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DOES THE DRD4 “PERSONALITY GENE” DIFFER BETWEEN URBAN AND RURAL MOUNTAIN CHICKADEES?

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DOES THE DRD4 "PERSONALITY GENE" DIFFER BETWEEN URBAN AND RURAL MOUNTAIN CHICKADEES?

BY

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ABSTRACT

Urbanization is altering natural habitats worldwide, forcing species to adapt or acclimate to these new environments to survive. One species that successfully resides in both rural and urban habitats is the mountain chickadee (*Poecile gambeli*). In Kamloops, BC, populations of mountain chickadees thrive in both habitats, with no observed difference in reproductive success. Previous studies have investigated whether there are behavioural differences between urban and rural mountain chickadees, finding evidence that anti-predator and neophobic behaviours (elements of animal personality) differ between these populations. Personality is heritable, making it possible to study whether the behavioural differences observed are a result of adaption or acclimation. Polymorphisms in the dopamine receptor D4 gene (*Drd4*) have been associated with variation in personality traits in great tits (*Parus major*), particularity with traits such as boldness and novel-seeking behaviour. Specifically, a single nucleotide polymorphism (SNP) at the 830th nucleotide of the *Drd4* receptor gene (SNP830) has been associated with variation in personality, with bolder individuals expressing C/T or T/T genotypes at SNP830. To explore whether the *Drd4* gene might be linked to behavioural differences in mountain chickadees and whether the frequency of the *Drd4* polymorphism differs between urban and rural birds, we analyzed 58 individuals from Kamloops populations using sanger sequencing. We successfully validated methods for analyzing the *Drd4* gene and detecting *Drd4* polymorphisms. All individuals tested were CC homozygous, suggesting that the *Drd4* SNP830 gene has little influence on behavioural variation seen in this population. Future studies will investigate other genes that have been linked to personality variation, such as the serotonin transporter gene (SERT), that might influence these behaviours.

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INTRODUCTION

Urbanization is rapidly increasing worldwide and to persist species must adapt or acclimate to novel selective pressures present in the changing environment (Kozlovsky et al. 2017). However, the ability of species to respond and cope with urbanization is highly variable, even across closely related taxa. Species can generally be categorized based on their urban tolerance group: urban avoiders, urban adapters, or urban exploiters (Humphrey 2023). Urban avoiders are generally not found in urban environments because their survival needs (e.g., food, nest sites) cannot be met in human-dominated landscapes (Humphrey 2023). Often, these are forest-dependent species such as small insectivores, bears, wolves, and cougars. Species that can survive and adapt in urban environments are referred to as urban adapters. Urban adapters utilize native and non-native vegetation to persist and include species such as raccoons (*Procyon lotor*) and great tits (*Parus major*) (Humphrey 2023). Lastly, species that thrive in urban habitats and exploit urban resources, such as brown rats (*Rattus norvegicus*) and pigeons (*Columba livia*), are referred to as urban exploiters (Humphrey 2023).

For urban adapter species that live and breed in urban environments, variation in individual personality may play an important role in determining success in this environment (Oers 2004). Personality refers to consistent heritable variation in an individual's behaviour associated with neophobia (fear of novel stimuli), exploratory, and cautious behaviour (Riyahi et al. 2017). For example, the heritability of exploratory behaviour in great tits ranges from 0.22 to 0.41 (parent-offspring regressions) (Dingemanse 2002), indicating a strong genetic component underlying variation in exploratory behaviour. Variation in personality can be especially important in fluctuating environments (Fidler et al. 2007) because these traits can be closely linked to overall fitness (Oers et al. 2004). Fluctuating environments exert diverse selective pressures on

individuals, thus maintaining variation as different personalities are favoured in different conditions (Dingemanse 2004).

