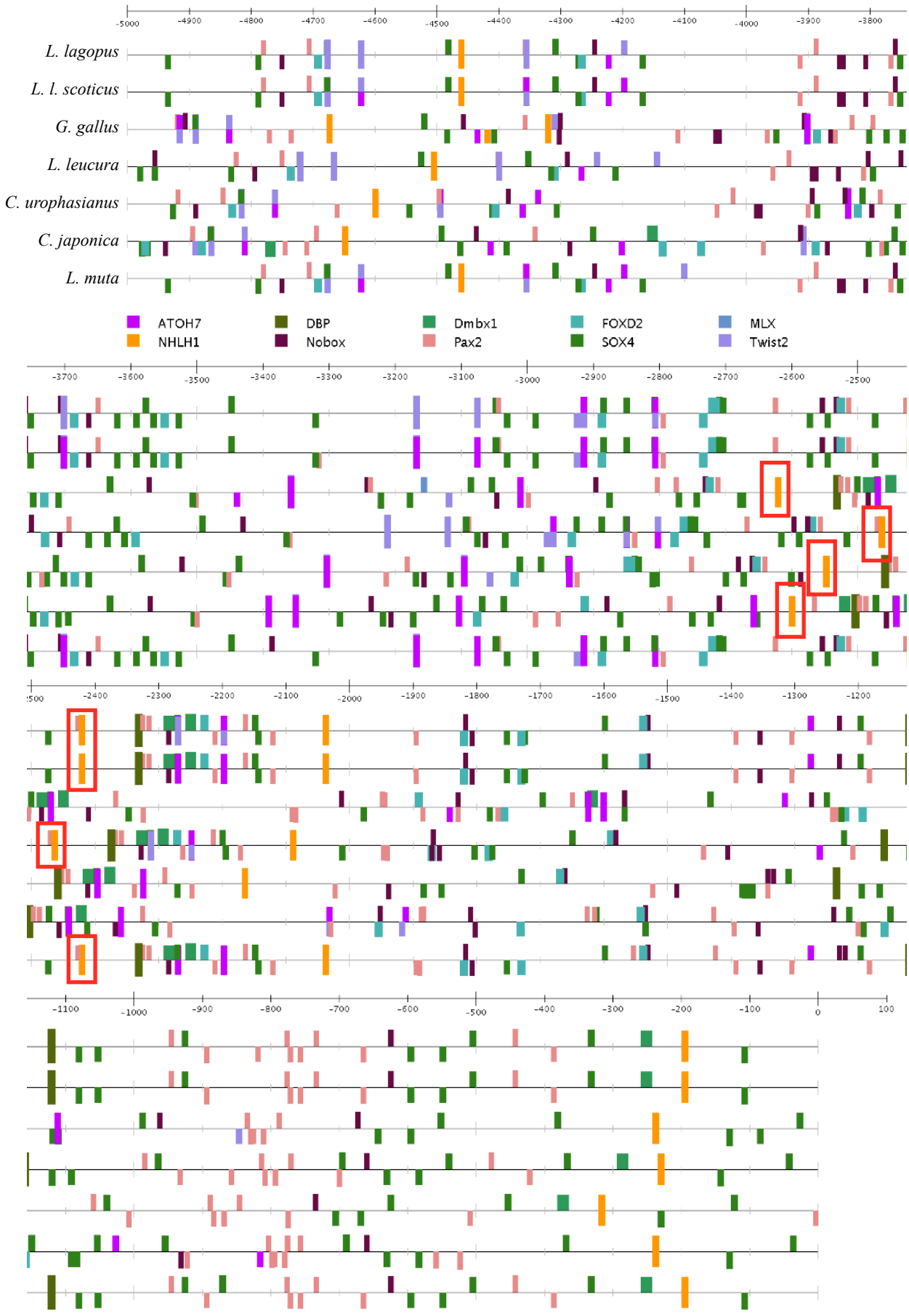

Figure 30. *Promoter regions of CORIN for L. lagopus, L. l. scoticus, G. gallus, L. leucura, C. urophasianus, C. japonica & L. muta. Red boxes indicate TFBS common to SCC species only.*

CREB1

The sequence for *C. urophasianus* matches no other species, while *G. gallus* and *C. japonica* show frame shifts and mismatches, the Lagopus species all have matching promoter regions (figure 31). *L. l. scoticus* lacks a site for NR5A1 at -2650 that can be found in the other Lagopus species and a site for CENPB at -150 that can be found in all species except *C. urophasianus* (figure 32). *Nr5a1* (Nuclear receptor subfamily 5 Group A member 1) has a role in the development of endocrine systems and sex determination, there does not appear to be any relationship with melanogenesis (Büdefeld *et al*., 2012; Shimigori *et al*., 2010). *Cenpb* (Centromere protein B) has an important role in the formation of centromeres during mitosis. It therefore has a close relationship with MITF and MC1R as they have critical roles in chromosome and centromere stability, however this role is during UV induced melanogenesis in humans and may not translate into SCC in ptarmigan (Li *et al*., 2021; Saffery *et al*., 1999).

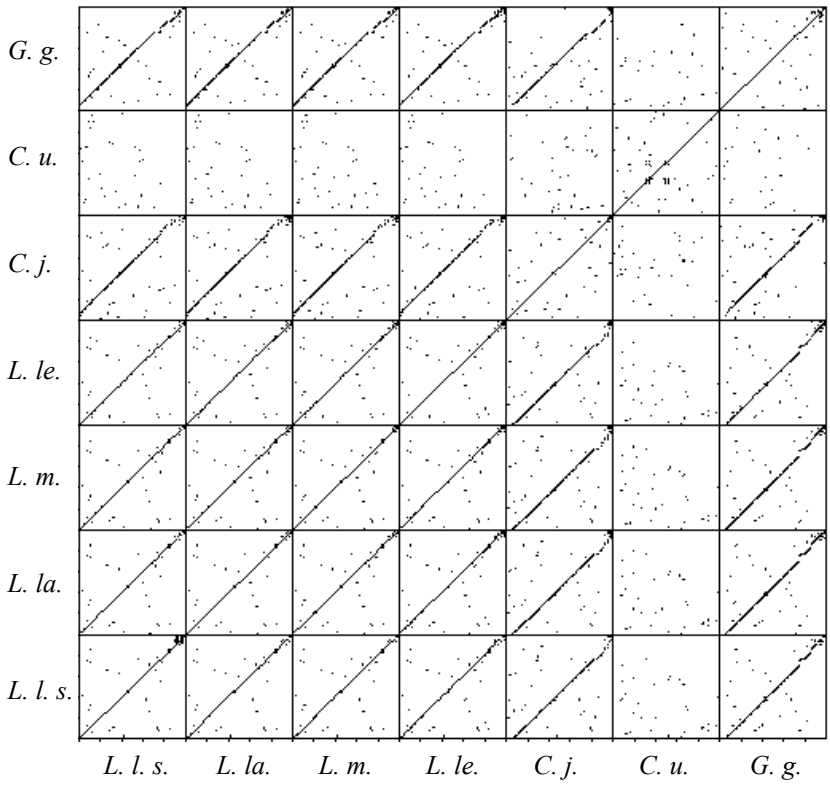

Figure 31. *Promoter polydot for CREB1 in galliform birds, G. g.=Gallus gallus, C. u.=Centrocercus urophasianus, C. j,=Coturnix japonica, L. le.=Lagopus leucura, L. m.=Lagopus muta, L. la.=Lagopus lagopus, L. l. s.=Lagopus lagopus scoticus.*

