SERT gene polymorphisms are associated with risk-taking behaviour and breeding parameters in wild great tits

Running title: *SERT* gene and risk-taking in great tits

Keywords: personality, candidate gene, serotonin, fitness, reproduction

Word count: 6870

Summary statement: Novelty exploration and breeding success in birds: the role of the serotonin transporter gene. What are the mechanisms underlying behavioural decisions and what role has genetic variation on individual behaviour?

Abstract

Individual differences in coping with potentially dangerous situations are affected by a combination of genetic and environmental factors. How genetic polymorphisms and behavioural variations are related to fitness is unknown. One of the candidate genes affecting a variety of behavioural processes, including impulsivity, anxiety and mood fluctuations in both humans and other vertebrates is the serotonin transporter gene (*SERT*/*SLC6A*). The aim of this study was to assess an association between *SERT* genotypes and novelty seeking, risk-taking behaviours and breeding parameters of great tits (*Parus major*) in a natural environment. We associated polymorphisms in the promoter exonic regions of the *SERT* gene with parental risk-taking related behaviour and fitness traits. Our results show that (i) risk-taking behaviour in our great tit population is linked to single nucleotide polymorphisms in the *SERT* gene exon 3 and exon 8; (ii) the genotype-behaviour associations are consistent at the presence of different stressors; (iii) polymorphisms in exon 8 could be associated with fitness-related traits, such as the start of egg-laying and hatching success. We showed for the first time that genetic variability of *SERT* plays an important role in shaping individual decision-making that affects fitness consequences in a wild population. However, the results are based on one population and on the polymorphisms that are in one single gene. Therefore, replication studies are needed in order to confirm these preliminary results.

Introduction

A combination of genes, the environment and experience-based learning is known to be responsible for variation in human and animal behaviour (Carere and Masterpieri, 2013). However, the molecular mechanisms underlying behavioural differences between individuals in natural animal populations, and to what extent variation in these mechanisms cause fitness differences is not well-understood. Although the gene-behaviour associations are environment- and condition-dependent (Adriaenssens and Johnsson, 2010), behavioural traits consistently differ between individuals of the same population (see Carere and Masterpieri 2013 for review) and have a significant genetic basis (see Laine & van Oers 2017 for a review). Hence, it is important to conduct experiments in different situations, habitats and across different years to evaluate the repeatability of behaviours and assess the generality of previously reported genetic effects on behavioural variation. Moreover, in order to understand evolutionary change in phenotypes, a connection between genes, behaviour and individual fitness should be tested in the same study system as well (see a review by van Oers et al., 2005). Growing evidence has suggested that variation in many behavioural traits such as fear, anxiety and novelty seeking are associated with dopamine- and serotonin-related signalling affected by genes (Korsten et al 2010., Van Oers et al 2005., Serretti et al 2006).

The role of serotonin transmission in shaping fear-related behavioural variation is widely studied in humans and laboratory animals (Canli and Lesch, 2007; Gryglewski et al., 2014; Murphy et al., 2003) and also in free-living populations (Champoux et al., 2002; Serretti et al., 2006). Alterations in the expression of neurotransmitter-related genes could change serotonergic signalling and thus ultimately shape behaviours where serotonin has been implicated (O´Leary and Cryan, 2010). A candidate gene that is central to the regulation of extracellular and synaptic serotonin concentration is the serotonin transporter gene (*SERT*). Variation in *SERT* gene expression has been associated with differences in the *SERT* protein availability and in modulating active reuptake of serotonin between synapses (Lesch et al., 1996). *SERT* gene polymorphisms in humans have been repeatedly linked with different psychiatric states like depression, anxiety and suicidal behaviour (Murphy et al., 2008). Also, serotonin transport affects parental behaviour through behavioural decisions and by prolactin secretion controlled by neural pathways containing serotonin (see e.g. Bakermans-Kranenburg and Ijzendoorn, 2008; Freeman et al., 2014). However, the relationships found between behavioural traits and genetic

differences in serotonin-related genes in humans have not always been consistent (Savitz and Ramesar, 2004).