Though personality is complex and influenced by many different genes, variation in exploratory behaviour has been directly linked to variation in the dopamine receptor (*Drd4*) gene (Fidler et al. 2007; Timm et al. 2015; Korsten et al. 2010). The *Drd4* receptor is responsible for regulating the dopaminergic system, which plays an important role in motivation and reward processing (Muda et al. 2018). Consequently, the *Drd4* gene may influence behaviours and personality, such as risk-taking (Muda et al. 2018). This is important because in a rural environment, exhibiting cautious behaviour may be advantageous as it aids in avoiding risky situations. However, in urban settings, excessive caution may impede exploration of potential new habitats or food sources. Evidence indicates that the *Drd4* gene influences behaviour across taxa, including birds (Fidler et al. 2007; Timm et al. 2015; Korsten et al. 2010), humans (Kluger et al. 2002), dogs (*Canis familiaris*) (Ito et al. 2004) and horses (*Equus caballus*) (Momozawa et al. 2005).

In birds, great tits have received considerable attention with respect to the *Drd4* gene. Fidler et al. (2007) identified a single nucleotide polymorphism (SNP) at the 830th nucleotide of the *Drd4* receptor gene (SNP830) and determined that individuals can be CC homozygous, CT heterozygous or TT homozygous at *Drd4* SNP830. Fidler et al. (2007) found evidence that the SNP830 region within the *Drd4* gene is related to variation seen in bird's exploratory behaviour. Over four generations, Fidler et al. (2007) selected birds based on their slow and fast early exploratory behaviour (EEB). EEB was used as a measure of novelty seeking behaviour, where individuals were rated on a scale of 0-10 based on the time taken to visit four different trees. Additionally, the researchers assessed the birds' responses to a novel object placed on a perch,

rating the bird on a scale from 0 (did not touch the perch) to 5 (pecked the novel object). After four generations, the authors examined differences in allele frequencies between the fast and slow EEB groups, finding that birds with fast EEB scores displayed the T allele at a higher frequency when compared to birds with slow EEB. Specifically, homozygous CC individuals appeared at a frequency of 0.3 in the fast EEB group and 0.9 in slow EEB group. Heterozygous CT individuals were more common in the fast group, with a frequency of 0.7 compared to 0.1 in the slow EEB group. Homozygous TT individual appeared at a frequency of 0.1 in the fast EEB and were absent in the slow EEB group. Further supporting these findings, Timm et al. (2018) also provided evidence linking the SNP830 variant in the *Drd4* gene to exploratory behaviour in great tits. However, Timm et al. (2018) found that heterozygous CT individuals displayed greater exploratory behaviour, demonstrated by approaching the novel object more frequently when compared to CC homozygous and TT homozygous individuals.

Because urban environments are highly variable with frequent novel stimuli, Riyahi et al. (2017) hypothesized that bolder birds (and those with T alleles) should be more frequently found in urban environments. Indeed, when comparing urban and rural great tits, Riyahi et al. (2017) found that urban great tits displayed greater exploratory behaviours and boldness compared to rural birds. In addition, Riyahi et al. (2017) determined that the allele frequency of the *Drd4* SNP830 polymorphism differed significantly between the rural and urban populations (Riyahi et al. 2017). In contrast to Fidler et al. (2007), however, Riyahi et al. 2017 found that urban birds displayed the T allele at a lower frequency compared to the rural birds, suggesting that the role of *Drd4* SNP830 polymorphisms may be population- and context-specific.

Though links between personality and *Drd4* polymorphisms in European tit species have been demonstrated, whether *Drd4* polymorphisms are associated with personality in con-familial

North American chickadee species remains unresolved. A previous study examining boldness in Black-capped Chickadees (*Poecile atricapillus*), Carolina Chickadees (*P. carolinensis*), and their hybrids found that boldness was not associated with variation in the *Drd4* genotype (Heuermann 2020). Therefore, it remains unclear if the *Drd4* gene is associated with behaviour in North American chickadees. Mountain chickadees (*Poecile gambeli*) thrive in both urban and rural environments with no apparent difference in reproductive success between these two populations (Marini 2017). It is currently unclear if mountain chickadees are successful in urban habitats because of behavioural flexibility and an ability to quickly acclimate to novel environments or if urban populations are adapting to this new environment—a situation which would result in a change in allele frequencies between the urban and rural populations. In addition, differences in allele frequencies (e.g., in the frequency of C and T alleles) could also arise if bolder chickadees differentially settle in urban environments.