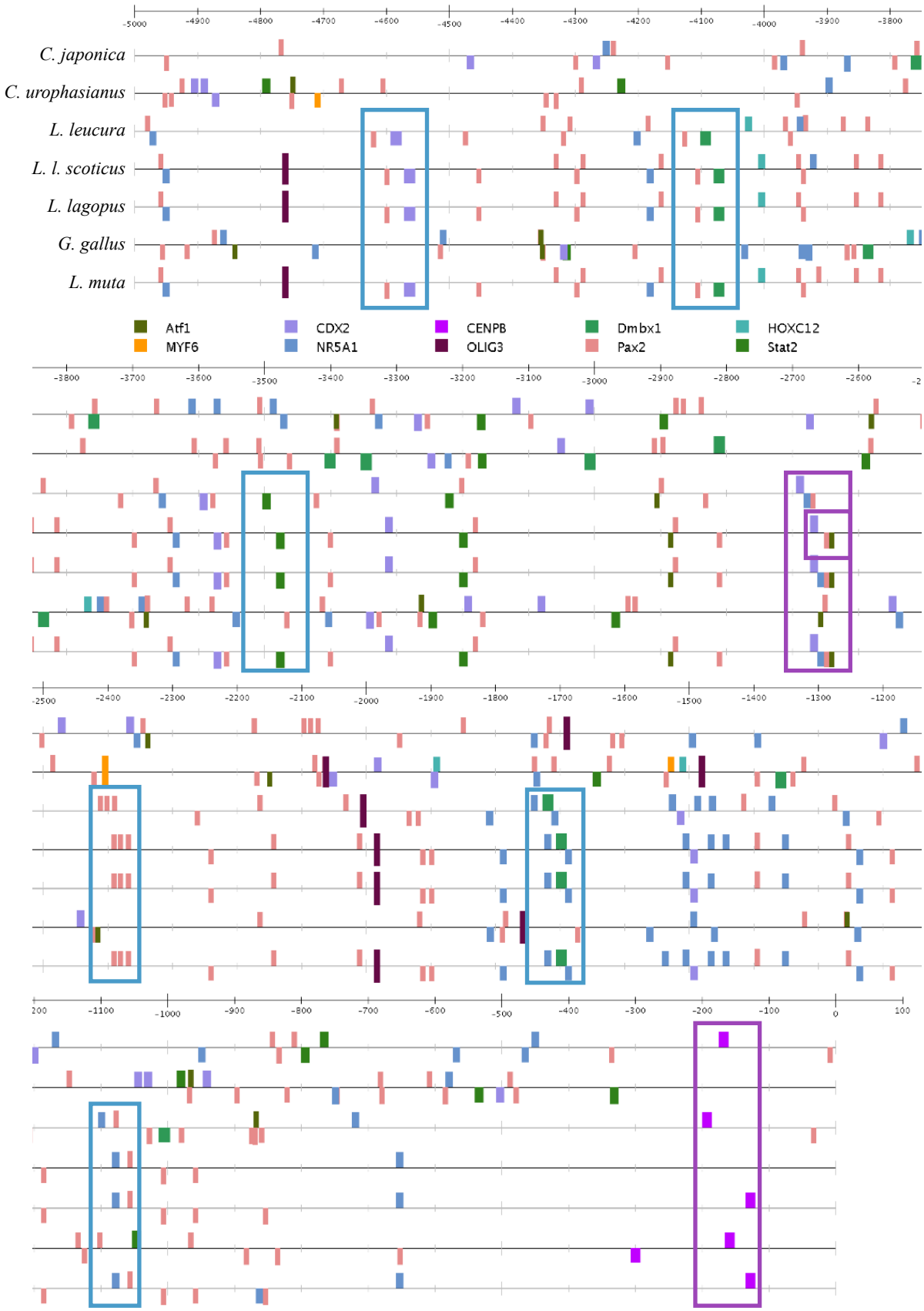

Figure 32. *Promoter regions of CREB1 for C. japonica, C. urophasianus, L. leucura, L. l. scoticus, L. lagopus, G. gallus & L. muta. Blue boxes indicate TFBS common to Lagopus spp. but not other galliforms. Purple boxes indicate TFBS that show differences between L. l. scoticus and other Lagopus spp. but not similarity between L. l. scoticus and non SCC galliforms.*

MITF

L. lagopus matches no other species, however the other species all match each other with some frame shifts between *C. japonica* and *G. gallus* and the Lagopus species (figure 33). There is a TFBS for PAX2 at -4200 to -3900 that can be found in all species except *L. l. scoticus* and *L. lagopus*, although *L. lagopus* has a site at -4100 that could function in the same role. There is a similar lack of a site for PAX2 at -3650 to -3250 in both *L. l. scoticus* and *L. lagopus*. At -2100 *L. l. scoticus* lacks binding sites for both PAX2 and TBX3 that can be found in the other species, with the possible exception of *L. lagopus* (figure 34).

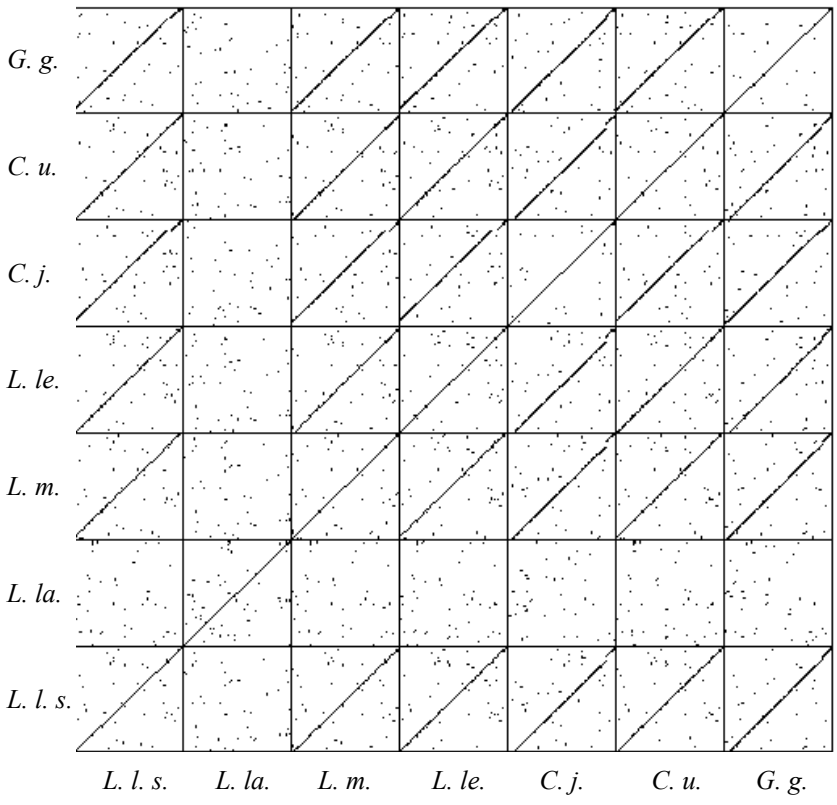

Figure 33. *Promoter polydot for MITF in galliform birds, G. g.=Gallus gallus, C. u.=Centrocercus urophasianus, C. j,=Coturnix japonica, L. le.=Lagopus leucura, L. m.=Lagopus muta, L. la.=Lagopus lagopus, L. l. s.=Lagopus lagopus scoticus.*