In this study we used the candidate gene approach to explore the association of *SERT* gene polymorphisms with behaviour in a natural population of breeding great tits. For birds, *SERT* gene variation was found to be different in urban compared to rural populations of blackbirds (*Turdus merula*) (Mueller et al 2014). Also, Riyahi et al (2015) have provided evidence that these polymorphisms in great tits (*Parus major*) differ between environments. Finally, *SERT* gene in dunnocks (*Prunella modularis*) affects female behaviour, where homozygous females took fewer risks than heterozygotes (Holtmann et al 2016).We hypothesize that the serotonin transporter gene plays an important role in affecting great tit individual novelty seeking and fear-related behaviours. Moreover, since individual differences in behavioural decisions could affect fitness, we explored the link between genetic polymorphisms and breeding parameters. For this, we determined the polymorphic regions of the *SERT* gene (exons) and the promoter region. From these we identified 9 single nucleotide polymorphisms (SNPs) for testing the relationships between behavioural phenotypes and genotypes. We used samples originating from two different years, in order to examine the generality of the effect. Specifically, we predicted that the parental latency to feed nestlings at the presence of different novel objects varies between genotypes. A previous study has shown that the delay in nest visitation and nestling provisioning rates are repeatable characteristics in great tits (Pagani-Núñez and Senar, 2013; Vrublevska et al., 2015). Hence, this trait can be regarded as a potential candidate trait for assessing personality in the wild. Second, we predicted that variation in breeding parameters such as the first egg date, clutch and brood size are associated with these genetic variants, probably via consistent behavioural differences between individuals. This assumption is based on previous studies reporting that reproductive success covaries with serotonergic signalling and maternal care in mice (Lerch-Haner et al. 2008) and in relation to personality variation (Smith & Blumstein 2008).

Materials and methods

The study was conducted in southwest Estonia (58 ° 7 ′ N, 25 ° 5 ′ E) at Kilingi-Nõmme study site during springs 2012 and 2014 at the breeding season (April to June). The area (about 50 km^2) consists of deciduous (*Betula sp, Alnus sp* and *Salix* sp) and coniferous (*Pinus sylvestris* and *Picea abies*) forest patches. Great tits in our study population breed in nest-boxes mounted on tree trunks at a height of 1.5 – 1.8 m.

The nest boxes were checked throughout the breeding period to obtain data on lay date of the first egg, clutch size, hatching date (hatch date = day 0 post-hatch) and number of hatchlings and nestlings (15-day-old). Adults (both male and female) were caught using nest-box traps during the second half of the nestling period (between days 7 and 15 post-hatch). Traps were checked every 15 minutes. Adults were ringed and weighed with a Pesola spring balance (precision 0.1 g). Captured adults were 1-2 years in age. Tarsus and wing length were measured using sliding calipers (with precision to 0.1 mm). Blood samples (~70 µl) were taken from the brachial vein with a sterile lancet and collected in a heparinized capillary tube. The blood was immediately stored at +4 °C, and afterwards centrifuged to separate cells from plasma and then stored at -80 °C until undergoing DNA analysis. Birds were ringed under Estonian Department of Environment Licence No. 11 and blood samples were taken under Animal Procedures Committee of the Estonian Ministry of Agriculture Licence No. 100.

Genotyping

DNA was extracted from the blood samples (2012) with a commercial kit (Roche High Pure PCR Template Preparation Kit 18) according to instructions from producer. Blood samples collected on 2014 were treated with the Puregene DNA Purification Kit. The concentration of received DNA was approximately 1 µg/ 1 ml. Primers were designed using the great tit genome (Assembly Parus_major 1.0.1; Accession number: SRS1185780; Laine et al., 2016). Primer sequences for *SERT* gene exonic regions (exons 1-13) were designed *de novo* (see Table 1).

123 **Table 1**

124 Primer sequences for *SERT* gene and sequence lenghts

Primer name		**5´** **3´**	Base pairs
Exon 1	Forward	CTCCTCGATAATTTGTTATACACGA	456
	Reverse	ATCTGATGGATTTAAGCATGGAAG	
Exon 2	Forward	GGTGTATCTGGAGTTTTAGGAAAG	134
	Reverse	ATGAGAAATCCCACAGTAATTCAG	
Exon 3	Forward	CAAGATTTGAAGTGATTTGAAGTGA	219
	Reverse	TATCTCTGAAGTCACAAGAAATGC	
Exon 4	Forward	CTTTATTGCTTGGATAGGAGTAGC	138
	Reverse	GAAATGTCGTGATTTTGAAAGCTG	
Exon 5	Forward	AAGCTAAATTGAGGGTGGACT	134
	Reverse	GCTCTCTGGGCAGGAACAAC	
Exon 6	Forward	ATTTTGAGGTAGACAACACAGGA	103
	Reverse	CTAAGATTGTGTCAGAAGTGCAA	
Exon 7	Forward	CAATCTGGCTTGTAATCATGGTA	127
	Reverse	GATACAGCCAGCATTCAATCC	
Exon 8	Forward	CAGCATGACAGTGACAAATCTC	112
	Reverse	GGACACTTTTACACAATACAGCT	
Exon 9	Forward	TCATTTTGCAACATATTTCCTAGTG	131
	Reverse	CACTAAATCCCCACCCTAAAGA	
Exon 10	Forward	CTCCTGTTACTTTTAGATGCCTG	96

