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Effects of two genes (DRD4 and SERT)
on great tit (*Parus major*) behaviour
and reproductive traits

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Effects of two genes (DRD4 and SERT) on great tit (*Parus major*) behaviour and reproductive traits

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I   **Timm, K**., Tilgar, V., Saag, P. (2015). DRD4 gene polymorphism in great tits: gender-specific association with behavioural variation in the wild. *Behavioral ecology and sociobiology*, *69* (5), 729–735.

II **Timm, K**., van Oers, K., Tilgar, V. (2018). SERT gene polymorphisms are associated with risk-taking behaviour and breeding parameters in wild great tits. *Journal of experimental biology*, *221*, 1–7.10.1242/jeb.171595.

III **Timm, K**., Mägi, M., Telve, K., Tilgar, V. (2018). The behavioural response of Great Tits to novel environment and handling is affected by the DRD4 gene. *Ibis*, 00–00.10.1111/ibi.12604.

IV **Timm, K.**, Tilgar, V., Koosa, K. The serotonin transporter gene could play a role in anti-predator behaviour in a forest passerine. *Manuscript*

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Author's contribution to the papers ('*' denotes a contribution equal to 0.33, '**' denotes a contribution of 0.66, '***' denotes the contribution of 1.00).

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Original idea	***	***	**	***
Study design	***	***	*	**
Data collection	***	***	*	**
Data analysis	**	**	*	**
Manuscript preparation	***	***	***	***

1. INTRODUCTION

Most of the theoretical and empirical research in behavioural genetics has been conducted on humans. However, understanding the animal behaviour, personalities and genetics behind these traits helps us understand the questions that are difficult to answer relying only on studies that focus on humans. Animal models provide the advantage of longitudinal research where personality can be more easily recorded and/or manipulated (Gosling et al., 2001). Personality is considered in this study as a persistent individual variation in behaviour that stays stable in different situations and environments over time (see also Carere and Maestripieri, 2013). A better understanding of animal personalities could therefore give valuable insight into the evolution of human behaviour and behavioural disorders, help to see the differences in vulnerability to stress in various environments and shed light on the causes of consistent behavioural differences between individuals. Most of the behavioural traits are at least partly heriditary, affect life-history traits and are subject to natural selection. Different behaviours do not evolve in isolation, but often in relation to each other which could generate trade-offs limiting behavioural plasticity and affect fitness (Sih et al., 2004). Behavioural traits are also a part of a complex system that involves the nervous system, neurotransmitters and genes. So far, research on behavioural genetics has mainly concentrated on the effect of **dopamine**, a neurotransmitter that is linked with novelty-seeking and **serotonin**, as well as harm avoidance and anxiety (Comings et al., 2000; Dalley and Roiser, 2012; Savitz and Ramesar, 2004). In the following sections, the effects of these neurotransmitters on behaviour is described more precisely.

Dopamine is involved in the brain's reward system, motor activity, cognition and also affects the release of other hormones and neurotransmitters (Neve et al., 2004). Alterations in dopamine transmission are related to Parkinson's disease, addiction and schizophrenia in humans (Howes and Kapur, 2009; Lawford et al., 2006). There are at least five subtypes of dopmaine receptors in humans (D1–D5) (Reif and Lesch, 2003). These receptors vary by their affinty to dopamine and regulate dopamine signaling in the brain (Neve et al., 2004). The dopamine receptor D1 shapes locomotor activity in rats (Rubinstein et al., 1997; Dulawa et al., 1999) while the D2 and D3 receptors are also partly related with prolactin and estrogen pathways and shape parental care (Sealfon and Olanow, 2000). The dopamine receptor D4 is related to many behavioural disorders in humans (Munafò et al., 2008) and motivational or exploratory behaviours in other animals (mainly in birds) (Fidler et al., 2007; Korsten et al., 2010). The D5 receptor is not essential for many dopamine-mediated behaviours, but might play a role in the activation of dopaminergic pathways and has been shown to play a role in male-female sexual behaviour (Kudwa et al., 2005).

The serotonergic system is involved in brain development, synaptic plasticity and also functions as a behavioural inhibitor in vertebrates (Reif and Lesch, 2003). It has been shown that the serotonin system affects a wide range of behaviours from anxiety to aggression, cognition, endocrine function, sex and

sleep (Hariri and Holmes, 2006). In addition, serotonin modulates the immune response as several serotonergic receptors have been studied in lymphocytes, monocytes and macrophages which suggests serotonin plays a role in immune cell function (Khan and Ghia, 2010). A key regulator in this system is the serotonin transporter that is responsible for the reuptake of serotonin from synaptic clefts and is widely used to study anxiety-related behaviours (Savitz and Ramesar, 2004). These transporters (5-HTT or SERT) are located in different parts of the nervous system including the central, peripheral and enteric systems. In brain neurons, serotonin amounts to approximately 10% of the total amount of serotonin in the body. Most of the serotonin is actually present in the enterochromaffin cells in the gastrointestinal tract which contain about 90% of the serotonin in the body (Kim and Camilleri, 2000). Even though blood platelets do not synthesize serotonin, they possess serotonin transporters and carry a high concentration of serotonin from the intestines (Watts et al., 2012). For instance, in humans and rats, the estimated concentration of serotonin in whole blood could be up to 250 ng/ml (Watts et al., 2012).

As serotonin and dopamine affect several behavioural traits, there is great interest in the mechanisms that play a role in the regulation of these neurotransmitters. Two main candidate genes have been associated with anxiety and novelty-seeking and are related to dopamine and/or serotonin neurotransmission. These are the serotonin transporter gene (SERT) and the dopamine receptor gene D4 (DRD4). Early studies (starting from the papers of Ebstein et al., 1996 and Lesch et al., 1996) have been followed by a flood of research on behavioural genetics concentrating on these two candidate genes. The DRD4 gene in humans is situated in chromosome 11 (Wang et al., 2004) and is expressed in the brain (e.g. in the hippocampus, hyothalamus, *substantia nigra* and prefrontal cortex) (Paterson et al., 1999). This gene is polymorphic in humans: variable tandem repeats (VNTRs) are studied in exon 3, the presence/ absence of the 7-repeat allele is related to behavioural phenotypes and a single nucleotide polymorphism (SNP) in the promoter region is associated with variation in gene expression (Munafò et al., 2008; Ronai et al., 2001). This VNTR polymorphism in exon 3 is also present in dogs, horses and non-human primates, but not in rats (Hejjas et al., 2007; Livak et al., 1995; Momozawa et al., 2005). In birds, a single nucleotide polymorphism (SNP) of the DRD4 gene is mainly studied in great tits (*Parus major*) (Fidler et al., 2007).

Variations in the DRD4 could be associated with novelty-seeking and impulsivity both in humans (Munafò et al., 2008) and other animals. Dopamine receptor activity in humans is associated with behavioural disorders such as addictive behaviour, Parkinson's disease and schizophrenia (Oak et al., 2000). The DRD4 gene affects impulsiveness in dogs (Hejjas et al., 2007), novelty-seeking in primates (Bailey et al., 2007) and hyperactive behaviour in mice (Avale et al., 2004). In birds, the DRD4 gene is one of the most studied when it comes to behavioural research, which can be traced back to the paper by Fidler and colleagues (2007) whose results indicated that this gene could play a role in exploratory behaviour. Later studies also infer that the DRD4 gene affects risk-taking and exploratory behaviours in different species and populations

(Garamszegi et al., 2014; Mueller et al., 2014; Holtmann et al., 2016). Some studies have also observed differences in the allelic variation of the DRD4 gene under different environmental conditions. For instance, there exists a significant difference in the DRD4 gene between urban and rural bird populations (Holtmann et al., 2016; Riyahi et al., 2017). However, not all studies have found a significant relationship between DRD4 genotypes and behavioural phenotypes (Edwards et al., 2015; Korsten et al., 2010; Rollins et al., 2015).

Genetic variation in the serotonin transporter gene (5-HTT or SERT) regulates the reuptake of serotonin by clearing it from extracellular space (Canli and Lesch, 2007). Both in primates and humans a polymorphism in the SERT gene forms two different alleles: a short (s) and a long (l) variant (Lesch et al., 1996). The presence of the S-allele is related to lower mRNA transcription and an increase in an aggressive behavioural response (Lesch et al., 1996; Davidge et al., 2004; Retz et al., 2004) but also negative emotions and depression (Caspi et al., 2003; Szily et al., 2008). In humans, the SERT gene is situated in chromosome 17 which is composed of 14 exonic regions (Murphy et al., 2004). The 5-HTTLPR (serotonin-transporter-linked polymorphic region) is only present in humans and higher non-human primates (Lesch et al., 1997). A relative loss in SERT gene functioning leads to changes in stress responsiveness, vulnerablity to environmental changes and an increase in anxiety levels both in humans and non-human mammals (Hariri and Holmes, 2006). Moreover, in humans SERT gene polymorphims affect several psychiatric states ranging in degree from depression to suicidal behaviour (Murphy et al., 2008). In mammals it has been shown that the SERT gene affects anxiety in mice (Holmes et al., 2003) and also the temperament of rhesus monkeys (Champoux et al. 2002). The SERT gene in birds has been less widely studied but the results infer that several polymorphisms in the gene are the main influence on behavioural traits such as impulsiveness, activity, neophobia and flight initiation distance (FID) (Abe et al., 2013; Riyahi et al., 2015; Holtmann et al., 2016). Furthermore, a deficiency in the serotonergic system of hens (*Gallus gallus*) is related to aggressive behaviour (Flisikowski et al., 2009).