Figure 34. *Promoter regions of MITF for L. l. scoticus, G. gallus, L. leucura, L. lagopus, C. urophasianus, L. muta & C. japonica. Purple boxes indicate TFBS that show differences between L. l. scoticus and other Lagopus spp. but not similarity between L. l. scoticus and non SCC galliforms.*

OCA2

L. l. scoticus matches with *L. muta* only, *L. leucura, C. japonica* and *G. gallus* mostly match each other with some frame shifts, the other species do not match each other (figure 35). As *L. l. scoticus* can only be compared to one other Lagopus species the differences between the promoter sequences might not correlate to differences in melanogenesis. *L. l. scoticus* has extra binding sites for PAX2 at -4700, -1600, -1050 & -100, and extra binding sites for ARID3A at -4450 and -1800 (figure 36). *Arid3a* (AT-rich interaction domain 3A) has multiple roles including: chromatin remodelling, epigenetic post-translational modification and cell cycle-regulated events. The allele responsible for the black-boned chicken variety, characterised by greater TYR expression, has a mutation that creates a putative binding site for ARID3A. However the relationship between ARID3A and melanogenesis has not yet been completely defined and mutations in chickens do not necessarily relate to SCC in ptarmigan (Yu *et al*., 2017).

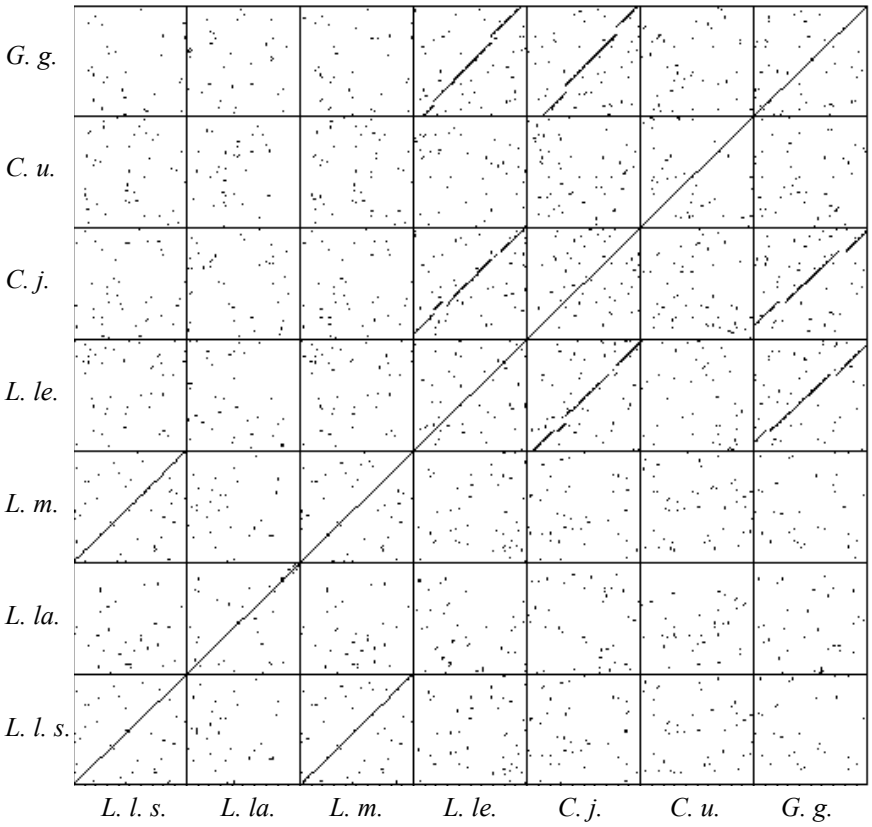

Figure 35. *Promoter polydot for OCA2 in galliform birds, G. g.=Gallus gallus, C. u.=Centrocercus urophasianus, C. j,=Coturnix japonica, L. le.=Lagopus leucura, L. m.=Lagopus muta, L. la.=Lagopus lagopus, L. l. s.=Lagopus lagopus scoticus.*