	Reverse	CTGTAAAATCCCCTTTGTTCACT	
Exon 11	Forward	GTCCTTAAAACTGTGCTTTAGTAAC	100
	Reverse	GCATCTAAAAGTAACAGGAGGTATA	
Exon 12	Forward	GGAGATACAGGAGCACTGCCA	167
	Reverse	AGCATTACTCCAGAAACAGCTACA	
Exon 13	Forward	TGCTCAGTTCAGCCTGTTGGA	74
	Reverse	CAGAGGCCTGAAACGCTCCT	
Promoter (Riyahi et al., 2015)			470
	Forward	CATCTTCTCCTTTGCTACAGCC	
	Reverse	ACAGAGCCTCAGAAGTTAGTTGA	

Primers for promoter region were used from Riyahi *et al.,* 2015. The PCR reaction mixture (25 µl) consisted of 2 µl (consisting 2 ng genomic DNA), 5 µl recombinant Taq Fire polymerase, 0.5 µl of each primer and water (17 µl) for the rest. Amplification was performed sequentially as follows: 95 °C for 3 min, 38 cycles at 95 °C for 30 seconds, 52 °C for 30 seconds, 72 °C for 1 minute and finally 72 °C for 4 minutes. All the samples were sequenced at the Estonian Biocentre where an Applied Biosystems sequencing platform is used. Received sequences were aligned using ChromasPro (Technelysium Pty Ltd, Aus) and visually examined for polymorphisms (SNPs). The SNPs were named according to the position in the exonic region/were taken from the former study (from Riyahi et al 2015). Construction of haplotypes followed the DNAsp programme (Librado and Rozas, 2009) and the linkage disequilibrium data was analysed with DNAsp 5.10 programme and with the online-calculator (Gaunt et al., 2007).

To test Hardy-Weinberg equilibrium, Chi-square tests were used. In order to take multiple testing into account, we calculated critical P-values by using Holm´s correction for the number of independent SNPs for testing deviations from Hardy-Weinberg equilibrium ($P_{critical}$ = 0.0062) and for testing associations of a genotype with latency behaviour and reproductive parameters ($P_{critical}$ = 0.0125, after omitting four SNPs that deviated from HW equilibrium).

142 Sample sizes (see Tables 1 and 2) vary between analyses because not all individuals were
143 successfully trapped and bled and were therefore not genotyped.
144
145 **Behavioural measurements**
146 The response of adult birds to a novel object was tested when nestlings were 7-13 days old.
147 A novel object (Eppendorf box) was placed on the rooftop of the nest box at the beginning of the
148 experimental phase (see Timm et al., 2015 for details). The experimental phase was preceded by
149 a control phase where the normal feeding rate of birds was recorded for 15 minutes. We
150 measured the feeding delay (latency in seconds to enter the nest box from the moment when the
151 novel object was placed). The experimental phase lasted 30 minutes. Overall, we tested the adult
152 (both parents) response to a novel object on 113 breeding pairs altogether (2012 and 2014). The
153 experiment (performed randomly in the population) was carried out between early morning (6
154 am) and early afternoon (2 pm), during the highest feeding activity. The behaviour of birds was
155 recorded with a digital video-camera placed on a tripod about 5-10 meters from the nest box.
156 In 2014, in addition to the response to the novel object, we also recorded the delay of
157 birds to enter the trap (116 individuals). When nestlings were at least 8 days old, a trap was
158 placed in the inner side of the nest box hole allowing birds to enter, but not to leave. The latency
159 to enter the nest box started from the moment bird was in sight of the camera (approximately 1-2
160 meters from the nest box; observed from the video recordings). All traps were checked after
161 every 15 minutes. The trap was considered to be a more hidden novel object as it is difficult to
162 see from a distance, but imposing significantly stronger effect at proximity.
163
164 **Statistical analyses**

165 Data was analysed using R statistical software version 3.1.2 (R Development Core Team
166 2014). Linear Mixed-Effect Models (LMM) with restricted maximum likelihood (REML), type
167 III sum of squares and Satterthwaite approximation for degrees of freedom were conducted using
168 “Anova” from the car package and “lmer” function in the lme4 package. F-statistics were
169 obtained using the “lmerTest” package. R package “compute.es” was used for calculating effect
170 sizes (Fisher’s z).
171 Responses to a novel object and a trap during the breeding time are potentially affected
172 by several confounding factors. First, in the analysis using data over two different years, we

examined the effect of genotype, parental sex, genotype by sex interaction, sex order (two levels: the first or second parent to enter the nest box), treatment (two levels: control, novel object), habitat (deciduous versus coniferous) and brood size (as a covariate) on the latency to enter the nest box. Year, individual ID (to account for repeated measures) and brood identity (female and male parent form a breeding pair) were also included in this model as random factors. Secondly, in the analysis on the latency to enter the nest box in response to the nest box trap, we examined the effect of genotype, parental sex, sex order, habitat (deciduous versus coniferous) brood size (as a covariate) and brood identity (as a random factor) and we included the interaction between genotype and sex. In none of these above models, sex order and the habitat type (two levels: deciduous or coniferous habitat) were significant and they were therefore omitted from final models. Third, using data on different breeding parameters (separate models for the start of egg-laying, clutch size, number of hatched young, number of fledged young), we examined the effect of genotype, parental sex, genotype by sex interaction, habitat, brood size as fixed factors and individual ID, brood identity and year as random factors.