Smith et al. (2021) conducted a study comparing anti-predator behavioural responses to the presentation of a snake predator model atop nests in both urban and rural environments. Rural mountain chickadees exhibited a stronger anti-predator response than urban chickadees (Smith et al. 2021). However, because snakes are an uncommon nest predator in the region, the authors suggested that this differential response could be due to either low exposure to snakes in urban environments or the birds in both habitats simply perceiving the snake model as a novel object rather than a predator and the differential response resulting from differences in neophobia across habitats.

To test this idea, a follow up study by Heales et al. (2024) examined rural and urban mountain chickadees responses to both a simulated predator (squirrel model) and a novel stimulus (red plastic cup). As expected, mountain chickadees had a greater response to the predator model

than the novel stimulus. However, when presented with the novel stimulus, rural birds displayed more neophobic behaviour and spent less time in the nest. In contrast, the urban bird quickly re-entered the nest, and spent little time examining the novel stimulus. Urban birds presented with a predator model displayed a greater response compared to their response to the novel stimulus than the rural birds (Heales et al. 2024). These results suggest a difference in neophobic behaviour between urban and rural individuals, though it remains unclear if urban birds display less neophobic responses because of acclimation (frequent exposure to novel, man-made objects) or if urban birds are genetically distinct from rural birds, potentially with respect to variation in the *Drd4* SNP830 polymorphism.

Another potential gene that may influence behaviour is the serotonin transporter (SERT) gene. Grunst et al. (2021) found evidence that the SERT gene is associated with neophobia and exploratory behaviour. Furthermore, Timm et al. (2018) determined that SERT gene exon 3 and exon 8 were linked to risk taking behaviour in great tits.

If *Drd4* SNP830 polymorphisms are associated with personality differences between the rural and urban mountain chickadees populations, then allele frequencies of the *Drd4* gene may vary between these populations. In this study we examined the allele frequency of the *Drd4* gene SNP830 polymorphism in urban and rural mountain chickadee populations. Using 3 years of data from 30 chickadees from urban environments and 28 chickadees from rural environments, we tested whether *Drd4* SNP830 polymorphisms varied by habitat type. In addition, we explored the potential for future research on the SERT gene by trying to amplify the SERT gene. This study aims to provide insight into whether the mountain chickadee's ability to thrive in urban habitats is a result of acclimation or if we can detect differences in genotypes between these habitats.

MATERIALS AND METHODS

Birds Studied

Adult mountain chickadee feathers were collected from 46 nesting boxes in both urban and rural locations across Kamloops, BC, Canada during the 2014 to 2016 breeding seasons. To obtain DNA samples, adult birds were captured at the nest while feeding nestlings and two tail feathers were collected (see Bonderud et al. 2017 for more detail). Capture and sample collection was done under a TRU Animal Care permit and CWS Master Bander permit 10834. We examined 58 mountain chickadee DNA samples consisting of both male and female (see table 1). Rural mountain chickadee feathers were collected from Kenna Cartwright Park which is an 8 km^2 area consisting mainly of a Great Basin grassland habitat and some forests (Bonderud et al. 2017). Rural mountain chickadee nesting boxes were erected in the forest, which contains mature ponderosa pine (*Pinus ponderosa*), and Douglas fir (*Pseudotsuga mensiesii*) (Bonderud et al. 2017). Urban mountain chickadee feathers were collected from nesting boxes located throughout 37 km^2 of urban and suburban habitats in Kamloops (including Thompson Rivers University, urban parks, and backyards of participating residents). The urban habitat mainly consisted of immature Douglas fir trees and various species of native and non-native deciduous trees and shrubs (Bonderud et al. 2017).

Figure 1. Rural (left) and urban (right) nestling sites.

Table 1. Sex of urban and rural mountain chickadees examined.

	Male	**Female**	**Unknown**
Rural	9	15	4
Urban	8	18	4

PCR Optimization

To determine the primers and optimal PCR conditions for examining the *Drd4* gene polymorphisms in mountain chickadees, we extracted DNA from a tissue sample from a deceased nestling. Using a Qubit dsDNA HS Assay Kit and a Qubit fluorometer (Themo Fisher Scientific), we determined that our nestling sample contained 27.7 µg/mL of DNA. We used this DNA as a positive control to test different primers and find the optimal PCR condition for developing genetic assays for mountain chickadees.