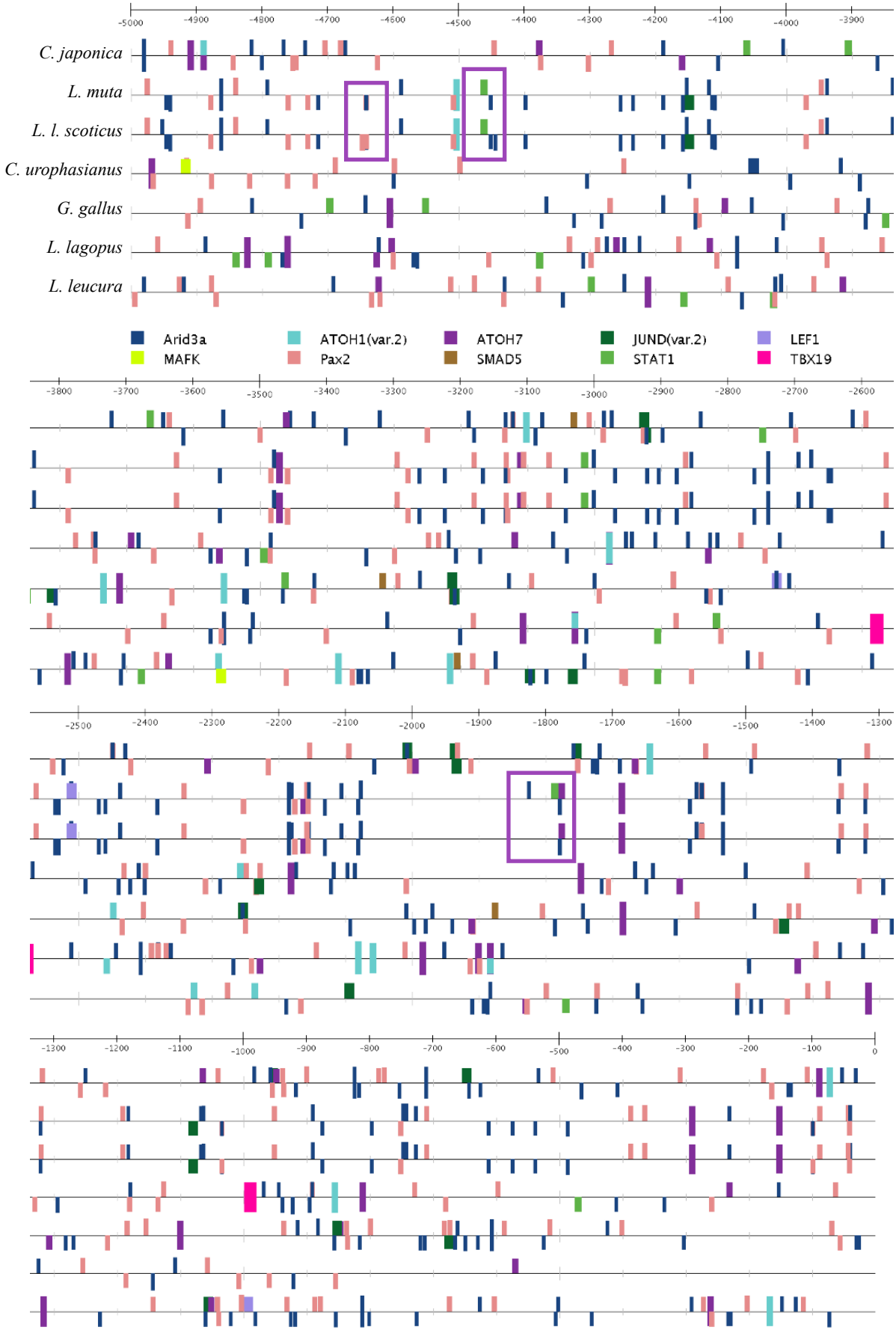

Figure 36. *Promoter regions of OCA2 for C. japonica, L. muta, L. l. scoticus, C. urophasianus, G. gallus, L. lagopus & L. leucura. Purple boxes indicate TFBS that show differences between L. l. scoticus and other Lagopus spp. but not similarity between L. l. scoticus and non SCC galliforms.*

SLC7A11

There is a clear difference between ptarmigan and other galliform birds, all the Lagopus species match each other but not the other galliforms and the other galliforms match each other with obvious frame shifts (figure 37). Once again *L. l. scoticus* lacks a PAX2 binding site that can be found in the other species, including the other galliform birds at -800, *L. l. scoticus* also lacks a site for FEV at -350 that can be found in the other Lagopus species (figure 38). *Fev* (fifth Ewing variant) codes for a transcription factor involved in serotonergic neuronal development and is implicated in Sudden Infant Death Syndrome (Broadbelt *et al*., 2009).

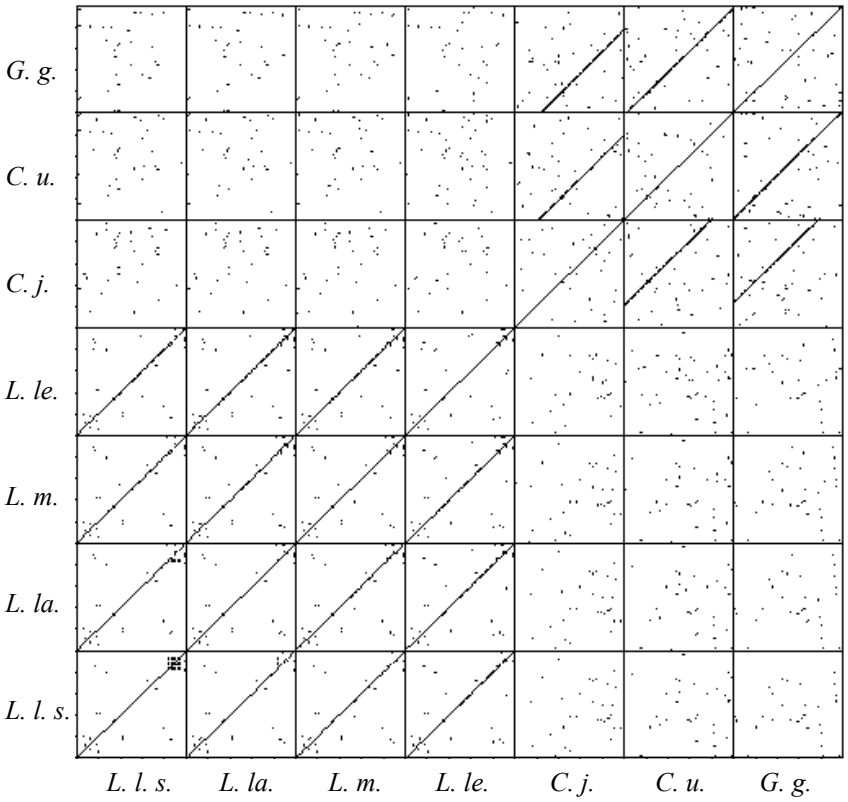

Figure 37. *Promoter polydot for SLC7A11 in galliform birds, G. g.=Gallus gallus, C. u.=Centrocercus urophasianus, C. j,=Coturnix japonica, L. le.=Lagopus leucura, L. m.=Lagopus muta, L. la.=Lagopus lagopus, L. l. s.=Lagopus lagopus scoticus.*