Pairwise post hoc comparisons were carried out with Tukey HSD post hoc multiple comparison tests (R package lsmeans; Lenth, 2014). Latency to enter the nest box was log (base)-transformed before the analysis to meet normality assumptions of residuals from the models. Breeding parameters were normalized using Box-Cox power transformation (R package MASS) (Venables and Ripley, 2002). Standardized β values were obtained from models with standardized variables. In order to cover allelic and genotypic effects, we used a general genetic model (that retains the 3 distinct genotype categories), an additive model (assuming that each additional copy of the variant allele increases the response; genotypes coded as a continuous predictor) and an over-dominant model (wherein heterozygous individuals are tested against homozygous individuals; genotype categories coded as heterozygotes versus homozygotes). In order to take into account multiple testing, we calculated critical P-values by using Holm´s correction for the number of independent SNPs: $P_{critical} = 0.0125$.

Results

The *SERT* gene is polymorphic in our great tit population. Three SNPs were identified in the promoter region (SNP136, SNP290 and SNP478), one SNP in exon 1 (SNP187), two SNPs in exon 3 (SNP253 and SNP278) and three SNPs in exon 8 (SNP197, SNP407, SNP457). Other exonic regions (exons 2, 4, 5, 6, 7, 9-13) studied were monomorphic. SNP197 in exon 8 is non-synonymous (complex data) and could affect behaviour through amino acid change. SNP457 in exon 8 is also non-synonymous and results in an amino acid change from proline (Pro) to leucine (Leu). SNPs found in the promoter region are the same as the SNPs detected by Riyahi et al (2015), but the number of significant SNPs varies between the populations, as Riyahi and colleagues found more polymorphic nucleotides (11) in this gene region.

Four SNP variants in our population deviated significantly from Hardy-Weinberg equilibrium even after correcting for multiple testing (Table 2) and were removed from further analysis. All the other allele frequencies tended to stay similar over the years in all SNPs. Underrepresented genotypes were both present in low frequencies in 2012 and 2014 (Table 2). We found no complete linkage disequilibrium between SNPs (all $R^2<0.2 \pm 0.2$). Confidence intervals for promoter region were as follows: Kelly ZnS = 0.24; Rozas Za = 0.24; Rozas ZZ = 0.03. For LD between exon and promoter, the CI are: Kelly ZnS = 0.00; Rozas Za = 0.00; Rozas ZZ = 0.00

Table 2

Alleles, protein coding and minor allele frequency (MAF) of each SNP for the *SERT* gene in Kilingi-Nõmme population over 2 years (2012, 2014). Population sample size: 232 [111 individuals (2012) and 121 individuals (2014)].

SERT locus	major/minor allele(s)	Location	MAF (minor allele frequency)	df	χ^2	HW P-value	Holds: $P_{critical}$= 0.0062 yes/no	Protein coding
SNP136	a/g	Promoter	20.3%	2	6.594	0.036	yes	
SNP290	a/g	Promoter	30.5%	2	18.02	0.000	no	

SNP478	c/t	Promoter	29.5%	2	22.50	0.000	no	
SNP187	a/t	exon 1	29.4%	2	4.425	0.109	yes	synonymous
SNP253	c/t	exon 3	18.3%	2	6.448	0.039	yes	synonymous
SNP278	a/g	exon 3	20.7%	2	15.99	0.000	no	non-synonymous
SNP197	c/t	exon 8	41.0%	2	0.163	0.921	yes	non-synonymous
SNP407	a/t	exon 8	36.2%	2	19.18	0.000	no	Synonymous
SNP457	a/g	exon 8	48.7%	2	2.408	0.299	yes	non-synonymous

Response to novel objects

In the complete sample (combined data of 2012 and 2014), no effects of sex and treatment on the delay in nest visitation were detected (Table 3). There was a positive correlation between the latency in the control and novel object phase [$\beta = 0.36 \pm 0.07$ (SE), $P < 0.001$, $N = 87$ nests; corrected for sex, brood size and sex order]. We also detected significant intra-individual repeatability for the latency between the control and novel object phase (repeatability = 0.67, $F_{1, 381} = 5.13$, $P = 0.024$; sex: $F_{1, 381} = 1.53$, $P = 0.22$). SNP253 in exon 3 was significantly associated with the delay in nest visitation (Table 3, Figure 1). Birds with a CC genotype resumed feeding significantly earlier than those with a CT genotype. There was also a tendency for an association between SNP197 in exon 8 and the delay in nest visitation, but this relationship was not significant after correcting for multiple testing (Table 3). The explained variance in the feeding delay by SNP253 in exon 3 was 11% (R-squared effect size, see Figure 2 for effect sizes). Due to low the frequency of the minor allele at SNP253 (exon 3), additive and over-dominant models were not fitted for this site (Table 3).