As the results from previous studies conducted with these candidate genes vary across populations and are sometimes contradictory, it is important to replicate the behavioural experiments in different populations and environments. The results from animal populations studied in captivity are often not replicated in a natural environment. Replication studies are important in order to understand the extent of variation in these behavioural responses and effects of the candidate genes underlying these variations. Understanding the effects of changing and novel environments on behavioural responses and reproductive traits provides an opportunity to understand individual adaptation in various situations and the role of genetic polymorphisms behind their behavioural variation and breeding success. However, the effects of dopamine and serotonin on breeding in birds has not been studied even though both systems could affect relationships between behavioural response and reproduction. The dopamine system is involved in reward-seeking, motivation and is implicated in sexual and pair-bonding behaviours in humans (Eisenberg et al., 2007a; Melis and

Argiolas, 1995; Young and Wang, 2004). In addition, polymorphisms in the DRD4 gene in humans are associated with sexual desire and arousal (Zion et al., 2006) and could be related to the season of birth in humans (Eisenberg et al., 2007b). Serotonin is involved in a wide range of reproductive functions (Berger et al., 2009). For instance, this neurotransmitter regulates sexual behaviour functions in humans (Hull et al., 2004; Zion et al., 2006) and is involved in gonadotropin release in fish (Prasad et al., 2015). Moreover, the serotonergic system is important in parenting through its influence on mood and the release of oxytocin which modulates affiliative responses to partners and offspring (Bakermans-Kranenburg and van Ijzendoorn, 2008).

This thesis contributes to the research on behavioural genetics with original studies conducted in a wild population of great tits. **Given that dopamine and serotonin affect behavioural responses and are regulated by genetic mechanisms, the main focus of this thesis is to study the effect of two genes (DRD4 and SERT) on behavioural traits in a wild population during the breeding season (I-IV).** The behavioural responses were mainly studied in the field and concentrated on individual reactions to a novel object or a stressor. The behavioural traits studied in the current thesis are more or less related to risk-taking behaviour and are often described on a bold–shy behavioural axis (Réale et al., 2007). Boldness is broadly defined as the willingness to adapt to a degree of risk in return for foraging or reproductive benefits (Wilson et al., 1994). A bolder individual is more risk-prone, often inspects predators more closely and is more eager to explore novel objects. Shy individuals demonstrate more risk-averse behaviour and inspect potential danger and/or novelty from a distance (Wilson et al., 1994). In the past, exploratory behaviour (which includes boldness) in birds was mainly studied as a complex trait (early exploratory behaviour) including several responses to novelty (see e.g. Van Oers et al. 2005; Drent et al. 2003). In the current study, behavioural traits (different responses to novelty or danger) were studied in separate experiments as that enabled us to evaluate the effect of candidate genes on behaviour more precisely and provided an opportunity to study individual variation in behavioural traits more in depth. The behavioural experiments in this thesis were measured using a scale of fear responses to various novel objects ranging in threat-level from the camera set-up to the presence of a potential predator. The tested hypotheses were as follows:

1) Hypothesis I: the behavioural response to novelty in birds is affected by the DRD4 gene both in the wild and the aviary (I, III).
Earlier findings from research on the DRD4 gene in birds have shown contradictory results (Korsten et al., 2010; Edwards et al., 2015). There are several reasons why replicating results is not always successful. Genetic diversity could be reduced by the bottleneck, traits are often complex, and other genetic and epigenetic factors can play a role. First, in order to test the generisability of former research conducted in the aviary (see e.g. Fidler et al. 2007; Korsten et al. 2010) where the effect of the DRD4 gene on behaviour is present, a field experiment with an unselected population was conducted during the breeding season (**I**). Secondly, as the experiments in the natural habitat and

during the breeding season could have a strong impact on individual behaviour, similar tests were also replicated in the aviary and before the beginning of egg-laying to control for the effects of the breeding season and the natural habitat (**III**). To test the hypothesis, individual behaviour in the presence of a novel object (camera, small pink box, test room, potential intruder) was studied.

2) Hypothesis II: individual reactions to novel or dangerous situations is related to the SERT gene in the wild (II, IV).
The serotonin transporter gene (SERT) affects stress responsiveness, anxiety and individual adaptation to environmental changes (Hariri and Holmes 2003). As several polymorphisms in the SERT gene play a role in behavioural reactions when facing novelty or stressful situations, it was assumed that individual responses to behavioural experiments is also related to this gene. The SERT gene has not been widely studied in birds and testing the generalisability of previous results is essential. As shown before, a reduction in the SERT gene's functioning strongly affects stress responsiveness and anxiety (Hariri and Holmes 2006). Therefore, stressful situations were included in the behavioural experiments (facing predator during incubation and entering the trap) in addition to experiments with novel objects (camera set-up and pink box).

3) Hypothesis III: differences in DRD4 and SERT genes are related to reproductive traits (II, IV).
Most of the research was conducted during the breeding season of birds in order to study the relationship between behavioural responses, genotypic variations and reproductive traits. Both dopamine and serotonin systems are good candi-dates for studying relationships with reproductive traits and behaviour. As beha-vioural traits shape breeding success through decisions that include the start of reproduction, picking a suitable partner and nesting site, it was predicted that the genes influencing behaviour could also be either indirectly or directly related to reproductive traits. Understanding the relationships between genes, behaviour and reproduction enables researchers to study potential selection mechanisms in different populations. It could also suggest why certain geno-types are underrepresented or even missing in some populations.

2. METHODS

2.1 Model species

In the current study the great tit (*Parus major*) was used as the model species. Great tit behavioural ecology is well known. Its behaviour matches well with the patterns described in other bird species and the individuals can be easily bred in captivity and is often selected for various behavioural lines (Groothuis and Carere, 2005; Verbeek, 1994). This allows manipulating rearing conditions both in the aviary and in the wild (as the birds breed in nest boxes) and gives the opportunity to study genetic differences, developmental plasticity and the functional significance of several aspects of bird behaviour. One advantage of using the great tit model is the possiblity to study reproductive traits and their correlations with behavioral reactions under natural conditions. This approach is not common but is essential to understanding the selection pressures leading to maintenance of different behavioral profiles in the same population (Groothuis and Carere, 2005).

The great tit is a small passerine that belongs to the family *Paridae*. Great tits are monogamous territorial birds, distributed all over the Palearctic region (Perrins, 1965), that nest in treeholes and artificial nest boxes. In Estonia (the study region) the great tit population during the breeding season is estimated to be up to 400,000 breeding pairs and during winter around 1 million birds overall (Elts et al., 2009). Great tits are the largest representatives of the *Paridae* family in Estonia. Their body length is around 15 cm and their body mass around 15–20 grams. The genders can be easily distinguished visually: males have bright yellow belly with a bold black stripe in the middle whereas females are paler and the middle stripe is narrower or interrupted.

Birds start their nest-building in the middle of April. The nest is built in a tree cavity or nest box and contains moss at the bottom and is lined with animal fur. The brood typically consists of around 6–12 pale pink eggs with rusty spots. Sometimes second broods occur in June–July. In the current thesis, only data from the first brood has been used. According to our data (**I**, **II**, **IV**) females started egg-laying around April 29–30. The mean clutch size was 11 eggs (the smallest clutch was 6, and the biggest was 14). Eggs were incubated by females for approximately 12 days and the nestlings were fed by both parents until the age of 17–21 days (**I**, **II**, **IV**). The greatest danger to nestlings in this study area are the spotted woodpeckers (*Dendrocopos major*), adults of which mainly consume a sparrowhawk (*Accipiter nisus*) diet.

Inhabited nest boxes were checked regularly in order to confirm the start of egg-laying, clutch size, incubation period, hatching date and the number of hatchlings and fledglings. Predated or deserted nests were not included in the final analysis. Adults were captured from the nest boxes when the nestlings were at least 8 days old (2012 and 2014) in order to avoid the nestlings being negatively influenced by the feeding interruption. In 2013 adults were caught before the start of egg-laying in the late evening (captured between 21:00 and

00:15) for the aviary experiment and released the day after. Adults were weighed with a Pesola spring balance (±0.1 g), while the tarsi and wing length were measured with sliding callipers (±0.1 mm). All the nestlings from studied nest boxes were ringed and weighed. Their tarsi and wings were measured at the age of 15 days (right before fledging and leaving the nest box). Blood samples from adults (2012–2014) and a pilot sample of nestlings (2016; *unpublished data*) were taken after 10–15 minutes of capture from the brachial vein into a heparinized capillary tube. Capillary tubes were centrifuged on the same day (10 minutes, 10,621 × G) in order to separate blood cells and plasma for hormone (corticosterone) analysis. All the samples were first preserved at – 20 °C, later at –70–80 °C.

2.2 Study site

The behavioural experiments were conducted in southwest Estonia (58° 7′ N, 25° 5′ E) at the Kilingi-Nõmme study site (Figure 1) during the springs of 2012, 2013 and 2014. The area (about 50 km^2) consists of deciduous (*Betula sp, Alnus sp* and *Salix* sp) and coniferous (*Pinus sylvestris* and *Picea abies*) forest groves. The great tits in the study population breed in nest boxes mounted on tree trunks at a height of 1.5–1.8 m.