We tested the SNP830 forward primer (5′ AAGCTGAGAGGCTGCATCTATGG 3′) and the reverse primer SNP830 (5′ATCCCACTGTTCATCCCACACTC 3′). The PCR reactions consisted of 5 µM of SNP830F, 5 µM of SNP830R, 12.5 µL of 2x GoTaq G2 Green Master Mix (Fisher Scientific), 7.5 µL of sterile PCR water, and 2.5 µL of genomic DNA which created 25 µL reactions. We amplified the genomic DNA using the following reaction conditions: initial

denaturation at 94°C for 4 min, 27 cycles of 94°C for 30 s, 61°C for 30 s, and 72°C for 30 s, and a final extension of 72.0°C for 1 min. The SNP830 primers and the reaction conditions were chosen based on a previous study examining the DRD4 gene in great tits (Fidler et al. 2007).

Furthermore, we tested a PCR assay targeting the serotonin transporter (SERT) gene using SERT F (5′CATCTTCTCCTTTGCTACAGCC3′) and the SERT R (5′ACAGAGCCTCAGAAGTTAGTTGA3′) primers. We also ran PCR reactions using *Drd4* exon 1F (5′ CGGAGTAGACGTAGAGGGGCAGGAC 3′) and DRD4 exon 1R (5′ GCTCCTCCCGCGGCTCGGCGGGCA 3′) primers. Both PCR reactions were run under the following reaction conditions: initial denaturation at 94°C for 5 min, 94°C for 30 s, 35 cycles of 62°C for 30 s, 72°C for 1 min, and a final extension of 72.0°C for 5 min.

We further tested the SNP830F and SNP830R primers under different reaction conditions: initial denaturation at 94°C for 4 min, one cycle of 94°C for 30 s, 35 cycles of 55°C for 30 s, and 72°C for 30 s, and a final extension of 72.0°C for 1 min.

Finally, we tested several different annealing temperatures for the SNP830 primers to determine if higher temperatures would successfully amplify the *Drd4* target, to evaluate whether we could optimize PCR conditions to be compatible with downstream sequencing steps (BigDye® Direct cycle Sequencing Kit protocols). The reaction conditions were 95°C for 5 min, 95°C for 30s o, 35 cycles of annealing at 50°C, 53°C, 55°C, 58°C, 61°C, or 63°C /30 s, 72°C for 1 min, and final extension of 72.0°C for 5 min.

The products of all PCR reactions were run on a 2% agarose gel (consisting of 50 mL of TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0), 1g agarose, 2 µL of Red Safe nucleic acid stain, and 5 µL of the PCR product) for 40 min at 80V.

Sample Processing and Sequencing

After determining the range of annealing temperatures that successfully amplify the mountain chickadee DNA, we were able to apply a Sanger sequencing assay targeting *Drd4* SNP830 to examine 58 mountain chickadee DNA samples. We extracted genomic DNA from feather samples using the standard protocol for the QIAamp DNA Mini Kit. We also used previously extracted DNA from Bonderud et al (2017), who extracted feather DNA using QIAamp DNA Micro Kit.