Table 3

General (non-specified), additive and over-dominant genetic models for feeding delay in great tits in 2012 and 2014. Effects in bold remained significant after correction for multiple testing. Interactive effects with P > 0.05 were omitted from final models.

	Promoter SNP136		Exon1 SNP187		Exon3 SNP253		Exon8 SNP197		Exon8 SNP457	
	F	P	F	P	F	P	F	P	F	P
1. General models										
Treatm	0.01	0.90	0.15	0.69	1.17	0.28	0.39	0.53	0.09	0.77
SNP	0.49	0.61	0.05	0.94	7.48	**0.007**	4.41	0.014	0.01	0.98
Sex	1.43	0.23	3.46	0.07	2.47	0.12	1.08	0.30	1.86	0.18
brood size	0.21	0.64	0.55	0.46	0.53	0.47	0.18	0.67	0.01	0.98
Treatm*SNP	-	-	-	-	6.18	0.014	-	-	3.53	0.033
N (pairs)	89		89		88		88		81	
2. Additive models										
SNP	-	-	0.01	0.92	-	-	3.89	0.05	0.01	0.95
Treatm*SNP	-	-	-	-	-	-	-	-	5.61	0.020
3. Over-dominant models										
SNP	-	-	0.02	0.87	-	-	0.44	0.51	0.04	0.95

In 2014 we also measured the time it took birds (N = 50 nests) to enter the nest box in response to the nest box trap. The latency in the control phase was positively related to the latency in the novel object phase (β ± SE = 0.40 ± 0.10) and trapping phase (β = 0.48 ± 0.12). A positive association was also found between the latency in the novel object and the trapping phase (β = 0.24 ± 0.11, N = 50 nests), while correcting for sex, brood size and sex order. The latency to enter the nest box differed significantly between all three treatment-phases ($F_{2, 216.0}$ = 19.84, P < 0.001), being the longest in the trapping (mean ± SE: 847 ± 59 s), intermediate in the novel object on the nest box (494 ± 53 s) and the shortest in the control (281 ± 58 s) phase (all post-hoc tests P < 0.01). In the trapping phase, females delayed nest visitation for a longer time than males ($F_{2, 17, 53}$ = 13.52, P = 0.002) and the latency differed between SNP197 genotypes (exon 8) ($F_{2, 19, 92}$ = 6.06, P = 0.008; post-hoc tests; CC vs. CT: p = 0.029; CC vs. TT: p = 0.011,

CT vs. TT: p = 0.99; Figure 3). We also found an association between SNP253 in exon 3 and the delay in nest visitation ($F_{2, 43} = 3.83$, P = 0.029) in response to the nest box trap, although this relationship did not remain significant after correcting for multiple testing.

Breeding parameters

Egg-laying started significantly earlier in deciduous than in coniferous forests ($F_{1, 100.1} = 18.25$, $p < 0.001$). SNP457 in exon 8 was significantly associated with the start of egg-laying in the additive model [Table 4, Fisher's z (effect size) = 0.36; confidence limits (CL): lower = 0.14, CL upper = 0.58]. Females with AG (mean ± SE = 26.62 ± 0.31, N = 61; post-hoc p = 0.004) and AA genotype (26.64 ± 0.52, N = 21; post-hoc p = 0.024) started egg-laying earlier than those with GG genotype (28.00 ± 0.50, N = 23), while no difference was observed between AG and AA genotypes (p = 0.99). Clutch size was on average larger in deciduous than in coniferous forests ($F_{1, 106, 85} = 8.50$, P = 0.003, N = 113 broods), but was not related to any *SERT* polymorphism (all P-values > 0.21) (Table 4).

Brood size at hatching did not depend on habitat ($F_{1, 91.35} = 3.26$, P = 0.07, N = 94 broods) and did not associate with any SNP in a general or an additive genetic model (Table 4). However, in an over-dominant model, SNP197 in exon 8 was associated with the number of hatched young. This relationship was sex-specific **(**sex: $F_{1, 42.7} = 1.09$, P = 0.30, sex × SNP: $F_{1, 44.16} = 4.28$, P = 0.044**)** and significant in females only ($F_{1, 73.0} = 7.43$, P = 0.008; Table 4). Post-hoc analysis among females showed that CT-heterozygotes had larger broods at hatching than CC- and TT-homozygotes combined (mean ± SE = 10.79 ± 0.56 versus 9.86± 0.54 offspring). Brood size on day 15 post-hatch (almost fledging) was neither related to habitat [$F_{1, 95, 45} = 4.80$, P = 0.029 ($P_{critical} = 0.0125$), N = 98 broods], nor to any of the genetic polymorphisms (all P-values > 0.08; Table 4).