2.3 Behavioural studies

Behavioural traits that are expressed consistently over time and across situations in different environments are termed personality or temperament (Groothuis and Carere, 2005; Réale et al., 2007). In the current thesis, several behavioural traits are studied, namely boldness, risk-taking behaviour, exploration and activity. In the current thesis the aforementioned behaviours are viewed together as individual response is often a combination of all these behaviours. These are the traits that are often correlated with each other and differentiating these behaviours could be complicated (Sih et al., 2004). For instance, bold indi-viduals are usually also more active, take more risks and learn faster, whereas shy individuals are not eager to take risks and are less active (Sneddon, 2003). The overview of studied behavioural traits measured over the years is presented in Table 1.
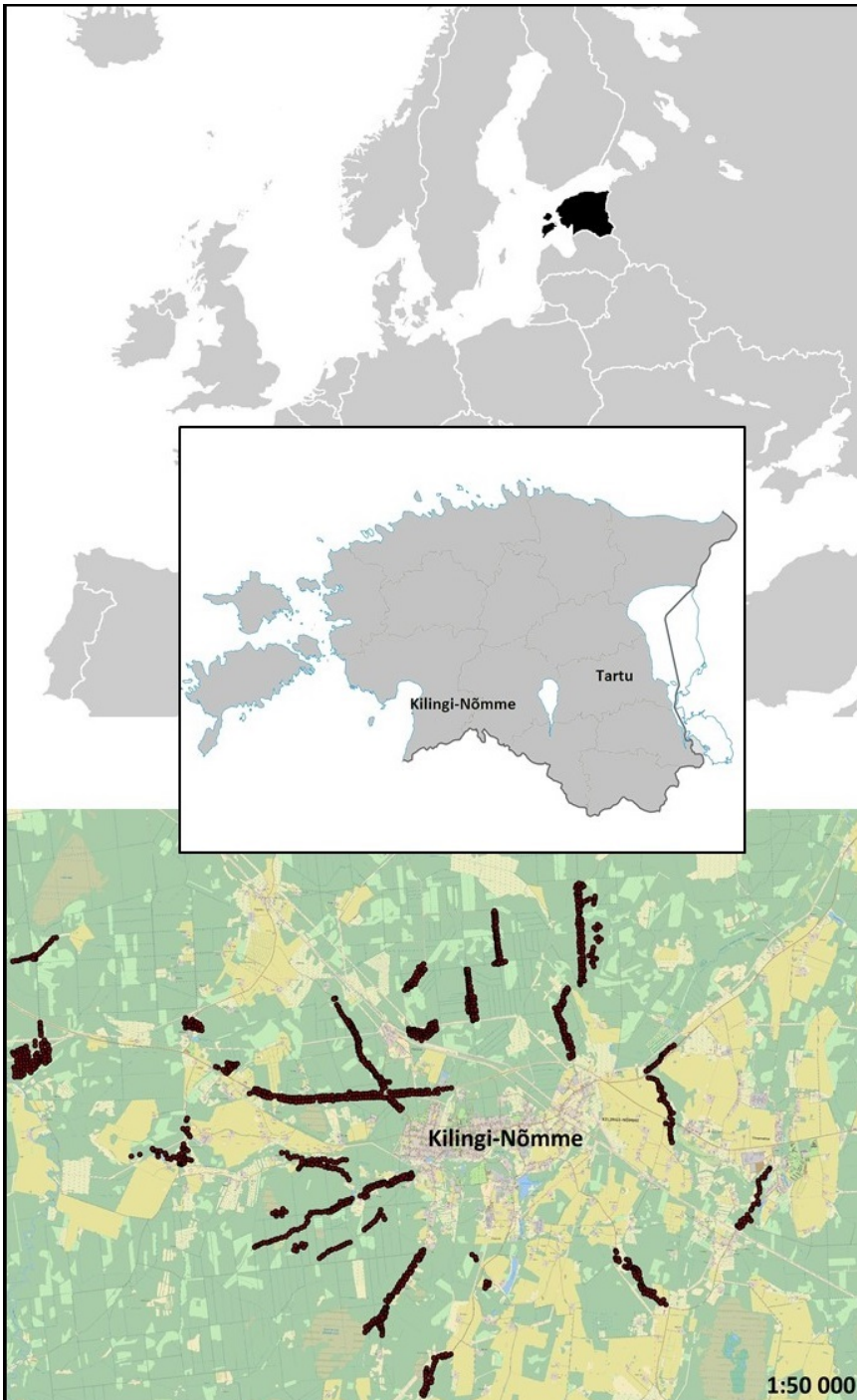
Figure 1: Study area of Estonian (Kilingi-Nõmme) rural great tit population. Nest boxes are marked with a dot. Green areas are forests, yellow patches refer to arable land.

Table 1: Overview of behavioural traits measured in different years.

Behavioural trait	Mean	Standard deviation	Year	Gene studied	Paper/ manuscript
Exploration* (females)	8.35 seconds	7.13	2012	DRD4	Paper I
Exploration* (males)	8.05 seconds	7.19	2012	DRD4	Paper I
Exploration** (both sexes combined)	1.76 Visits to novel object	2.87	2013	DRD4	Paper III
Exploration*** (females)	1.13 (minutes)	2.14	2012, 2014	SERT	Paper II
Exploration*** (males)	1.1 (minutes)	2.28	2012, 2014	SERT	Paper II
Anti-predator behaviour (females)	59.4% of individuals hissed actively		2012	DRD4, SERT	Paper IV
Delay to enter trap (females)	4.12 (minutes)	6.1	2014	SERT	Paper II
Delay to enter trap (males)	4.18 (minutes)	6.89	2014	SERT	Paper II
Distress calls	77.6% of individuals made distress calls		2013	DRD4	Paper III

* In 2012 exploration was measured by observing the feeding delay (latency to enter the nest box) at the presence of a novel object. The delay measurement started from the moment that a bird made visual contact until it entered the nest box (**I**).
** In 2013 exploration in the aviary before the start of the egg-laying period was studied (**III**). Visits to the novel object and number of trees visited were observed.
*** In 2014 exploration was studied by observing the feeding delay (latency to enter the nest box) at the presence of a novel object. The delay measurement started from the moment that the novel object was placed (**II**).

2.3.1 Anti-predatory behaviour

At the beginning of the breeding season during incubation, female reaction to a potential intruder was observed (2012, **IV**). A spotted woodpecker dummy was placed on the nest box as a potential threat to the eggs and female. The reactions to the threat were twofold: about half of the females stayed calm in the nest without giving any reaction. The other half reacted to the danger with anxious hissing and wing-flapping (**IV**). Both reactions stayed stable over time (Koosa

and Tilgar, 2016) and therefore it was assumed that this behaviour could have a genetic background. In this experiment no males were tested as the experimental design required the presence of the bird in the nest box. Still, the males do hiss (*pers. obs*) and could be studied in aviary conditions in the future.

2.3.2 Novel object experiments

In all the experiments, the birds' entrance into the nest box was videotaped. The main experiment was conducted during the feeding period of the nestlings (aged 7–10 days). In the control phase, the normal feeding rate and reaction to the camera set-up in great tits was measured. In the second part of the experiment, a novel object was placed on the nest box roof and the changes in the latency to the first nest visitation was measured (in 2012 and 2014). In 2012, the latency to enter the nest box (i) was measured from the moment that a bird entered the sight of the camera and (ii) from the moment of placing the novel object on top of the nest box. In 2014, the latency measures started from the moment of placing the novel object (pink plastic microtube rack $13.3 \times 13.3 \times 5$ cm) on top of the nest box. This behavioural change in the length of feeding interruption could reflect individual variations in risk-taking, exploration and/or ability to cope with novelty. Similar previous experiments were conducted in the aviary conditions where the effect of the DRD4 gene on such behaviour was measured (e.g. Fidler et al., 2007). In the third part of the experiment, the effect of the trap on the first nest visitation was also studied when the nestlings were older than 10 days (in 2014).

2.3.4 Aviary experiment

In 2013 an aviary experiment was conducted before the start of the egg-laying period (**III**). The observation room ($4.0 \times 2.4 \times 2.3$ m) was adapted from Dingemanse and colleagues (2002) with slight modifications (Figure 2). The aviary contained five artificial wooden trees. Besides the trees, birds could land on the edges of sliding doors, the main door, the camera and the one-way observation screen. Along the 4.0 m wall is one sliding door enabling the researcher to place the cage next to the aviary and the bird to enter the room. In the front is the entrance door and the one-way screen for observation.

The aviary experiment was conducted in order to exclude the impact of breeding activities and the effects from the natural environment on individual behaviour. For instance, the number of nestlings could influence parental motivation to return to the nest box. In the aviary experiment (for trial room set-up, see Dingemanse et al., 2002 and Figure 2), wild-caught birds were used. Behavioural trials in the aviary were conducted the morning after capture and every individual performed two behavioral trials. First, the bird entered the aviary that contained artificial trees (novel environment trial) and second, a novel object was added to the trial room (pink plastic microtube rack $13.3 \times 13.3 \times 5$ cm) on

top of the artificial tree. All behavioural trials were videotaped and the number of trees visited, jumps, flights, pecks and calls were recorded (**III**). Finally, the rate of vocalizations made during the release was studied.
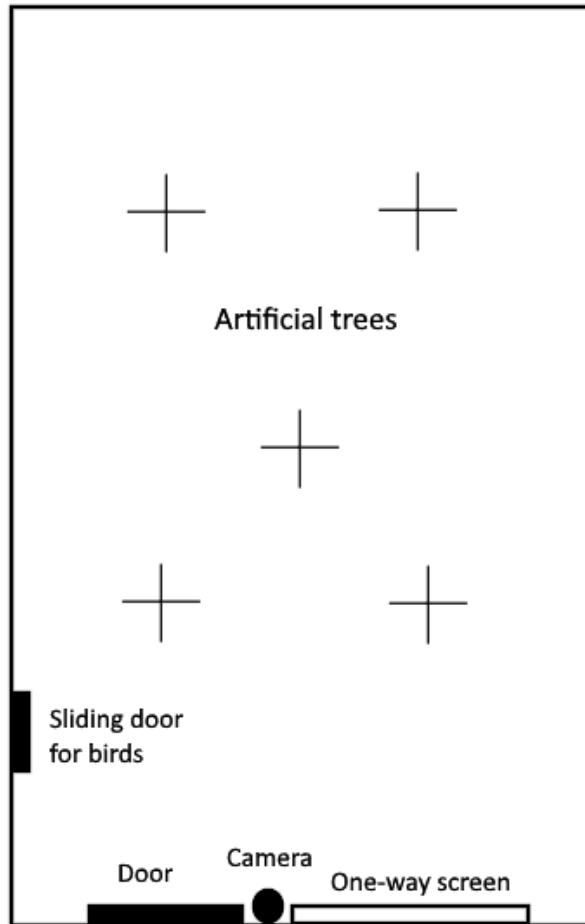

Figure 2: The set-up of the aviary.