We examined the polymorphism located at SNP830 *Drd4* gene in mountain chickadees using BigDye® Direct cycle Sequencing Kit. *Drd4* primers were modified to contain an additional M13 priming site sequence to make the assay compatible with the BigDye® Direct cycle Sequencing Kit; therefore, the DNA was amplified from genomic DNA using M13_SNP830 forward primer (0.8 µM): 5′ TGTAAAACGACGGCCAGTAAGCTGAGAGGCTGCATCTATGG 3′ and the M13_SNP830 reverse primer (0.8 µM):
5′ CAGGAAACAGCTATGACCATCCCACTGTTCATCCCACACTC 3′. The PCR reactions consisted of 0.8 µM of M13_SNP830F, 0.8 µM of m13_SNP830R, 5.0 µL of BigDye® Direct PCR Master Mix, 2.5 µL of sterile PCR water, and 1.0 µL of genomic DNA for a total volume of 10.0µL and were run under the reaction conditions: 95°C/10 min, one cycle; 96°C/3 s, 62°C/15 s, 68°C/30 s, 35 cycles; 72.0°C/2 min, hold; 4.0°C/∞, hold. Cycle sequencing was performed on the amplified DNA following BigDye® Direct cycle Sequencing Kit protocol. Big Dye® Direct M13 Forward Primer (1.0µL) and Big Dye® Direct Sequencing Master Mix (2.0µL) was added to the 10 µL amplified DNA and the following thermocycler conditions: 37°C/15 min, hold; 80.0°C/2 min, hold; 96.0°C/1 min, hold; 96.0°C/10 sec, 50°C/5 sec, 60°C/75 sec, 25 cycles. The sequencing reactions were then purified using ethanol/EDTA precipitation. The plate containing the

sequencing reactions was centrifuged in a swinging bucket centrifuge for 10 seconds at 1,000 x *g*. The adhesive Film from the plate was removed, and 10µL of the sequencing reaction was transferred into new plate. To each reaction, 2.5µL of 125mM EDTA solution and 30µL of absolute ethanol was added for a total volume of 42.5µL/well. The plate was resealed, vortexed for 10 seconds, and then centrifuged for 10 seconds at 1,000 x *g*. It was then incubated at room temperature for 15 minutes and centrifuged at 1,870 x g (4°C) for 45 minutes. The adhesive film was removed, and absorbent paper was placed into the centrifuge. The plate was carefully inverted onto the absorbent paper and centrifuged at 185 x g for 1 minute. The plate was then left to dry at room temperature for 10 minutes while protected from light. The samples were then analyzed in using a SeqStudio Genetic Analyzer (ThermoFisher Scientific).

Analyses

We analyzed the sequencing electropherograms in Sequencing Analysis Software v7 (ThermoFisher Scientific). We confirmed the quality of the electropherograms visually in the region of interest and located the polymorphism using the leading sequence, which was determined using NCBI gene sequence of the great tit (*Parus major*): we recorded whether the individual was CC homozygous, CT heterozygous or TT homozygous. Using the leading sequence, ′GCCCCTATGCCCGT′, we were able to locate the polymorphism which occurred at roughly 117 base pair (bp). Directly following the leading sequence, the sequence ′GCCGGC′ or ′GCTGGC′ appeared. If the individual was CC homozygous the sequence following the leading sequence was ′GCCGGC′. If the individual was TT homozygous the sequence following the leading sequence was GCTGGC. If the individual was heterozygous, it would have both a C and T present.

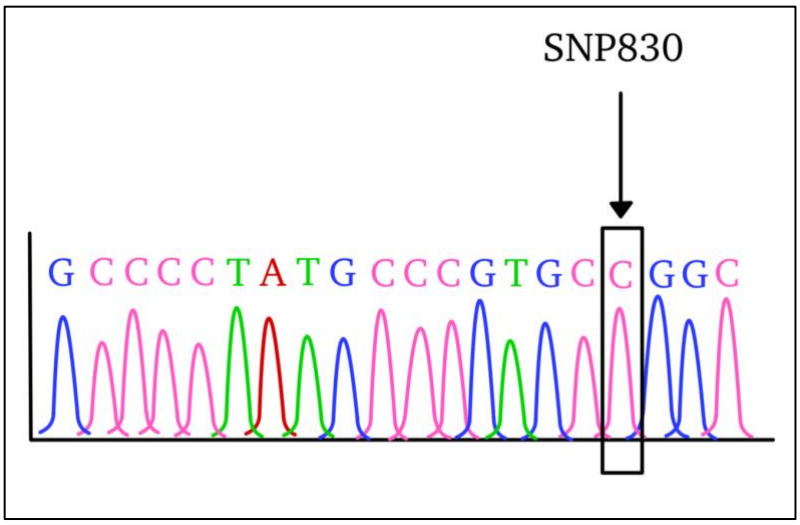

Figure 2. Sanger sequence electropherogram showing individual C/C homozygous.