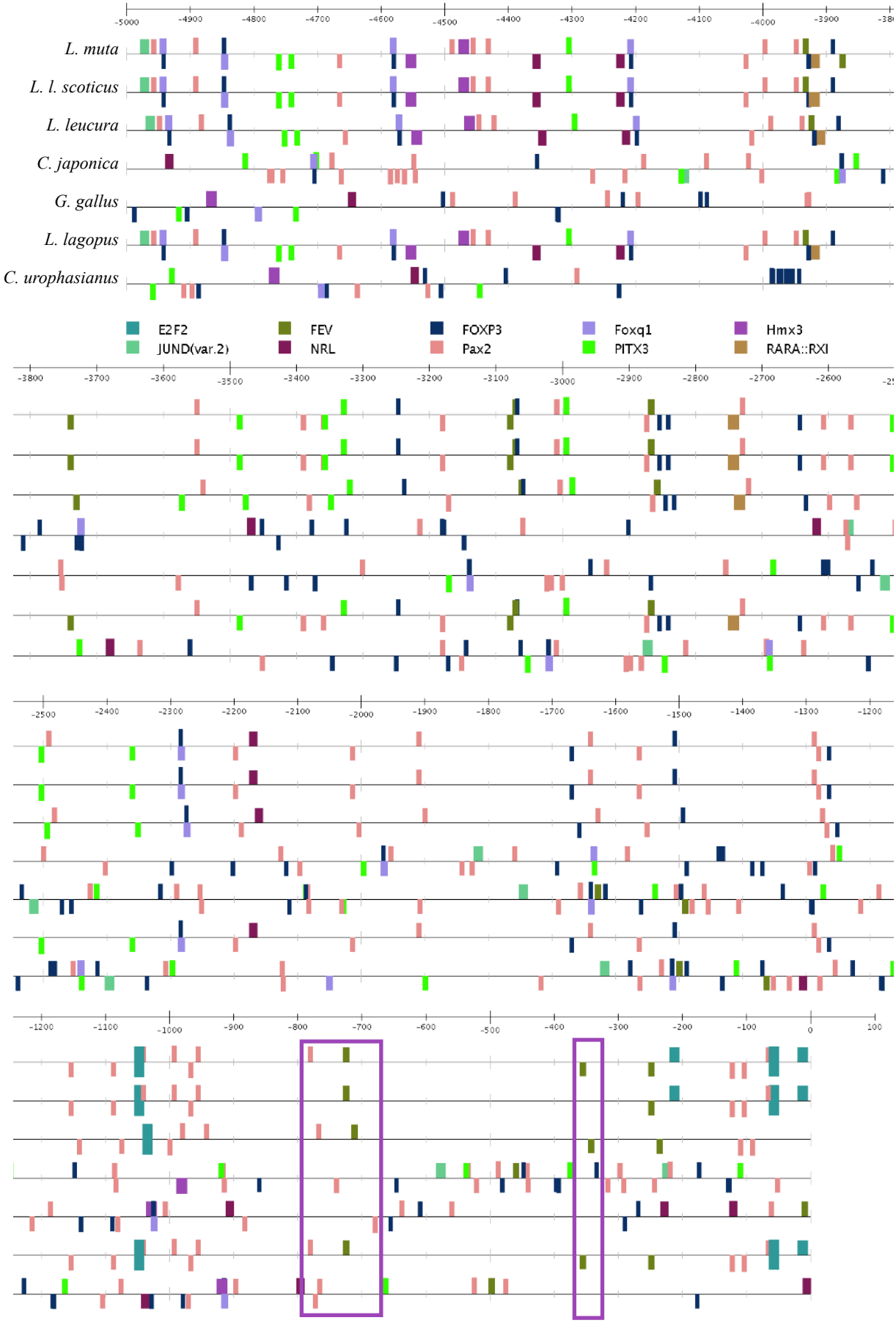

Figure 38. *Promoter regions of SLC7A11 for L. muta, L. l. scoticus, L. leucura, C. japonica, G. gallus, L. lagopus & C. urophasianus. Purple boxes indicate TFBS that show differences between L. l. scoticus and other Lagopus spp. but not similarity between L. l. scoticus and non SCC galliforms.*

SLC45A2

All species have matching sequences, except *C. japonica* which has a partial match and notable frame shifts with all other species (figure 39). There is a binding site for PAX2 that can be found between -2400 and -1800 in non SCC species, including *L. l. scoticus*, but not in Lagopus species that undergo SCC (figure 40).

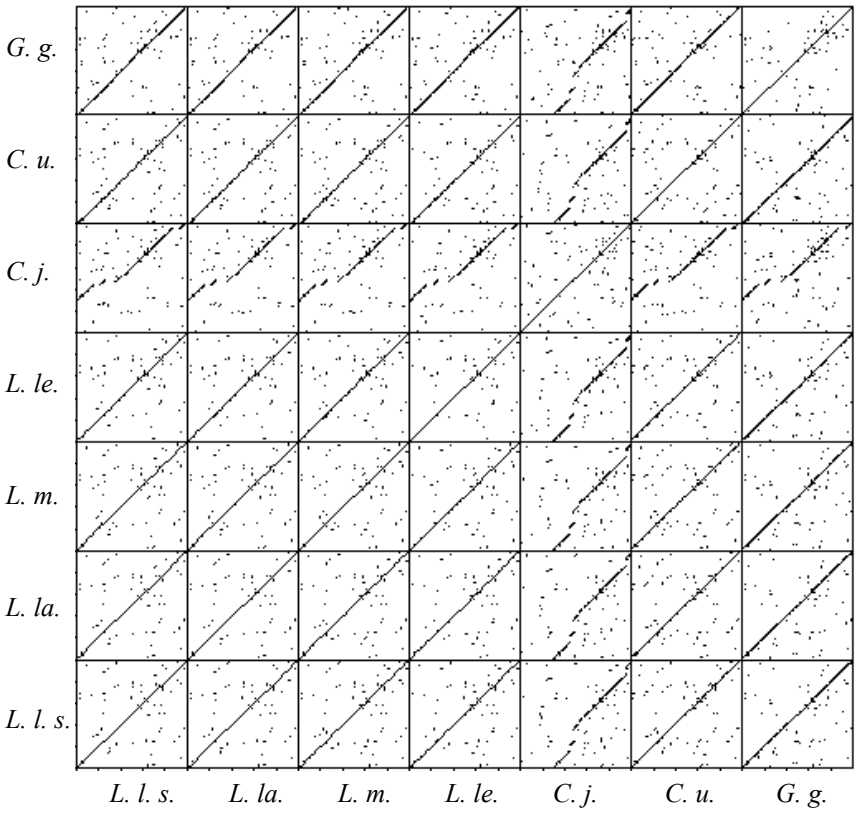

Figure 39. *Promoter polydot for SLC45A2 in galliform birds, G. g.=Gallus gallus, C. u.=Centrocercus urophasianus, C. j,=Coturnix japonica, L. le.=Lagopus leucura, L. m.=Lagopus muta, L. la.=Lagopus lagopus, L. l. s.=Lagopus lagopus scoticus.*

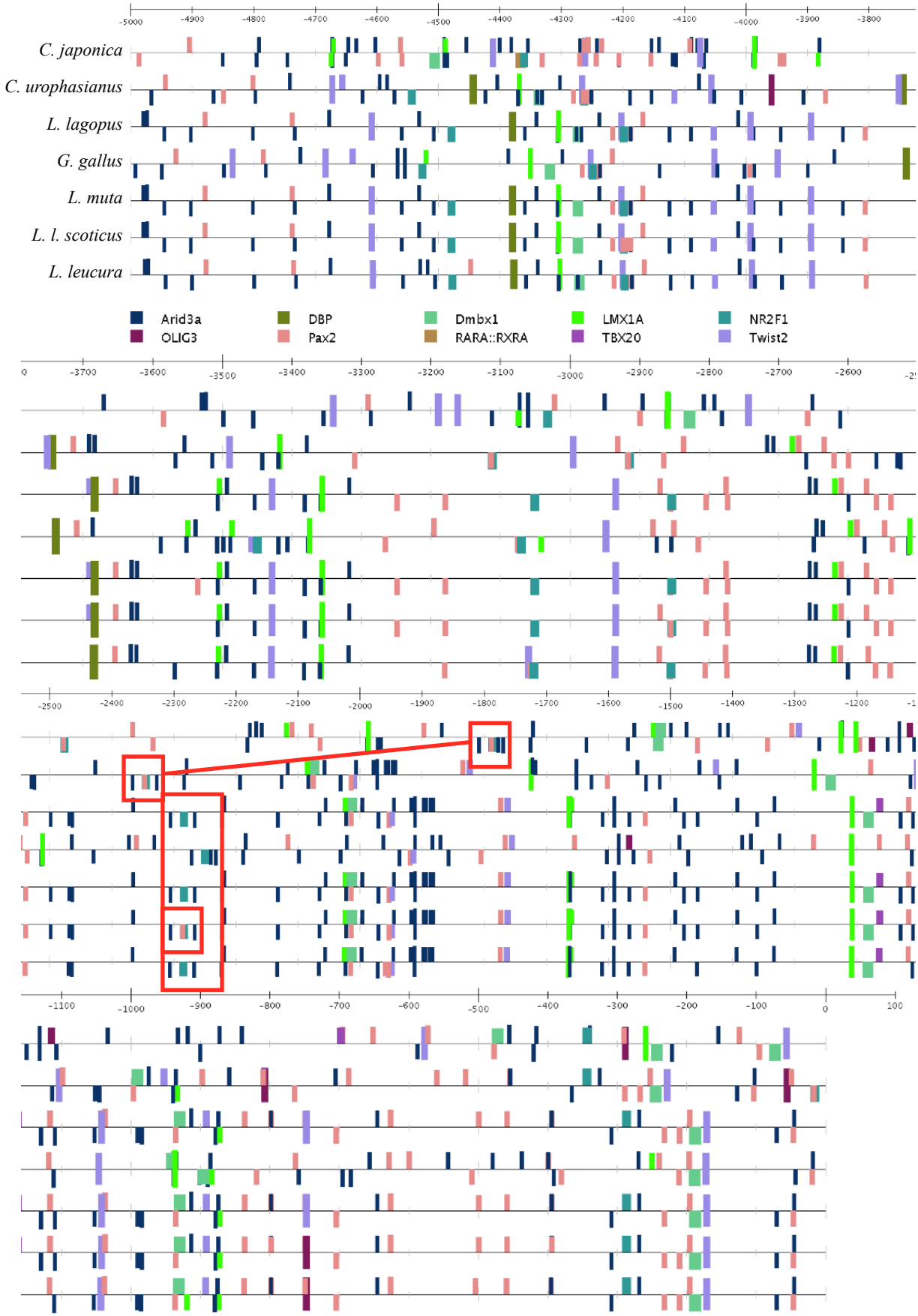

Figure 40. *Promoter regions of SLC45A2 for C. japonica, C. urophasianus, L. lagopus, G. gallus, L. muta, L. l. scoticus & L. leucura. Red boxes indicate TFBS common to SCC species only.*