2.4 Genotyping

Two candidate genes were tested: the DRD4 (SNP830) gene and the SERT gene where all the SNPs in exonic regions and the promoter region were studied. Heritability was not studied as the blood samples from nestlings were taken occasionally in some years, but not in others. Also, the epigenetic mechanisms such as methylation were not studied.

For the DNA extraction from blood cells, a commercial kit (Roche High Pure PCR Template Preparation Kit 18, Roche, Basel, Switzerland) and the Puregene DNA Purification Kit (Qiagen, Hilden, Germany) were used. The

quality of the obtained DNA was tested with Nanodrop Nucleic Acid Quantification and the concentration of received DNA was approximately 1 μg 1 ml–1. All the samples were sequenced at the Estonian Biocentre where an Applied Biosystems sequencing platform is used.

In the DRD4 gene analysis, the same methods and primer sequences were applied from earlier studies (Fidler et al., 2007, **I**, **III**). The PCR reaction mixture (10 μl) consisted of 1 μl genomic DNA (~10 ng), 5 U/μl recombinant Taq polymerase (nucleotides added), 0.2 mM dNTP-s 1× reaction buffer, 2 mM MgCl2, 0.8 μM primer and water for the rest. Amplification was performed sequentially as follows: 94 °C for 4 min., 30 cycles at 94 °C for 30 s., 61 °C for 30 s., 72 °C for 40 s. and 72 °C for 4 min. SNP830 has an allele-specific restriction place for enzyme Nae I (Pdi I, Fermentas). For RFLP analyses 0.5 μl of restriction enzyme, 1× reaction buffer and 3.5 μl water (Fermentas) were added into 10 μl of PCR product and incubated for 5 h. at 37 °C (**I**, **III**). DNA fragments were separated using electrophoresis on a 3% agarose gel for 1 h. at 140 V. Band profiles were photographed and homozygotes and heterozygotes were initially detected by visual observation.

In the SERT gene analysis (exonic regions) primers were designed for all the exonic regions and the promoter area using the great tit genome (Laine et al., 2015, **II**). This analysis enables researchers to conduct further analysis with the SERT gene as a set of primers for studying SERT gene exonic regions is now available (**II**). Primers for the promoter region were applied from the previous study (Riyahi et al., 2015). In all the analyses, the PCR reaction mixture (25 μl) consisted of 2 μl genomic DNA, 5 μl recombinant Taq Fire polymerase (nucleotides added), 0.5 μl of each primer and water for the rest (17 μl). Amplification was performed sequentially as follows: 95 °C for 3 min., 38 cycles at 95 °C for 30 s., 52 °C for 30 s., 72 °C 1 min. (**II**). After that, samples were kept at 72 °C for 4 min.

The sequences were aligned with the ChromasPro 1.7.6 programme and then visually examined for polymorphisms. The presence of polymorphic regions was confirmed on both forward and reverse sequences. Construction of haplotypes followed the DNAsp programme (Librado and Rozas, 2009) and the linkage disequilibrium data was analysed with the DNAsp 5.10 programme and with an online calculator (Rodriguez et al., 2009).

2.5 Hormone analysis

For the corticosterone analyses, the Correlate EIA kit (Cat No 900-097, EnzoLifeSciences, Assay Designs, USA) was used to measure plasma CORT levels (**III**, see details in Tilgar et al., 2016). Plasma dilution and the concentration of the steroid displacement buffer (SDB) were optimized and validated for measuring great tits: 1.5% of SDB (10 lL 1: 100 SDB, which equals 0.15 lL of raw SDB) was added to 10 lL of raw plasma (**III**). All standards and samples were analysed in duplicate (**III**).

2.6 Statistical analysis

In statistical analysis both Statistica 8.0 and R (version 3.1.2) were used (for details, see **I–IV**). For testing the Hardy-Weinberg equilibrium, Chi-square tests were used. Three different models were used to cover allelic and genotypic effects. The general genetic model includes three distinct genotype categories in the analysis. In the additive model, genotypes are coded as continuous predictors and each additional copy of the variant allele increases the response. In the over-dominant model, heterozygous individuals are tested against homozygous individuals and genotype categories are coded as heterozygotes versus homozygotes.

If the data was not normally distributed, the transformations were used (e.g. logarithm; see **I–IV** for details) in order to meet normality assumptions of residuals. If the the variance was much larger than the mean, a GLZ with negative binomial distribution was applied (O'Hara and Kotze, 2010, **I**, **III**). General linear models (GLM), generalized linear models (GLZ) and linear mixed-effect models (LMM) were used in the studies (**I–IV**).

2.7 Ethics of the experiments

The experiments conducted in the current thesis comply with the laws of Estonia and are approved by the Animal Procedures Committee of the Estonian Ministry of Agriculture and by the Estonian Department of Environment with following licences:

1. Licence no 11
   Licence to ring birds, permission granted by Estonian Environmanetal Board.
2. Licence no 100
   Granted permission to collect blood samples from great tit nestlings and adults. This research complied with the licence by not exceeding the number of sampled individuals and volume of blood samples taken from birds. Permission was granted by the Animal Procedures Committee of the Estonian Ministry of Agriculture.
3. Licence no 108
   Permission to conduct behavioural experiments was approved by the Animal Procedures Committee of the Estonian Ministry of Agriculture. The studies complied with the conditions stated in the licence.

3. RESULTS

The DRD4 gene polymorphism in the Estonian great tit population (I, III)
All the DRD4 exon 3 SNP830 genotype frequencies were in the Hardy-Weinberg equilibrium (sample sizes: CC = 39, CT = 56 and TT = 16) in 2012 (**I**). The low numbers of TT genotyped individuals corresponded with previous studies (e.g. Fidler et al., 2007). While comparing the genotype frequencies in 2012–14, the CC homozygote showed lower numbers in 2013 and 2014 than in 2012 (Figure 3). Still, the genotype frequencies met the Hardy-Weinberg equilibrium in 2013 and 2014 (N_{2013}: CC = 6; CT = 42; TT = 36; N_{2014}: CC = 12; CT = 68; TT = 24), (**I**, **III** + *unpublished data from 2014*).

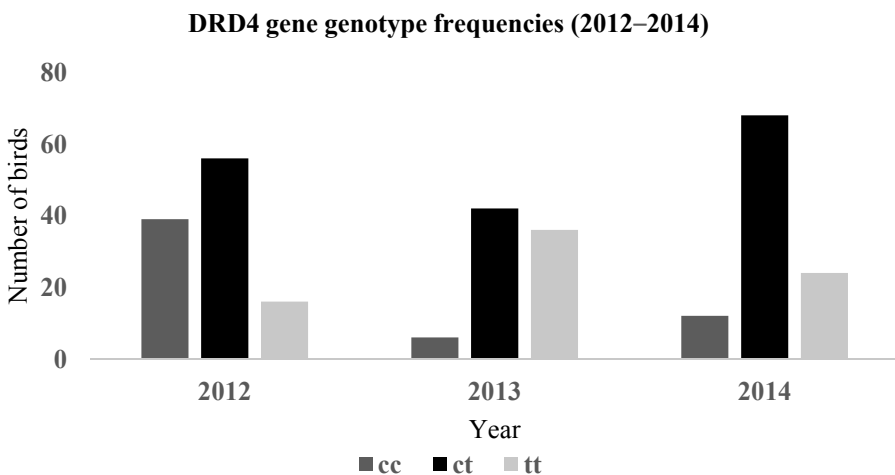**DRD4 gene genotype frequencies (2012–2014)**

Figure 3: DRD4 SNP830 frequencies in Estonian rural (Kilingi-Nõmme) population over three years (2012–2014). CC and TT are homozygotes, CT is heterozygote.

The SERT gene polymorphisms in the Estonian great tit population (II, IV)
Alongside the DRD4 gene, the serotonin transporter gene (SERT) was studied. Altogether 13 exonic regions and the promoter area of the great tit chromosome 19 from serotonin transporter region were analyzed (**II**). Out of these, 9 significantly polymorphic regions (SNPs) were found, present in the promoter region and exons 1, 3 and 8 (see Table 2, modified from Paper **II**). Other exonic regions in the current population were monomorphic. The three SNPs studied are synonymous (Table 2), while the rest of the polymorphisms were non-synonymous and their behaviour could be influenced by changes in amino acids (Table 2). Allele frequencies of the SERT gene polymorphisms in the current population tended to stay similar over two years (2012 and 2014). However, some of the SNPs were constantly not in the Hardy-Weinberg Equilibrium (Table 2). Violation of HWE indicates that some genotypes are constantly missing in both years.

The number of significantly polymorphic SNPs in the SERT gene varies between different geographic populations. Riyahi and colleagues (2015) found more polymorphic SNPs in the promoter area of the same gene in a Spanish great tit population than the present study. Therefore, a pilot study (from 2016, *unpublished data*) was conducted where the allele frequencies of the SERT gene SNP234 (promoter region) were studied in rural and urban populations both in adults and nestlings (Figure 4). In Estonia, this particular SNP is polymorphic in the urban population (Tartu). However, in rural population (Kilingi-Nõmme) almost no variance among nestlings and adults was found.

Table 2, adapted from Paper II: Alleles, protein coding and minor allele frequency (MAF) of each SNP for the SERT gene in the Kilingi-Nõmme population over 2 years (2012, 2014). Population sample size: [232]: 111 individuals (2012) and 121 individuals (2014). Major/minor alleles are the most common and least common alleles in the population. HWE refers to the Hardy-Weinberg Equilibrium.