RESULTS

The SNP830 primers successfully amplified the DNA which allowed us to determine the optimal PCR temperatures. However, the *Drd4* exon 1 primers (figure 3) and the SERT primers did not amplify the targeted DNA.

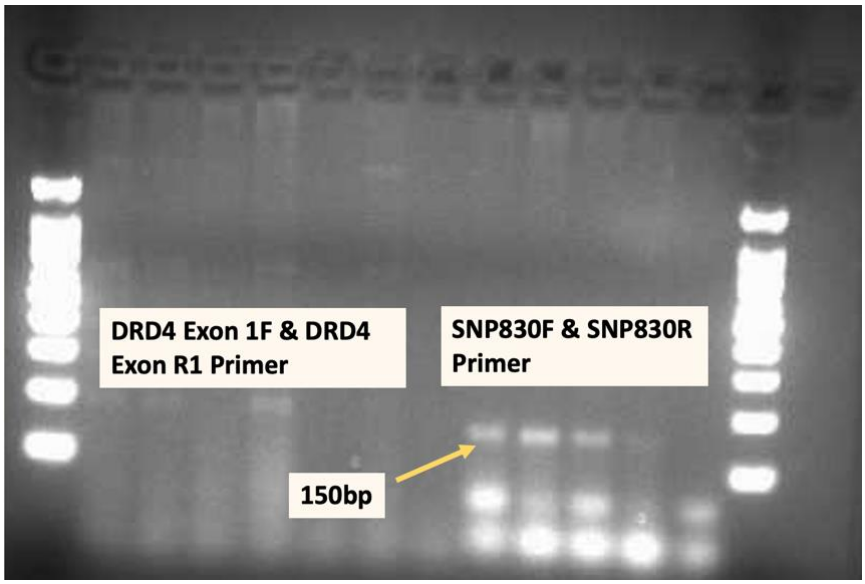

Figure 3. Agarose gel displaying the DNA amplified by the *Drd4* Exon 1 primers (with annealing temperature of 62°C) and the SNP830 primers (with annealing temperature of 62°C).

The SNP830 primer amplified DNA at annealing temperatures 50°C, 53°C, 55°C, 58°C, 61°C (see figure 4).

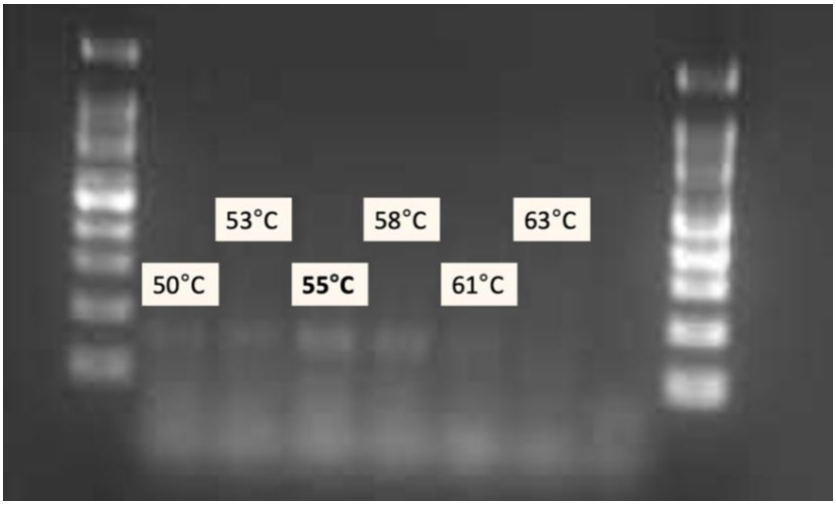

Figure 4. Resulting gel showing the different annealing temperature using our positive control DNA, which revealed that optimal annealing temperate is 55°C.

All individuals tested were CC homozygous for *Drd4* polymorphism (Table 1). Therefore, there was no difference in allele frequence between the two populations examined.

Table 2. Frequency of each *Drd4* polymorphisms in both rural and urban mountain chickadees.