TBX19

There is no available sequence for *C. japonica* and so it cannot be compared to the other species. All Lagopus species have matching sequences and *C. urophasianus* shows a partial match with a frame shift, *G. gallus* does not match any of the other species (figure 41). There are multiple binding sites for RHOXF1 that differ between *L. l. scoticus* and the other Lagopus species. At -4200 there is an extra site that seems more similar to the sites found in *G. gallus* than in the other species, while at -3900, -2900 & -2750, *L. l. scoticus* lacks a site that can be found in the other Lagopus species with some similarity between *G. gallus, C. urophasianus* and the Lagopus species, excluding *L. l. scoticus* (figure 42). This suggests that TBX19 and by extension POMC are under different regulation in ptarmigan that undergo SCC and those that do not. However as POMC affects so many different systems it is possible that these differing promoter regions are related more to different behaviours than melanogenesis.

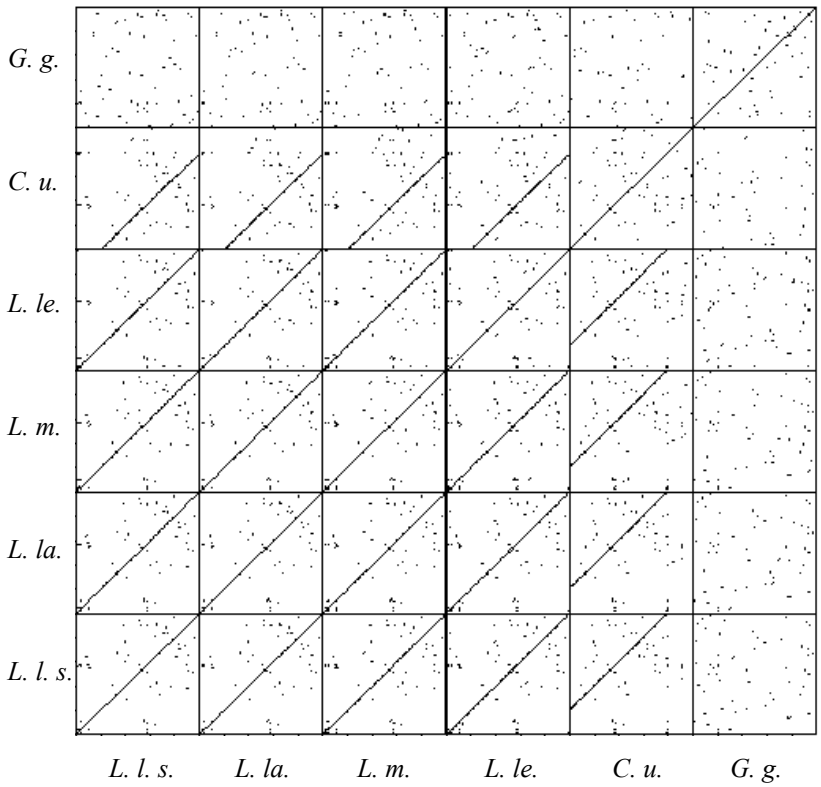

Figure 41. *Promoter polydot for TBX19 in galliform birds, G. g.=Gallus gallus, C. u.=Centrocercus urophasianus, C. j,=Coturnix japonica, L. le.=Lagopus leucura, L. m.=Lagopus muta, L. la.=Lagopus lagopus, L. l. s.=Lagopus lagopus scoticus.*