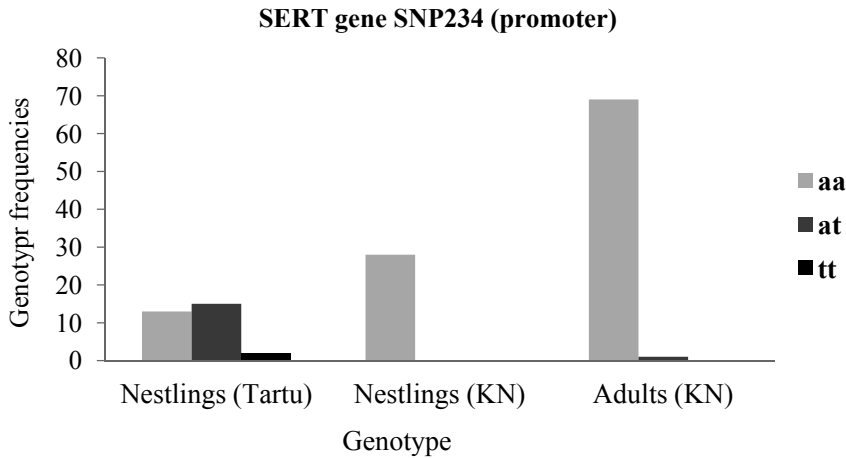| SERT locus	major/minor allele(s)	Location	Minor allele frequency	HWE holds: y/no	Protein coding
SNP136	a/g	Promoter	20.3%	Yes	
SNP290	a/g	Promoter	30.5%	No	
SNP478	c/t	Promoter	29.5%	No	
SNP187	a/t	exon 1	29.4%	Yes	synonymous
SNP253	c/t	exon 3	18.3%	Yes	synonymous
SNP278	a/g	exon 3	20.7%	No	non-synonymous
SNP197	c/t	exon 8	41.0%	Yes	non-synonymous
SNP407	a/t	exon 8	36.2%	No	synonymous
SNP457	a/g	exon 8	48.7%	Yes	non-synonymous

SERT gene SNP234 (promoter)

Figure 4: SERT gene SNP234 genotype frequencies in Estonian great tit populations (2016). Tartu is urban, Kilingi-Nõmme (KN) is a rural area. AA and TT are homozygotes, AT is heterozygote.

Behavioural variation and the SNP830 in the DRD4 gene (I, III)

Behavioural response towards a novel object in natural population of great tits is associated with the DRD4 gene SNP830 (**I**). Males with the T allele (TT or CT) resumed food forraging faster than birds with a CC genotype. During the experimental phase where the novel object was placed on top of the nest box, the feeding delay was longer than the control phase (camera set-up) for both genders, even though female behaviour was not related to the DRD4 gene. However, female behaviour was negatively related to brood size (**I**). Therefore, the potential effect of the breeding season on female behaviour could play a role.

In 2013 a behavioural experiment was conducted in captivity before the start of egg-laying (**III**). The response to the novel object (activity and exploration) in the aviary was significantly related with the DRD4 gene in both genders. The number of visits to a novel object and the number of artificial trees visited correlated significantly with the DRD4 genotype. Birds with the CT genotype explored the novel object more often than those with the TT genotype while no differences were observed between the CC and CT genotypes or between the CC and TT genotypes (**III**).

Behavioural response and stress hormone analysis in relation to the DRD4 gene SNP830 (III)

Individual vocal response during the release correlated with DRD4 poly-morphism. Post hoc analysis revealed significant differences between the CT and TT genotypes ($P = 0.04$), but not between the CC and CT ($P = 0.29$) or CC and TT ($P = 0.96$) genotypes. The highest probability to give alarm calls was in CT heterozygotes. It was also asked if stress-induced corticosterone levels could be related to DRD4 polymorphism on site SNP830. Among females (as there were too few males for the analysis) there exists a relationship between CORT

levels and the polymorphism in the DRD4 gene. The lowest CORT levels were detected in CT heterozygotes while TT homozygotes had the highest levels of CORT (post hoc $P = 0.032$). The corticosterone levels were negatively correlated with the probability of making alarm calls during release (**III**).

Behavioural variation and the SERT gene polymorphisms (II)
The SERT gene is related to the variation in behavioural response to different types of stressors: a camera, a novel object and a nest box trap (**II**). The latency to enter the nest box was significantly different between all three stressors ($P < 0.001$) and had longest delay during the trapping phase and the shortest delay in the presence of the camera (control phase). SNP253 in exon 3 was significantly correlated with the behavioural response to the presence of a novel object. CC homozygotes resumed their feeding behaviour significantly earlier than CT heterozygotes. Owing to the low frequency of the minor allele (T), it was not possible to analyse TT homozygotes properly. The behavioural response to the trap correlates with the SNP197 in exon 8. CC homozygotes entered the nest box after a longer delay compared to CT and TT individuals (**II**). It is important to note that there was also a tendency for an association to exist between SNP197 in exon 8 and a delay in nest visitation during the novel object phase and SNP253 (exon 3) during the trapping phase, but these relationships were not significant after correcting for multiple testing (**II**).

Behavioural variation in anti-predator behaviour and relationship with the DRD4 and SERT genes (IV)
SNP830 in the DRD4 gene had no effect on female behaviour to the presence of an intruder ($P = 0.93$, **IV**). The SNP187 in SERT gene exon 1 correlates significantly with female behavioural response (**IV**). Pair-wise post hoc tests showed a significant difference between the AT and TT genotypes of the SNP187 ($p = 0.007$), while not between the AA and TT genotypes ($p = 0.18$), probably due to the very small sample size of the AA genotype. TT homozyogtes tended to pursue a more active defence strategy whereas the heterozygotes and AA homozygotes remained passive.

Relationships of candidate genes with reproductive traits (I, II, IV)
Polymorphisms in the SERT gene were related to some reproductive traits (start of egg-laying, number of hatched young), but not to others (clutch and brood size, brood size during the fledgling stage). The SERT gene SNP457 in exon 8 was significantly associated with the start of egg-laying in the additive model in the novel object experiment (**II**). Females with the AG and AA genotype started egg-laying earlier than those with the GG genotype, whereas no difference was observed between the AG and AA genotypes (**II**).

The number of hatched young is associated with the SNP197 in the SERT gene exon 8. This relationship was sex-specific and significant in females only (**II**). Heterozygous CT females had larger broods at hatching than CC or TT homozygotes. Here the over-dominant model was used (**II**). No direct relationship between the DRD4 gene and any reproductive traits was found (**I**).

4. DISCUSSION

A changing environment demands a change in individual behaviour. One of the factors limiting a behavioural response to a novel environment lies in the genes that affect neurotransmitter functioning (Carere and Maestripieri, 2013). The aim of the current thesis was to study individual responses to novel or dangerous situations and the influence of candidate genes related to neurotransmitters that affect behavioural variation. Both genes studied (DRD4 and SERT) were polymorphic in the Estonian great tit population and are therefore suitable for studying the mechanisms behind individual variation in behavioural traits (**I–IV**). In the current study, the DRD4 and SERT genes affected behavioural traits that are essential for individual survival, adaptation and breeding success (e.g. risk-taking, exploration, nest defence) (**I**, **IV**).

As both genes (DRD4 and SERT) are polymorphic in several great tit populations (see also Korsten et al., 2010 and Riyahi et al., 2015), it seems that the effect of these genes is widespread even though in some populations no relationship with behaviour was found (Korsten et al., 2010). Therefore, these gene regions are important in other bird species as well and can be used as general candidates for behavioural variation in future studies. Moreover, these genes could be related to reproductive traits and might affect population structure through selective forces.

The DRD4 gene and novelty-induced behavioural responses (I, III)
The DRD4 gene affects individual behaviour during breeding season in the Estonian great tit population (**I**). Males with a homozygous TT genotype and CT heterozygotes were faster to return to the nest box at the presence of novel object compared to individuals carrying the CC genotype. Thus, individuals with a T allele are considered to be bolder with risk-taking and are more exploratory (see also Fidler et al., 2007). However, in females no relationship between DRD4 genotypes and behaviour in the natural environment was found. Possibly, the environmental factors might shield the effect of the genotype as the brood size affects female motivation to feed the nestlings (**I**). Therefore, the experiment that was conducted during the breeding season showed also the effect of the breeding season on individual behaviour in females. Formerly, the association between SNP830 in the DRD4 gene and exploratory behaviour in wild populations was shown to be independent of sex (Korsten et al., 2010). However, in their study, first-year birds who had not bred were used and the experiment was conducted during the non-breeding season (Korsten et al., 2010). In the current study with Estonian great tits an experiment was also replicated before the start of the breeding season and in captivity in order to control for the possible influence of the environment (**III**).

In the aviary experiment (in captivity), both male and female exploratory behaviours were related to the DRD4 gene genotypes: CT heterozygotes were bolder and more exploratory than homozygotes (TT and CC). In the former experiment where individual behaviour was measured in the wild, carriers of

the T allele (CT and TT) were quicker to resume feeding behaviour (**I**). Therefore, it seems that the TT homozygotes in the aviary were not as exploratory as shown before (**I**, **III**). Differences in exploratory behaviour and risk-taking possibly occur due to different experimental set-ups. Still, the presence of the T allele in the Estonian population is related to bolder and more exploratory behaviour similar to other populations as well (Fidler et al., 2007; Korsten et al., 2010), suggesting an effect from the overdominance of the T allele (Fidler et al., 2007, **I**).

Individual stress response and the DRD4 gene (III)

A change in environment is often stressful for an individual (Badyayev, 2005). Stressful events and environments modify the integration of several regulatory systems, including neurotransmitters (Badyayev, 2005). As individual reaction to novelty was related to the DRD4 gene, it was assumed that variations in stress levels among individuals could also be influenced by the DRD4 gene. As shown before, corticosteroid hormones are essential for cognitive performance (de Kloet et al., 1999) and the intensity of alarm calling in great tits is also associated with exploratory behaviour (Hollander et al., 2008). According to Krams and colleagues (2014) alarm calls could reflect individual anxiety levels. In the current study, individual alarm calling was observed during the release after the aviary experiment in order to observe individual stress levels.