DRD4 Polymorphism	**C/C**	**C/T**	**T/T**
Rural	28	0	0
Urban	30	0	0

DISCUSSION

This study examined variation in the *Drd4* gene, which is associated with personality variation, to explore whether differences in behaviour observed between urban and rural Kamloops mountain chickadees are a result of acclimation or genetic differences between populations. In order to analyze polymorphisms in the *Drd4* gene, we evaluated several primer pairs previously used for genotyping great tits to examine the same genes in mountain chickadees. Though several primer pairs did not amplify DNA from our mountain chickadee samples, we successfully

validated a method for analyzing the *Drd4* SNP830 sequence in mountain chickadees—a region that has shown strong associations with personality in European great tits. Using this method, we successfully examined *Drd4* SNP830 alleles in 58 mountain chickadees from urban and rural populations; however, we did not detect any genetic variation in the *Drd4* SNP830 in the sampled population: all individuals tested in both populations were homozygous for the CC allele.

Given the lack of variation we detected in our population, it is unlikely that variation in the *Drd4* SNP830 polymorphism has an important influence on behaviour in our population of mountain chickadees. Our results suggest that differences in behaviour responses in the rural and urban population observed by Smith et al. (2018) and Heales et al. (2024) were not influenced by the *Drd4* SNP830 gene. Even though we did not find evidence that suggests the *Drd4* gene is responsible for the behavioural differences seen in the Kamloops mountain chickadee populations, behavioural traits are likely influenced by a combination of many genes and environmental factors (Grunst et al. 2021). As such, further research should be conducted that investigates other potential genes that are associated with personality variation such as the serotonin transporter gene (SERT).

The SERT gene has been associated with personality variation in the great tits, influencing neophobia, and exploratory behaviour (Grunst et al. 2021). Specifically, Riyahi et al. (2015) investigated the SERT SNP234 polymorphism and found a significant correlation with novelty seeking behaviour in great tits. Their study revealed that individuals homozygous for the T allele at SERT SNP234 were quicker to approach novel objects compared to those who were TA heterozygous. Furthermore, Riyahi et al. (2015) observed a higher frequency of TT homozygous individuals in urban environments.

Initial attempts to amplify the *Drd4* gene using the *Drd4* exon 1 primers and the SERT gene using the SERT primers were unsuccessful. Further testing of these primers at different

annealing temperature is needed to confirm that these primers fail to amplify mountain chickadee DNA. Because these primers were designed for great tits, it is possible that these primer sets failed because they were not specific enough for the DNA template or were unable to amplify the target DNA (Biocompare 2013). In future studies, designing primers specific to mountain chickadee DNA could potentially improve amplification success (Biocompare 2013).

Though many genes are associated with variation in personality and behaviour, our results are consistent with behavioural plasticity, rather than genetic differentiation, in driving differences in behaviour between urban and rural mountain chickadees, as we found no genetic difference of the *Drd4* gene between the rural and urban populations. Previous research has suggested that the mountain chickadees ability to reside in harsh, higher elevation climates is associated with enhanced problem-solving abilities compared to those found in at low elevation, which may be attributed to behaviour plasticly (Kozlovsky et al. 2015). Therefore, it is possible that mountain chickadees are able to survive and utilize urban habitats due to their behavioural plasticity.

CONCLUSION

In this study, we successfully amplified the *Drd4* gene in mountain chickadees. Surprisingly, however, we found no evidence of a polymorphism at this gene, with all individuals being C/C homozygous at SNP830. Future studies should investigate other potential genes that could influence personality and behaviour such as the SERT gene. If it is determined that SERT polymorphisms are present in the Kamloops mountain chickadee population, then we can use data that has been previously collected on mountain chickadees behavioural response to predator models and novel stimuli to determine if exploratory, boldness and novelty seeking behaviour is correlated with the polymorphism of the SERT gene in these populations. However, because we found no difference in allele frequency of the *Drd4* gene, our data our consistent with the idea that

variation in behaviour between urban and rural chickadees may arise through behavioural flexibility and acclimation to novel, anthropogenic environments.

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