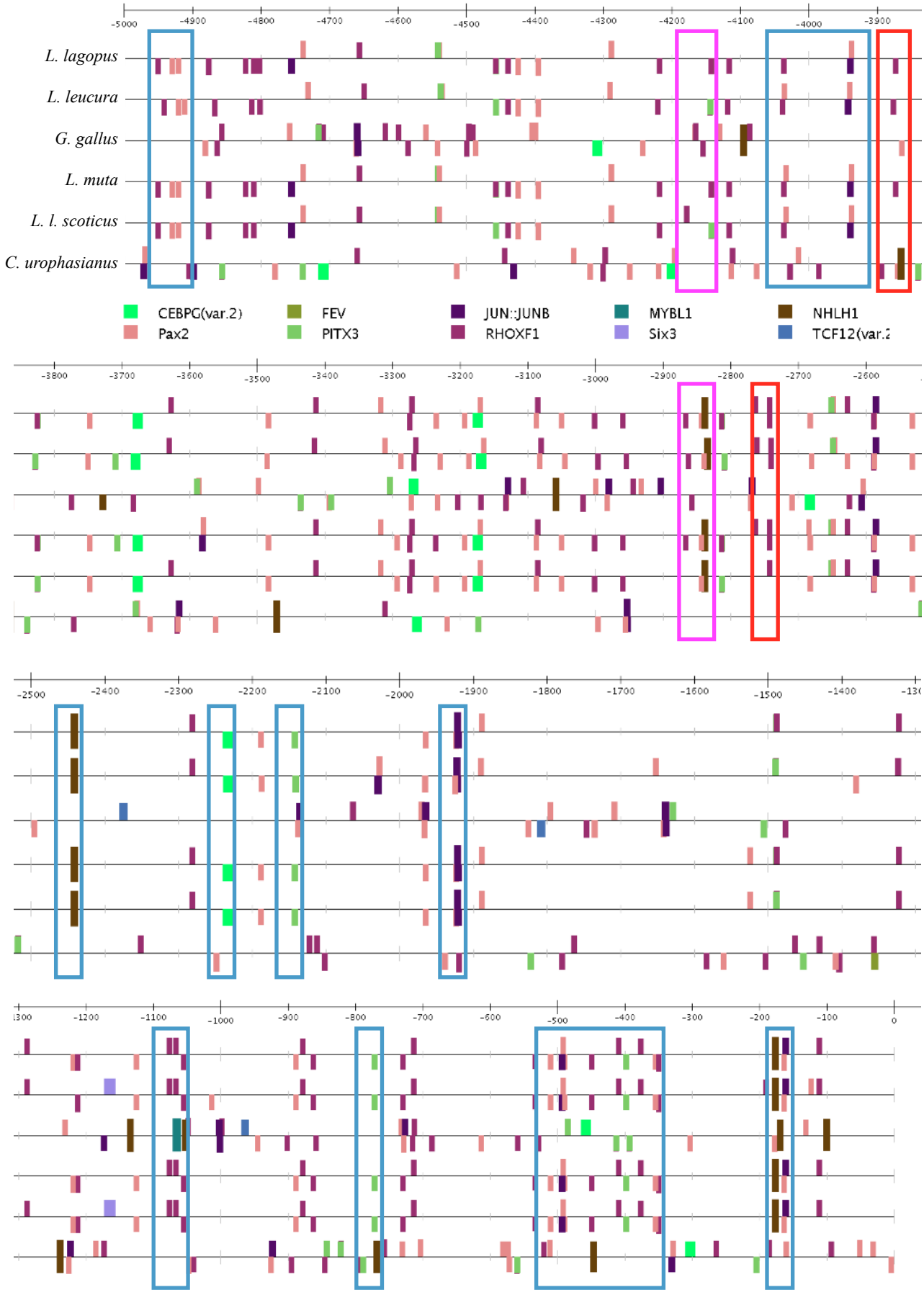

Figure 42. *Promoter regions for TBX19 in L. lagopus, L. leucura, G. gallus, L. muta, L. l. scoticus & C. urophasianus. Blue boxes indicate TFBS common to Lagopus spp. but not other galliforms. Red boxes indicate TFBS common to SCC species only. Purple boxes indicate TFBS that show differences between L. l. scoticus and other Lagopus spp. but not similarity between L. l. scoticus and non SCC galliforms.*

Table 9. Summary of TFBSs that differed between *L. l. scoticus* and other Lagopus species.

Transcription Factor	Gene Name	Extra Site in *L. l. scoticus*	Missing Site in *L. l. scoticus*	Role
PAX2	Paired Box 2	SLC45A2	PC1, MC1R, TYR, CORIN, MITF, OCA2, SLC7A11	Development of the urogenital tract, eyes, and CNS
MEIS1	Meis Homeobox 1	PC1	-	Development & transcriptional regulator of Pax6
TBX3	T-Box Transcription Factor 3	PC1	MITF	Development of mammary glands, lung mesenchyme & inner ear
RHOXF1	Rox Homeobox Family Member 1	POMC, TBX19	TBX19	May be involved in reproduction esp. spermatogenesis
SOX4	Sry-Box Transcription Factor	-	PC2	Skin development & wound repair
MEOX2	Mesenchyme Homeobox 2	-	TYR	Limb muscle differentiation
NOBOX	Newborn Ovary Homeobox Encoding Gene	TYRP1	-	Oogenesis & folliculogenesis
NR5A1	Nuclear Receptor Subfamily 5 Group A Member 1	-	CREB1	Sex determination
CENPB	Centromere Protein B	-	CREB1	Facilitate centromere formation
ARID3A	At-Rich Interactive Domain Containing Protein 3A	-	OCA2	Embryonic patterning & cell cycle regulation
FEV	Fifth Ewing Variant Transcription Factor	-	SLC7A11	Neuronal development

4. Discussion

As stated earlier the aim of this thesis is to investigate SCC in ptarmigan and expand our knowledge of how this is regulated.
The hypotheses regarding this aim are as follows:

1. Melanogenesis is regulated through the expression of key genes of melanogenesis: POMC, PC1, PC2, MC1R, ASIP, TYR, TYRP1 & DCT.
2. These genes will be up-regulated during the production of brown feathers and down-regulated during the production of white feathers.
3. This difference in gene expression will be detectable in the skin and feather follicles of ptarmigan during their moult between brown and white plumage.
4. The promoter regions of the genes involved in melanogenesis (table 1) will differ between *L. l. scoticus* and the ptarmigan species that undergo SCC.

To test the first three hypotheses qPCR was performed on skin samples from new and old feathers of *L. lagopus* and *L. muta* during the autumn moult between the autumn and winter morphs. The relative gene expression was calculated and a student's t-test was used to determine significance. The results of the t-tests (tables 7 & 8) show that only TYRP1 had a significantly higher gene expression in brown skin samples than white ones, which aligns with the second hypothesis. However this is only true for *L. muta*, the *L. lagopus* samples showed no difference in expression for any gene. Although MC1R had a general trend of lower expression in white samples (figure 11), the p-value was low but not significant (p=0.056, table 8).

L. muta and *L. lagopus* are very closely related species; based on their mitochondrial DNA the two diverged around 65,000 years ago, with over 4% of a mid-Sweden population of *L. lagopus* showing signs of hybridisation with *L. muta* (Quintela *et al*., 2010). Based on this, it seems unlikely that the method of regulating melanogenesis would be different between species, especially as their phenotypes are so similar at the time when the samples were taken, during the autumn moult to a white morph. As the two species are so similar it is unexpected that there is a significant difference for TYRP1 in *L. muta* but not *L. lagopus* (p =0.028 and 0.902, respectively). The difference between them is more likely to be an artefact of the sample size, the *L. muta* samples came from one individual while the *L. lagopus* samples came from five different birds.

There is no reason, however, to believe that TYRP1 being down-regulated during the autumn moult is a false positive. This would mean that the first stages of melanogenesis do occur; α-MSH binds with MC1R and starts a signalling cascade using cAMP and CREB1. However, without TYRP1, tyrosinase is not stabilised and DHICA will not be oxidised, this means that although most of the pathway is intact melanin will not be produced. Siberian hamsters also display SCC and tyrosinase activity increases during both of their moults (Logan & Weatherhead, 1980). This suggests that ptarmigan and hamsters may have a similar mechanism of post-tyrosinase inhibition of melanogenesis during the autumn moult into their winter morphs.

The near significance of MC1R might be due to ptarmigan selectively increasing and decreasing the amount of receptors within each melanocyte as part of their annual melanogenesis cycle. By removing the receptor from the cell membrane the melanocyte can not be stimulated to produce melanin, or not stimulated to produce as much. Moreover, if the MC1R gene is able to influence the other genes in the melanogenesis pathway, both up- and down-stream of itself, in the same manner

as in the morphs of the barn owl (San-Jose et al., 2016). Then inhibition and expression of MC1R would have a cascade effect on melanogenesis as a whole and seasonal expression could affect phenotype through a relatively small difference in gene expression.

There is the possibility that gene expression is localised entirely to the follicles and that this study could be refined in the future with smaller, more accurate, samples. The skin samples taken were about $1cm^2$ and contained multiple follicles, if the goal is to compare relative gene expression between feather follicles then ideally individual follicles should be used for RNA extraction and purification. This method was not used for this thesis as the goal was to maximise potential RNA extraction, however as this did not produce very clear results it is not necessarily the best method. Although, Ferreira *et al*., (2020) used a similar experimental design to compare brown and white morphs of hares and took skin samples measuring $2cm^2$. They found significant differences in gene expression with the main differences between these two studies being the number of samples, the size of the follicles and the thickness of the skin. Birds have larger follicles and thinner skin so it is possible that regulation is localised to the follicles and extra skin provides unnecessary extra RNA.

Ferreira *et al*., (2020) also used a different system to evaluate which samples to use, setting a minimum RIN score of 8; similarly San-Jose *et al*., (2016) set a minimum score of 9. This study did not use a similar method as this would reduce the sample size too much and the RIN scores were only used as a reference for the Tapestation values. However larger sample sizes would negate this need and could potentially lead to more accurate results as only the samples with a greater integrity of RNA would be used.