The results demonstrate that individual vocal behavior varied significantly among individuals and was related to the DRD4 gene (**III**). Most of the alarm calls were produced by the CT heterozygotes rather than the TT and CC homozygotes. As anxiety is positively correlated with stress-induced corticosterone levels (Sapolsky et al., 2000), individuals who produce more alarm calls should also have higher levels of stress hormone (Krams et al., 2014). In the current study, measuring the main stress hormone in birds (corticosterone) levels after capture indicated that CT heterozygotes had the lowest levels of stress hormone compared to other genotypes. Thus, producing more alarm calls does not reflect increased hormonal stress response in this population. However, similar to Riyahi and colleagues (2017), our results could show that the greater probability of alarm calls produced could reflect bolder behaviour in great tits. Therefore, the results of the current study also infer that individuals who conducted more calls during release are probably bolder and not as stressed as individuals with higher corticosterone levels. Furthermore, these results indicate that boldness and stress response in novel or changing environment are likely co-ordinated together by the stress hormone levels and the genes underlying them.

The SERT gene and novelty-induced behavioural response (II)

The SERT gene in birds has not been widely studied. Therefore, in the current study, all the gene regions in the SERT gene including introns, exons and promoters were analysed. The results showed that the SERT gene plays an important role in behavioural variation in different contexts during the breeding season. A somewhat similar behavioural trait as in the study with the dopamine receptor gene D4 was examined using an experiment with a novel object. The

results showed that individual behaviour in response to novelty is also affected by the SERT gene. Moreover, several polymorphic regions, not just one SNP in the SERT gene different regions are important in shaping individual behavioural phenotype. These SNPs are present in exonic regions 1, 3 and 8 (**II,** Table 2). As these SNPs were close to each other, correlations between these regions were studied. However, in this study no correlation or linkage disequilibrium (LD) between the SNPs was found. As the linkage disequilibrium in natural population is dependent on genetic diversity in the genome, LD studies in the current population need a denser genome marker map in order to detect linkage associations (Backström et al., 2006).

A polymorphism in the exonic region 3 correlates with behavioural reaction towards different types of novelty: a camera, novel object and trap. The longest delay before entering the nest box and resume feeding the nestlings was in the presence of the nest box trap. The shortest delay occurred during the control phase (camera). In all the set-ups, CC homozygotes were bolder and resumed feeding behaviour significantly earlier than CT heterozygotes while the novel object was present. As the TT homozygotes were underrepresented, behavioural variations in these genotypes were not studied. However, if the CC homozygotes are more adaptive in the changing environment as these individuals were faster to return to the nest box, it could infer that these individuals are more successful at raising offspring in variable conditions.

Secondly, an SNP in exon 8 (SNP197) seemed to affect individual behavioural response towards the trap placed on the nest box, even though this SNP is not related to the behavioural response to the presence of a camera or novel object (box) (**II**). The longest delay of entry was present in the CC homozygotes, longer than those birds who carried the T allele (TT or CT). In this study, a trap is considered to be a more threatening object than a novel object placed on top of the nest box as it impedes entry. Therefore, the SERT gene polymorphisms play a role in situations that are more stressful than the DRD4 gene that affected behavioural responses only in the presence of a novel object. Indeed, in humans and rats, serotonin levels are causally related to behavioural flexibility in different situations (see the overview by Coppens et al., 2010). Therefore, the results from the current study also indicate that serotonin and the SERT gene are important factors in shaping behavioural variation and flexibility in birds.

The SERT gene and anti-predatory behaviour (IV)
In addition to behavioural measurements during the feeding period of the nestlings, female reactions to intruders at the time of incubation was studied. The attack of a potential predator is considered to be more threatening than a mere novel object. Any animal whose behaviour enables it to avoid or deter predators while being attacked will have a higher possiblity of surviving and will be more successful at raising offspring (Lind and Cresswell, 2005). Understanding variation in persistent behavioural phenotypes enables us to study individual success and vulnerability in changing and stressful environments. The response to the intruder (hissing behaviour) was shown to be

consistent over time (Koosa and Tilgar, 2016) and reflects individual boldness in birds (Krams et al., 2014). In the current thesis, individual response (hissing or non-hissing) was related to the polymorphism in the SERT gene exon 1. TT homozygotes tended to perform a more active defence strategy (hissing) against intruders. AT heterozygotes and AA homozygotes stayed more passive (non-hissing). Similar to the experiment with the trap, the correlation with the SERT gene and the anti-predatory response point out that this gene has an important effect on the behavioural reaction during a threatening situation. It is also essential to note that only the SERT gene polymorphism in exon 1, but not in exonic regions 3 and 8, affects the behavioural response. It indicates that different regions of the SERT gene are important in creating behavioural reactions in dangerous situations and in novel environments.

The effect of candidate genes on reproductive traits
Behavioural traits shape reproduction through individual decisions in choosing suitable nesting sites and partners, as well as decisions regarding the start of egg-laying, parenting and much more. In the third hypothesis it was presumed that if the behavioural decisions correlated with the genes behind them, then these genes could also have an impact on individual reproductive success. As most of the behavioural experiments were conducted during the breeding season, several traits related to reproduction were studied. Previous studies have shown that the reproductive value of the brood could explain the anti-predatory response in females (Tilgar and Kikas, 2009). Moreover, the start of egg-laying could be related to predation risk (Byrkjedal, 1980) and parental investment could play an important role during the breeding season (Fontaine and Martin, 2006). As shown in **Paper I**, females were probably more motivated to feed offspring than males, as the brood size was related to the female behavioural response. However, neither the brood size nor the number of fledglings were related to the DRD4 or SERT genes. Also, in the study during the incubation period where female reaction towards the woodpecker intruder was observed, brood size had no effect on this trait at all even though this behaviour could be important in female and/or nestling survival (**IV**; Lind and Cresswell 2005; Montgomerie and Waterhead 1988).

Still, for the first time it was shown that the SERT gene polymorphisms could be associated with the timing of breeding as the SNP457 in exon 8 might play a role in initiating egg-laying (**II**). Heterozygous birds (AG) and homo-zygous AA females started egg-laying earlier than GG homozygotes. Adapting the time of breeding helps ensure the birds' procurement of a sufficient amount of food to feed their nestlings (Noordwijk et al., 1995). Therefore, the rising spring temperature in Estonia (Jaagus et al., 2014) could also shift the cater-pillar peak and shape the individual adaptation of great tits in a changing environment as shown by Visser and colleagues (1998). If the carriers of the A allele start egg-laying earlier, this might infer that these individuals could be faster to react to the shift in the environment.

The second polymorphism in exon 8, SNP197 was significantly related with the number of hatched young when heterozygotes had larger broods compared

to homozygotes (**II**). This SNP was also related to individual behaviour in the presence of the trap where CT heterozygotes were bolder at entering. The larger broods could indeed be a source of motivation to enter the nest box more frequently. Thus, the mutual effect between brood size, behavioural response and the SERT gene could also affect genotype frequencies in the population.

When it comes to the second candidate gene, the DRD4 gene, SNP830 was not directly related to any reproductive trait, but could have had some indirect effects as the brood size was related to female behaviour. Therefore, dopamine and serotonin are involved in shaping individual reproductive success in great tits and including reproductive traits in future studies of behavioural genetics is essential. Also, if these genes do indeed play a role in the breeding season, it would enable us to study population structures and fluctuations of different genotypes. Variations in reproductive traits could reflect some components of fitness and are possibly subject to selection pressures.

Genotype frequencies in different populations
The DRD4 gene was studied over three different years (2012–2014) (**I**, **III**, **IV**). The genotype frequencies in the Estonian (Kilingi-Nõmme) population of great tits varied slightly over the years but this variation was not statistically significant. The most frequent was the CT heterozygous genotype. TT and CC homozygote frequencies varied over the years (Figure 3). Compared to earlier studies, the observed frequencies of the DRD4 gene in Kilingi-Nõmme population were similar to other great tit populations (Fidler et al., 2007; Korsten et al., 2010). For example, in former studies, the TT homozygotes were constantly underrepresented, similar to the results gained in 2012 from the Estonian population (Fidler et al., 2007; Korsten et al., 2010; **I**).

Allele frequencies in the SERT gene were studied in 2012 and 2014 (**II**, **IV**). Some genotypes (homozygotes) were constantly missing in both years. Underrepresented genotypes or variation between years in the population could be affected by several factors. In the wild population, migration and selection pressures shape the population structure. Moreover, mutation rates, genetic drift and gene flow could vary over the years (Hartl et al., 1997). For instance, the frequencies of the SERT gene polymorphisms in two distant populations (Estonia and Spain) differ significantly (Riyahi et al., 2015; **II**). Some of the SNPs found in the study conducted by Riyahi and colleagues (2015) were not present in the Estonian population (e.g. SNP234 in the promoter is not polymorphic) (**II**). However, this SNP234 could play an important role in shaping behavioural response (Riyahi et al., 2015). Therefore, a pilot sample of nestlings and adults was studied both in urban and rural populations of great tits in 2016 in order to detect potential variations in this particular region of the SERT gene (SNP234) (*unpublished data*). In the Kilingi-Nõmme population (rural), the main study area, almost no variation in this gene region was present in the population. However, in the urban nestlings, the SNP234 is polymorphic (Figure 4, *unpublished data*). Similar to Riyahi and colleagues, the T allele in SNP234 is more present in urban populations (Riyahi et al., 2015). These results

infer that the genotype frequencies in Estonian population also vary and the role of local adaptations in shaping genotypes cannot be underestimated.

Potential mechanisms of behavioural variation

Several SNPs in the current study were synonymous, meaning that a change in DNA sequence does not change the protein sequence. It could be that these SNPs are just neutral or non-functional. However, synonymous polymorphisms could have functional effects: these codons often define mRNA secondary structure, expression and transcription (Oak et al., 2000; Shabalina et al., 2013). SERT gene synonymous polymorphisms could also act as transcriptional enhancers (Fiskerstrand et al., 1999). These effects are often mediated through linkage with other non-synonymous SNPs as well (Chamary et al., 2006). Moreover, the neurotramitter functioning is often part of a greater network as shown in rats where dopamine and serotonin levels affect behaviour simultaneously (Van Erp and Miczek, 2000).