Table 4

General (M1), additive (M2) and over-dominant (M3) genetic models for breeding traits in great tits in 2012 and 2014. Only SNP-related effects are shown. Effects in bold remained significant after correction for multiple tests. Day 1 = April 1st.

	Promoter SNP136		Exon1 SNP187		Exon3 SNP253		Exon8 SNP197		Exon 8 SNP457	
	F	P	F	P	F	P	F	P	F	P
(a) Egg-laying onset (Models M1, M2, M3: SNP + habitat, N = 104 broods)										
SNP (M1)	1.05	0.35	0.66	0.52	0.47	0.62	0.92	0.40	4.21	0.021
SNP (M2)	-	-	0.98	0.33	-	-	0.02	0.90	**8.04**	**0.005**
SNP (M3)	-	-	1.04	0.31	-	-	1.63	0.20	3.28	0.07
(b) Clutch size (Models M1, M2, M3: SNP + habitat + sex + habitat + SNP × sex, N = 104 broods)										
SNP (M1)	0.71	0.49	1.40	0.26	1.60	0.21	0.59	0.56	0.23	0.80
SNP (M2)	-	-	0.87	0.36	-	-	0.57	0.45	0.15	0.70
SNP (M3)	-	-	2.64	0.12	-	-	0.70	0.42	0.37	0.69
(c) Brood size on day 1 (Models M1, M2, M3: SNP + sex + habitat + SNP × sex, N = 94 broods)										
SNP (M1)	2.55	0.08	0.42	0.66	1.43	0.49	1.92	0.16	0.13	0.87
SNP (M2)	-	-	0.90	0.34	-	-	1.75	0.19	0.49	0.48
SNP (M3)	-	-	1.70	0.20	-	-	**8.17**	**0.006**	0.39	0.68
Sex*SNP (M3)	-	-	-	-	-	-	4.28	0.044	-	-
SNP (M3) (females only)							**7.43**	**0.008**		
SNP (M3) (males only)							0.30	0.64		
(d) Brood size on day 15 (Models M1, M2, M3: SNP + sex + habitat + SNP × sex, N = 114 broods)										
SNP (M1)	0.81	0.67	1.67	0.20	2.05	0.06	0.24	0.79	0.19	0.83
SNP (M2)	-	-	2.09	0.15	-	-	0.42	0.52	0.14	0.71
SNP (M3)	-	-	0.01	0.90	-	-	0.98	0.32	3.34	0.042

Discussion

In this study, we determined whether SNPs in the coding regions of *SERT* gene could predict behavioural differences in a natural population of great tits. We found that SERT plays indeed an important role in individual behavioural response and this effect is consistent over sexes and contexts. Also, the *SERT* gene could be related to fitness-related traits as one of the SNPs associated significantly with breeding parameters. In this study we associated genetic variability in four polymorphic exonic regions and the promoter area in the *SERT* gene to variation in the delay in feeding in response to a mild stressor (camera set-up in the control phase), a moderate stressor (novel object) and a strong stressor (nest box trap) and three fitness related traits (start of egg-laying, clutch size and brood size). Of those five regions, SNPs in exons 3 and 8 significantly associated with behavioural traits and SNPs in exon 8 with breeding parameters. SNP197 and SNP457 in exon 8 are non-synonymous and have the potential to affect behaviour through amino acid change. The polymorphism in exon 3 is synonymous, thus not altering the amino acid sequence and therefore it is less likely that the relationship between the SNP253 in exon 3 and parental reproductive behaviour represents a causal relationship. However, this affect could be mediated via linkage with other non-synonymous polymorphisms, effects on splicing or on transcription (Chamary et al., 2006).

Hardy-Weinberg principle was violated in some cases, indicating that some genotypes are constantly missing or underrepresented on both years (Table 2). There is a certain probability that sequencing errors may be the cause of this. However, these differences tended to stay similar over the years despite good-quality DNA and repeated sequencing. The likely cause of HWE deviations can be complex, especially in a wild outbred population. Mate choice, mutation rates, selection, genetic drift and gene flow are not stable over time as mating is not random, there is migration in open populations and some of the individuals may be more successful in passing on their genes to the next generation than others (Hartl et al., 1997). When we compare our results with an earlier study where the *SERT* promoter region was sequenced (Riyahi et al., 2015), we see that the number of detected polymorphisms in the promoter area differs between the studies. We assume that the main differences are caused by the differences in PCR product length, where especially the ends of sequences could vary due to the variation in sequence quality. Moreover, some SNPs found in the Spanish population (Riyahi et al., 2015) were not polymorphic in our population.