Aside from TYRP1 there does not seem to be any significant difference between *L. muta* and *L. lagopus*. The results from the Tapestation (table 3) do not suggest any advantage between field and laboratory sample taking. Provided the samples are submerged in RNAlater they can be left at room temperature for several hours before being frozen; the greatest impact on RNA concentration seems to be the extraction method. The most effective methods were those that reduced the amount of time that the sample was thawing once removed from the freezer, with the best being the use of liquid nitrogen or QIAzol (Table 2). Despite being the best method for RNA extraction there is still a large amount of variation in the resulting concentration, which is why the Tapestation values were then used to select the best samples and why other studies define minimum RIN scores.

The promoter analysis can be used to compare the relative merits of EMBOSS and CiiiDER analysis. The polydot graphs from EMBOSS give a clear idea of whether two sequences are similar or not, but only at the right word size value. At small values the threshold for comparison is too low and there is too much ‘background noise’ to see if the sequences are actually similar. While at large word sizes the threshold is too high and the sequences won’t produce a diagonal line even if they do show similarities; determining the right word size is done through trial and error.

Polydots show an overview of sequence similarity but do not get into specifics; CiiiDER however seems to show both an overview and specific differences. Most differing sequences can be seen at a glance and specifics can be seen if the sequences are visually compared. The drawback of CiiiDER is that the potential TFBSs are not necessarily the ones of most interest regarding that gene, but they may prove a useful starting point for further studies such as promoter-reporter assays.

The polydots and CiiiDER figures used in this thesis show that there are clear differences between the promoter regions of *L. l. scoticus* and other Lagopus species, the most obvious being the lack of PAX2 binding sites in seven of the fifteen sequences studied (table 9). As *Pax2* has an established role in melanogenesis it seems possible that its function extends beyond development and iris pigmentation. However, as *L. l. scoticus* is missing TFBSs that can be found in other Lagopus species it seems unlikely that *Pax2,* which promotes melanogenesis in the retina, is related to the absence of the white morph in *L. l. scoticus*. The expected result would be an extra binding site for TFs that promote melanogenesis and missing sites for TFs that inhibit it. P*ax2* does have an extra site in SLC45A2 but without further research it is impossible to tell the significance of any of these promoter differences.

The frameshift seen in the polydots may be caused by differences in the annotations of the sequences. The promoter regions were taken from just before the start of the first intron and it is possible that if they were taken from the 'ATG' codon at the transcriptional start site instead then the end point of the sequences would have a better match. However this is speculation and assumes that the first introns of the genes are different between species. Most of the misalignments in the polydots were between Lagopus species and other galliform birds which are less closely related.

As *L. l. scoticus* is a subspecies of *L. lagopus* any difference between their promoter regions is of significant interest for the seasonal regulation of SCC. The polydots show that most of the sequences are largely similar, except for MITF and OCA2 (figures 33 & 35). For the MITF promoter sequence the dissimilarity seems insignificant as *L. l. scoticus* has matching sequences in the other galliform species, while *L. lagopus* does not match with any of them. This difference may be caused by differences in the annotation causing the first intron to be a different length and the promoter region to start at different points in their respective genomes. The expected result of this though would be a frame shift and based on their CiiiDER outputs there is little to no similarity between *L. lagopus* and any other species, while the other species all share matching sequences of TFBSs (figure 34). It is possible that the reason that this region is only different in *L. lagopus* is because this region has changed in *L. lagopus* since the sub-speciation of *L. l. scoticus* but remained the same in *L. l. scoticus* and the other Lagopus spp. If this is the case it seems that this region has not had a notable impact on their physiology or behaviour and does not change the regulation of MITF. The polydot for OCA2 shows that most of the species are dissimilar to each other; *L. l. scoticus* only has a similar sequence to *L. muta* while *L. leucura* shows similarity to *C. japonica* and *G. gallus*, with frame shifts, but to no other species. These differences can be explained in the same way as with MITF and if this type of analysis is to be repeated it seems that it would be advantageous to repeat the comparisons from the transcriptional start site.

An important result that was noted during sample taking, but not described above, is that ptarmigan are not truly white in the winter. The plumaceous barbs (the lower, fluffy part of the feather that provides insulation, figure 3) were a light grey colour. This means that melanogenesis is not completely stopped in the autumn moult only decreased. The primaries also have black rachises (the upper part of the central shaft of the feather, figure 3), which suggests that the production of white feathers in the summer may be caused by regional inhibition of transporter proteins and that the melanosomes were not built into the keratin during feather growth. This theory of transport inhibition lines up with the results of the relative gene expression (figures 11 & 12) and the differences in the promoter regions for SLC45A2, SLC7A11 and OCA2 (table 9).

It is also possible that there is a difference in gene expression that cannot be seen due to a small sample size and that if this experiment were repeated with more individuals there would be a significant difference between brown and white groups. This is the case for all of the genes tested; larger sample sizes would improve the reliability of the results, being either significantly different or significantly similar. It should also be noted that qPCR by itself can only show the presence and relative abundance of a given gene and not where the gene is first expressed or modified. This could be the case for POMC, PC1 and PC2, whose presence in the skin could be after they have been expressed in the pituitary gland and secreted into the bloodstream. To remedy this, future work could use cultured melanocytes to show the expression of POMC within the follicle cells themselves.

This study is limited not just in a small sample size but also in the scope of the genes tested, while this was intentional it does leave room to improve for future studies. There are plenty of other genes that could be looked into, including the genes studied for promoter analysis, such as SLC7A11 and CREB1, and different primers and PCR protocols could yield different results.

The promoter analysis is dependent on the availability of genome sequences and their annotations. *C. urophasianus* lacks a sequence for PC1 (figures 15 & 16), while *C. japonica* lacks one for TBX19 (figures 41 & 42), these sequences are less important for this thesis as these species are not the focus. However other genes, namely KIT and KITLG, had to be excluded as there were no available sequences for *L. l. scoticus*. KIT is a tyrosine-kinase-based cell surface receptor specific to the KIT ligand and upon binding it will activate signalling pathways within the melanocyte, including MAPK (mitogen activated protein kinase) making it synergistic with the endothelin pathway of EDNRB (Arnheiter & Debbache, 2021; Kulikova, 2021). These genes, among others, play important or potentially important roles in melanogenesis and SCC but any difference in their promoter regions between SCC and non SCC galliforms is unknown.

5. Conclusion

The down-regulation of TYRP1 during the production of white feathers suggests that *L. muta,* like hamsters, have post-tyrosinase regulation of melanogenesis. It is possible that *L. muta* also have a peak in tyrosinase expression during moulting but that cannot be determined from these experiments. There are multiple differences in the promoter regions of the genes looked at in this thesis, most notably the common absence of TFBSs for PAX2 in *L. l. scoticus*. However the significance of any of these differences is unknown.

Based on the results from these experiments it is difficult to outright reject or accept the hypotheses. The difference in TYRP1 expression is only significant for one species, with only one individual sampled. Additionally qPCR does not give a direct answer to expression and a different method might give more accurate results on the up- or down-regulation of genes. The design of this study also misses out on the spring moult where ptarmigan start producing pigmented feathers again, a longer study would be able to compare gene expression at both the start and end of the winter morph and possibly the other pigmented morphs throughout the rest of the year. However, based on the data from these experiments and observations of ptarmigan feathers during dissections it seems plausible that melanogenesis itself is not regulated seasonally but instead the white morph is due to lack of melanin being transported to keratinocytes and built into feathers as they grow.

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