Candidate gene studies often use polymorphisms that are not causal and the effect of these polymorphisms shapes behaviour through linkage disequilibrium or epigenetic mechanisms (Bossdorf et al., 2008; van Oers et al., 2005; Nordborg and Tavare, 2002). Linkage disequilibrium is present when traits are affected by various gene regions, but a selective force prefers particular combinations (van Oers et al., 2005). As the DRD4 gene and SERT gene in great tits affect similar behaviours, potential linkage disequilibrium (LD) between these genes and between different SNPs within the SERT gene was studied. However, in the current study no linkage disequilibrium was found in the Estonian great tit population (**II**, **IV**). Previously, Mueller and colleagues showed that the linkage disequilibrium structure in DRD4 gene exon 3 region is conserved across four great tit populations and therefore does not explain heterogeneous associations (Mueller et al., 2013). When it comes to the SERT gene, Riyahi and colleagues (2015) observed LD between SNP440 and SNP478 in the promoter region. As in the Estonian great tit population, fewer SNPs were present in the promoter area (no variation in SNP440 at all) compared to Riyahi and colleagues and it was not possible to study LD in this gene region (**II**).

In addition to linkage disequilibrium, several epigenetic factors, mRNA functioning and RNA stability could affect the behaviour through genetic variation (Knapp et al., 1998; Oak et al., 2000). Possible gene–environment interactions are suggested in both genes, DRD4 and SERT (Riyahi et al., 2015). In the study by Riyahi and colleagues, it was found that the methylation levels of SERT gene are higher in urban populations (1–4%) than in rural areas. However, in the Spanish population, the DRD4 gene and its methylation levels were present, but not strongly involved in the exploratory behaviour (Riyahi et al., 2015). In a subsequent study, Verhulst and colleagues found an important effect of methylation on the DRD4 gene and behavior in great tit populations (Verhulst et al., 2016). In the current thesis, the role of epigenetics was not studied, but these effects could be relevant in Estonian great tit population as there exists a difference between urban and rural populations in the SERT gene (*unpublished data,* Figure 4).

5. CONCLUSIONS

Both candidate genes studied are polymorphic in Estonian great tits and correlate with behavioural traits. Most of the experiments were conducted in the wild and during the breeding season. F**irstly, exploratory behaviour in great tits was affected by the DRD4 gene polymorphism** both in the wild and in the aviary. Moreover, the DRD4 gene could play a role in individual anxiety levels as the production of alarm calls was affected by polymorphism of this gene. It is important to note that if the novel object experiment is conducted during the breeding season, the female behavioural response might be influenced by the brood size. Also, the DRD4 gene and behavioural response in stressful situations could be related to acute levels of corticosterone. **Secondly**, **individual reactions to novel or dangerous situations in the wild are related to the SERT gene polymorphisms**. Similar to the DRD4 gene, the SERT gene affects the reaction towards a novel object. Besides, the behavioural reaction to a potentially more dangerous object (e.g. a trap or predator) was correlated with the SERT gene polymorphisms. Therefore, the SERT gene seems to affect decision-making under stressful situations. **Thirdly, reproductive traits could be related to differences in the SERT gene, but are not related to the DRD4 gene.** The SERT gene SNP457 in exonic region 8 was related to the start of egg-laying and the second polymorphism in exon 8, SNP197 was significantly related with the number of hatched young. When it comes to the second candidate gene, the DRD4 gene, it was not directly related to any reproductive trait, but could have had some indirect effects through its influence on behaviour.

6. SUMMARY

Genetic studies give the opportunity to understand mechanisms potentially underlying individual behavioural variations. Behaviour varies between individuals but the individual responses to situations and environments often stays persistent. Adapting one's reaction to a situation or being behaviourally more plastic in a changing environment is costly and often limited. The limits of behavioural plasticity are affected by the environment itself, other behavioural traits and genes. Therefore, knowing the genes that affect behaviour and the neurotransmitter networks behind behavioural responses enables us to understand individual variations in behaviour. Studies of behavioural genetics also include behavioural disorders in humans, such as depression, anxiety and schizophrenia; adaptation in animals (e.g. reintroduction and colonization) and finally domestication and selective breeding in farming.

Behavioural traits are a part of a complex system that involves the nervous system, hormones, neurotransmitters and genes. Two main candidate genes that are widely studied have been associated with anxiety and novelty-seeking and are related to dopamine and/or serotonin neurotransmission. These are the serotonin transporter gene (SERT) and the dopamine receptor gene D4 (DRD4). Given that dopamine and serotonin regulate behavioural responses and are related to variations in individual genotypes, the main focus of this thesis was to study the effect of these candidate genes on behavioural traits in a natural population. As most of the experiments were conducted during the breeding season, reproductive traits were also included in the analysis.

To study this, different behavioural experiments were conducted both in the aviary and in the wild. The great tit was used as a model species that allowed us to conduct experiments both in the wild and in captivity. Individual behavioural variation was measured in the presence of a novel object, a nest box trap and during a simulated predator attack. Most of the behavioural experiments were conducted during the breeding season which enabled us to include reproductive traits in the analysis. For gene analyses, blood samples were collected for DNA separation and sequencing.

The results show that the candidate genes (DRD4 and SERT) are polymorphic among Estonian great tits. Moreover, individual behavioural responses to a novel object were affected by both candidate genes. The DRD4 gene is related to male behavioural response in the natural environment and in the aviary. In female great tits, there was no effect from the DRD4 gene on behaviour in the experiment conducted in the wild. However, while conducting the experiment in the aviary and before the start of the breeding season, a similar correlation between the DRD4 gene and behaviour was found in females as well. Therefore, the potential effect of the breeding season on female behaviour played a role as the brood size was related to the female behavioural response. As novel situations could be stressful, individual corticosterone (CORT) levels

were included in the experiments. Stress hormone levels were correlated with individual vocal response during release after the aviary experiment. Moreover, the CORT levels were related to the DRD4 gene. The lowest levels of acute CORT were observed in CT heterozygotes, suggesting that these individuals are less influenced by handling stress. Indeed, when facing a novel object, CT heterozygotes were bolder in approaching it. Also, when birds were released, CT heterozygotes conducted most of the alarm calls which could infer bolder behaviour.

The SERT gene polymorphisms were also related to the behavioural reaction to the novel object as a SNP in exonic region 3 correlates with a behavioural reaction in the presence of different types of novelty: a camera (inducing the shortest delay), an object on the nest box and the nest box trap (inducing the longest delay). Moreover, the SERT gene seems to play a significant role when the situation is more stressful. An SNP in the SERT gene exon 8 is only related to the behavioural response towards the trap on the nest box as the carriers of the T allele were bolder than CC homozygotes. Also, an SNP in the exonic region 1 is associated with individual risk-taking behaviour during the simulated predator attack during the incubation period. Behavioural adaptation could also affect the frequencies of the SERT gene polymorphisms. A pilot sample of urban and rural birds showed that one particular SNP (SNP234) that is present in former studies and relates to exploratory behaviour is missing in the Estonian rural population. In urban birds (especially nestlings), the SNP234 was present.

Most of the behavioural traits in the current thesis were conducted during the breeding season which enabled us to study several reproductive traits toghether with behavioural decisions that might shape breeding success in birds. Therefore, as the DRD4 and SERT genes are related to behavioural decisions in the presence of novelty, it was assumed that these genes could play a role in breeding success as well. However, the DRD4 gene was not directly related to any reproductive trait studied. The SERT gene polymorphism in exon 8 SNP457 was related to the start of egg-laying even though this particular SNP had no effect on behaviour. Also, the second polymorphism in exon 8, SNP197 was significantly related to the number of hatched young where heterozygotes had larger broods than homozygotes. This SNP also affected individual behavioural responses to the presence of the trap where heterozygotes were bolder at entering than homozygotes. Therefore, the SERT gene polymorphisms could be either directly or indirectly related to individual fitness as several gene regions in different tests seem to have some effect on breeding success.

In conclusion, the aim of this thesis was to study the effect of two candidate genes on bird behaviour and reproductive traits. The results show that the DRD4 and SERT genes are important in shaping behavioural decisions in the presence of different stressors and novelty. Moreover, the stress hormone levels could affect the behavioural response related to the DRD4 gene. The SERT gene might be either directly or indirectly related to reproductive traits. The study with the DRD4 gene also indicates that conducting experiments in dif-

ferent environments and seasons could have a significant influence on individual behaviour. Therefore, in subsequent studies, picking the study site and timing of the experiment should be considered carefully. Differences in the environment could be also the reason why no correlation between the candidate genes and behaviour was found in some populations. By understanding the effects of candidate genes on behaviour, it would be possible to expand future studies to the causal mechanisms that shape behavioural traits.

7. SUMMARY IN ESTONIAN

Dopamiini ja serotoniiniga seotud geenide (DRD4 ja SERT) mõju rasvatihase käitumisele ja sigimistunnustele

KOKKUVÕTE

Loomadel esinevaid püsivaid käitumistunnuseid nimetatakse käitumistüüpideks või ka isiksusteks. Siia kuuluvad näiteks julgus võtta riske, agressiivsus, sotsiaalsus ning ekstravertsus. Isiksuse avaldumist kujundavad lisaks keskkonnast tulenevatele teguritele ka geenid. Teades geene, mis kujundavad käitumist, on võimalik paremini mõista loomade kohanemisedukust erinevates keskkondades. Lisaks mõjutavad mitmed geenipiirkonnad inimesel ärevushäirete tekkimist, depressiooni ja ka skisofreenia avaldumist. Selleks, et uurida erinevaid käitumisega seotud aspekte ning geene, kasutatakse sageli mudelliike. Võrreldes inimesega on mudelina kasutatavatel liikidel enamasti oluliselt lühem elutsükkel, mis võimaldab lihtsamini uurida muu hulgas ka käitumise mõju sigimistunnustele ning jälgida tunnuste päritavust ehk säilimist põlvkonnast põlvkonda.