Behavioural variation

We found that two newly discovered SNPs in exon 3 and exon 8 potentially play an important role in novelty and risk-taking related behaviours of wild great tits. In our experiment, we manipulated nestling feeding behaviour by exposing parents to different novel stimuli. As a result, we found that the delay in feeding was the longest in the trapping phase, intermediate in the novel object phase and the shortest in the control phase. Given that feeding interruption after exposure to a stressor is a reliable measure of parental fear level (Tilgar et al., 2011; Van der Veen and Sivars, 2000), we have reason to expect parents to be differently stressed when facing a novel or threatening object. We also found that latency behaviours measured in the presence of different stressors were moderately correlated, suggesting that this trait is repeatable over context. In an earlier study, a polymorphism found in the *SERT* gene promoter region was found to associate with exploration behaviour in great tits (Riyahi et al., 2015), indicating a more general role for *SERT* in great tit personality traits.

We suggest that genotype-behaviour associations are relatively consistent over different situations. Despite varying fear levels, genotypic effects on the latency response remained the same in different experimental phases. Heterozygous individuals carrying CT genotype at the SNP253 (exon 3) tended to have significantly longer delays in feeding nestlings in all situations compared to homozygous CC genotype. In the case of SNP197 (exon 8), significant differences between genotypes emerged when the latency response during the most stressful manipulation (trapping phase) was measured. We found that homozygous individuals carrying CC genotype at the SNP197 had longer feeding delay than CT and TT genotypes.

Breeding success and *SERT*

We hypothesized that genetic differences in *SERT* may affect breeding success via changes in behavioural decision-making. Here we show for the first time that different *SERT* genotypes are associated with breeding time as well as offspring number at hatching. Firstly, starting egg-laying earlier could be beneficial especially in seasonal environments where the selection pressure on timing of reproduction exists as the nestling phase falls into the peak of caterpillars (Visser et al., 1998). Since *SERT* mainly affects behaviour-related traits, we suggest that the impact on breeding time could be linked to serotonin effects on feeding behaviour

(Magalhães et al., 2010), which is important for reaching breeding condition. Alternatively, it could be mediated via serotonin effects on hormonal levels, such as prolactin. In this respect, it has been shown that prolactin is secreted by anterior pituitary cells under the stimulatory control of vasoactive intestinal polypeptide which activity is controlled by neural pathways containing dopamine, serotonin and opioids (Freeman et al., 2000).

Second, the number of hatchlings was higher in CT heterozygotes compared to TT and CC homozygotes combined (overdominant model). In the great tit, a female incubates eggs alone and the male feeds her during incubation (Ridley, 2008). Thus, we can assume that heterozygotes can recover more rapidly from disturbing events and resume parental activities, such as incubation and seeking for food, after shorter delay than homozygotes. Genetic polymorphisms in these loci are non-synonymous, potentially affecting *SERT* enzymatic activity and neuronal information processing in the central nervous system (Freeman et al., 2000). In order to understand these effects in the future, we have to keep in mind that the potential impact of *SERT* and the polymorphisms are linked with fitness through behavioural decisions.

The impact of genotype on reproduction could indeed go via individual behavioural decisions (Dingemanse and Wolf, 2010). This can especially be true for reproductive traits that are more related to parental sensitivity to environmental stressors through decision-making (e.g., incubation or provisioning behaviour), rather than the traits that directly depend on the parental quality and food availability (e.g., clutch size and egg size). Hence, in a heterogeneous environment, individually consistent behaviours can be adaptive in a particular environment (e.g., reduced fearfulness can be related to increased parental care in stressful environments), but maladaptive in the other context; for example shy individuals can be less effective in finding novel food sources (Bell et al., 2007). Thus, it is likely that fluctuating natural selection translates genetically driven behavioural patterns into individual differences in fitness components. This could lead to the maintenance of genetic polymorphisms in natural populations.

Data Accessibility

DNA sequences: Genbank accessions: KP869099 and SRS1185780 (Laine et al 2016; Riyahi et al., 2015).

Acknowledgements

We kindly thank Jüri Parik for the help in sequencing and analysing data. We also thank Kaarin Koosa for the help in the field. We also thank all the reviewers for reading and commenting the manuscript. This study was financed by an Institutional Research grant no. IUT34-8 from the Estonian Ministry of Education and Research. Authors declare no competing interests.

References

Adriaenssens, B., and Johnsson, J. I. (2010). Shy trout grow faster: exploring links between personality and fitness-related traits in the wild. *Beh. Ecol.* **22** (1): 135-143.

Bakermans-Kranenburg, M. J., and van Ijzendoorn, M. H. (2008). Oxytocin receptor (OXTR) and serotonin transporter (5-HTT) genes associated with observed parenting. *Soc. Cogn. Affect. Neur.* **3(2)**, 128-134.