Käitumistunnuste avaldumine toimub läbi keerulise mehhanismi, kus oluline osa on virgatsainetel, mis mõjutavad otseselt närvisüsteemi tööd. Peamised sellised ühendid on 1) dopamiin, mis mõjutab uurivat käitumist, 2) serotoniin, mis reguleerib ärevust ja agressiivsust ning 3) noradrenaliin, mis seostub motivatsiooniga. Nii on käitumise uurimisel peamised kandidaatgeenid need, mille puhul on näidatud olulist mõju virgatsainete toimimisele. Et aga varasemates uurimustes on saadud käitumisgeenide mõjude osas vastukäivaid tulemusi, siis on oluline teha uuringuid erinevates keskkondades ja populatsioonides.

Praeguses teadustöös sooviti vastust küsimusele, kas varasemalt uuritud kaks geenipiirkonda, mis mõjutavad inimesel ning loomadel ärevust (serotoniini transporteri geen/SERT geen) ning uurivat käitumist või riskijulgust (dopamiini retseptori geen D4/DRD4 geen) omavad olulist mõju ka lindude käitumisele. Varasemad uuringud on näidanud, et DRD4 geen mõjutab rasvatihaste ja primaatide uurivat käitumist, koerte impulsiivsust ning hiirte hüperaktiivsust. Rasvatihasel, kes on üks paremini uuritud liike, pole aga kõigis populatsioonides seoseid DRD4 geeni ja käitumise vahel leitud. Seega uuriti praeguses teadustöös DRD4 geeni ja käitumise vahelisi seoseid nii välikatses kui ka aviaariumis (katseruumis), et mõista geeni mõju erinevates keskkondades. Teiseks, SERT geeni on kõige rohkem uuritud inimestel seoses erinevate käitumishäiretega (näiteks depressioon, ärevus ning suitsiid). Ka teistel imetajatel mõjutavad SERT geeni piirkonnad just ärevusega seotud käitumist. Lindudel on aga seda geeni veel võrdlemisi vähe uuritud, ehkki on näidatud, et mitmed SERT geeni piirkonnad mõjutavad lindudel impulsiivsust ning reaktsiooni uudsele objektile või olukorrale. Seega, töö teine uurimisküsimus keskendus SERT geeni ja käitumise vahelistele seostele lindudele loomuomases keskkonnas. Kolmandaks, kuna DRD4 ja SERT geenid on seotud ka sigimisnäitajate

ja kaaslase valikuga, uuriti teadustöös nende geenide mõju lindude pesitsus-edukusele.

Kui eelnevad käitumiskatsed ja geeniuuringud on tehtud enamasti labori-tingimustes, siis praegune töö viidi läbi loodusliku rasvatihaste populatsiooniga ning lindudele omases keskkonnas. See võimaldab genotüübi ja käitumise vahe-lisi seoseid laiemalt testida, sest laboris ei pruugi kõik käitumistunnused alati avalduda. Lisaks tehti suur osa katsetest pesitsusperioodil, et hinnata käitumis-geenide mõju sigimistunnustele. Läbiviidud käitumiskatsete eesmärgiks oli jäl-gida isendite hakkamasaamist uudse või stressirohke olukorraga. Põhikatses filmiti vanalindude toitmiskäitumist (pesapoegade toitmist) Edela-Eestis Kilingi-Nõmme katsealal. Katsetes vaadeldi nii tavalist toitmissagedust, kui hinnati uudsest objektist tingitud toitmispausi pikkust. Sarnane korduskatse viidi läbi ka kontrollitud tingimustes ja enne pesitsusaega, et välistada kesk-konnast tulenevad mõjutusi. Lisaks hinnati emaslindude reaktsiooni võimalikule sissetungijatele ning vanalindude käitumist lõksuminekul (tugev stressiolukord). Lisaks käitumiskatsetele määrati lindude genotüüp nii DRD4 kui SERT geeni osas.

Töö tulemusena selgus, et mõlemad geenid (DRD4 ja SERT) on Eesti rasva-tihaste populatsioonis polümorfsed, st geenipiirkonnad varieeruvad isendite vahel. Mõlemad geenid seostusid ka lindude käitumisega. DRD4 geen mõjutas isaslindude uurivat käitumist nii pesitsusajal vabas looduses kui ka laborikatses (aviaariumis). Emaslindudel ilmnes DRD4 geeni mõju käitumisele aviaariumi-katses enne pesitsusaega, aga mitte vabas looduses poegade toitmise ajal. Küll mõjutas emaslindude käitumist vabas looduses pesakonna suurus, mis tõe-näoliselt võis varjutada geeni mõju käitumisele. Lisaks uuriti selles katses ka lindude stressitaset. Kõige madalam stressihormooni tase oli nendel isenditel, kellel olid olemas DRD4 geeni erinevad alleelid (heterosügoodid). Uudsele objektile lähenedes olid need linnud kõige altimad ja kiiremad lähenejad ning vabastamisel tegid nad ka kõige sagedamini hoiatushüüde. Sarnaselt on vara-semalt rasvatihastel leitud, et hoiatushüüdude suurem hulk näitab tõepoolest julgemat käitumist. Ka madal stressihormooni tase viitab, et need indiviidid võiksid stressiolukorras paremini hakkama saada. Seega, vaatamata väikestele erinevustele aviaariumi ja katsete vahel, mida tehti vabas looduses, on tõe-näoline, et DRD4 mõjutab oluliselt lindude käitumisotsustuste kujunemist ning võib olla seotud ka stressitaseme kujunemisega.

Teiseks uuriti SERT geeni mõju tihaste käitumisele. Selgus, et rasvatihastel esineb selles geenis mitmeid polümorfseid piirkondi (SNPd), mis võivad käitu-mist mõjutada. Uuriva käitumisega tundmatu objekti suhtes oli seotud polü-morfism, mis paikneb geeni kolmandas eksonis. Et katses kasutati sama käitumistunnust, mida DRD4 geeni puhulgi, kontrolliti ühtlasi seoseid ka DRD4 geeni ja SERT geeni vahel. Ehkki sellised geenid võivad olla aheldatud ja koos päranduda, siis uuritud tihasepopulatsioonis sellist seost ei leitud. SERT geeni uuringud näitasid ka, et geen mõjutab enam neid olukordi, mis on tihaste jaoks stressirohkemad ja ohustavamad kui DRD4 geeni puhul. Näiteks leiti seos SERT geeni ekson 8 SNP197 ja lõksumineku vahel. Stressirohke olukord loodi

ka katses emaslindudega haudeperioodil, kus SERT geeni mõju käitumisele ilmnes siis, kui pesa ohustas võimalik sissetungija. Katses imiteeriti rähnitopisega rünnakut pesakastile ning leiti, et polümorfism SERT geeni eksonis 1 mõjutas seda, kas emaste reageering on aktiivne (susin ning rünnak topise suunas) või passiivne (ei tee midagi). Kokkuvõtvalt, serotoniini transporteri geen on oluline tegur lindude käitumise kujundamisel erinevates olukordades. Geen mõjutas nii reageeringut uudsele objektile, lõksule kui ka sissetungijale ning omas märkimisväärselt suuremat mõju käitumisele olukordades, mis põhjustavad rohkem ärevust.

Nii DRD4 kui SERT geeni puhul uuriti nende mõju lindude pesitsusedukusele. Seega vaadeldi lisaks erinevaid pesitsusega seotud tunnuseid nagu näitkes munade arvu, munemise algusaega, koorunud poegade arvu, pesakonna suurust ning poegade arvu lennuvõimestumisel. Enamik neist tunnustest polnud kummaski kandidaatgeenis käitumist mõjutavate polümorfismidega otseselt seotud. Küll aga leiti seos SERT eksonis 8 paikneva SNP197 ja pesakonna suuruse vahel. Suuremate pesakondadega olid heterosügoodid, kes ühtlasi näitasid käitumiskatses julgemat käitumist uudse objekti suhtes. Teine polümorfism (SNP457) eksonis 8 seostus koorunud poegade arvuga. See SNP pole küll käitumisega otseselt seotud, kuid mõjutas tõenäoliselt kuidagi otsust, millal munemist alustada. Nii võib arvata, et pesitsusedukus tihastel küll olla mõjutatud SERT poolt käitumise kaudu, kuid on vähe või pole seotud DRD4 geeniga.

Kokkuvõtvalt, töö tulemusena selgus nii DRD4 kui ka SERT geeni mõju tihaste uurivale käitumisele ning riskijulgusele. Oluline on märkida, et vabas looduses võib pesitsusaeg mõjutada DRD4 geeni ja käitumise vahelisi seoseid. Seega tuleks edasistes uuringutes omavahel võrrelda erinevaid keskkondi ning perioode, mõistmaks olulisi tegureid, mis käitumist võivad kujundada. Mõõtmised peamiselt laboritingimustes võivad olla ka ühtlasi põhjuseks, miks varasemates töödes pole seoseid DRD4 geeni ja käitumise vahel alati leitud. SERT geenis kirjeldatud polümorfismid osutavad geenipiirkondadele, mida pole varasemalt uuritud, kuid, millega tasub tulevikus edasi töötada, kuna need SNPd omavad mõju käitumisele. Seejuures on oluline silmas pidada neid polümorfisme SERT geenis, mis kujundavad pesitsusedukust ja suunavad seeläbi looduslikku valikut. Teades käitumist mõjutavaid geene, on võimalik keskenduda põhjuslikele seostele, mis kujundavad käitumisvastuseid. See omakorda aitab mõista kohanemisedukust nii tihastel, inimestel kui ka muudel loomaliikidel.

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