Bell, A. M., Backstrom, T, Huntingford, F.A., Pottinger, T. G and Winberg, S. (2007) Variable behavioural and neuroendocrine responses to ecologically relevant challenges in sticklebacks. *Physiol. Behav*. **91**, 15-25

Both, C., Dingemanse, N. J., Drent, P. J., and Tinbergen, J. M. (2005). Pairs of extreme avian personalities have highest reproductive success. *J. An. Ecol.* **74**(4), 667-674.

Canli, T., and Lesch, K. P. (2007). Long story short: the serotonin transporter in emotion regulation and social cognition. *Nat. Neurosci.* **10**(9), 1103-1109.

Carere, C. and Maestripieri, D. (2013). Animal personalities: behaviour, physiology, and evolution. University of Chicago Press.

Caspi, A., Sugden, K., Moffitt, T.E., Taylor, A., Craig, I.W., Harrington, H., McClay, J., Mill, J., Martin, J., Braithwaite, A. and Poulton, R., (2003). Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science* **301**(5631), 386-389.

Chamary, J. V., Parmley, J. L., and Hurst, L. D. (2006). Hearing silence: non-neutral evolution at synonymous sites in mammals. *Nat. Rev. Gen*. **7**(2), 98-108. DOI:10.1038/nrg1770

Champoux, M., Bennett, A., Shannon, C., Higley, J. D., Lesch, K. P., and Suomi, S. J. (2002). Serotonin transporter gene polymorphism, differential early rearing, and behaviour in rhesus monkey neonates. *Mol. Psych*. **7**(10), 1058-1063. DOI:10.1038/sj.mp.4001157

Dingemanse, N. J., and Wolf, M. (2010). Recent models for adaptive personality differences: a review. *Phil. Trans. Roy. Soc. B*. **365**(1560), 3947-3958.

Fidler, A. E., van Oers, K., Drent, P. J., Kuhn, S., Mueller, J. C. and Kempenaers, B. (2007). *DRD4* gene polymorphisms are associated with personality variation in a passerine bird. *Proc. Roy. Soc. B*. **274**:1619. DOI: 10.1098/rspb.2007.0337

Finn, D. A., Rutledge-Gorman, M. T., and Crabbe, J. C. (2003). Genetic animal models of anxiety. *Neurogen*. **4**(3), 109-135.

Freeman, M. E., Kanyicska, B., Lerant, A., and Nagy, G. (2000). Prolactin: structure, function, and regulation of secretion. *Physiol. Rev*. **80**(4), 1523-1631.

Garamszegi, L.Z., Mueller, J.C., Markó, G., Szász, E., Zsebők, S., Herczeg, G., Eens, M. and Török, J. (2014). The relationship between *DRD4* polymorphisms and phenotypic correlations of behaviours in the collared flycatcher. *Ecol. Evol.* **4**(8), 1466-1479.

Gaunt, T. R., Rodríguez, S., and Day, I. N. (2007). Cubic exact solutions for the estimation of pairwise haplotype frequencies: implications for linkage disequilibrium analyses and a web tool'CubeX'. *BMC Bioinformatics.* **8**(1), 428.

Gryglewski, G., Lanzenberger, R., Kranz, G. S., and Cumming, P. (2014). Meta-analysis of molecular imaging of serotonin transporters in major depression. *J. Cerebr. Blood F. Metabol*. **34**(7), 1096-1103.

Hartl, D. L., Clark, A. G., and Clark, A. G. (1997). Principles of population genetics (Vol. 116). Sunderland: Sinauer Associates.

Iwanir, S., Brown, A.S., Nagy, S., Najjar, D., Kazakov, A., Lee, K.S., Zaslaver, A., Levine, E. and Biron, D. (2016). Serotonin promotes exploitation in complex environments by accelerating decision-making. *BMC Biology.* **14**(1), 1.

Korsten, P., Mueller, J.C., Hermannstädter, C., Bouwman, K.M., Dingemanse, N.J., Drent, P.J., Liedvogel, M., Matthysen, E., van Oers, K., van Overveld, T. and Patrick, S.C. (2010). Association between *DRD4* gene polymorphism and personality variation in great tits: a test across four wild populations. *Mol. Ecol*. **19**(4): 832-843.

Laine, V.N., Gossmann, T.I., Schachtschneider, K.M., Garroway, C.J., Madsen, O., Verhoeven, K.J., De Jager, V., Megens, H.J., Warren, W.C., Minx, P. and Crooijmans, R.P. (2016). Evolutionary signals of selection on cognition from the great tit genome and methylome. *Nat. Comm*. **7**: 10474.

Laine, V. N., and van Oers, K. (2017). The quantitative and molecular genetics of individual differences in animal personality. In *Personality in Nonhuman Animals* (pp. 55-72). Springer, Cham.

Lenth, R.V. 2014. lsmeans: Least-squares means. R package version 2.00-5. DOI:10.18637/jss.v069.i01

Lesch, K. P., Bengel, D., Heils, A., and Sabol, S. Z. (1996). Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. *Science*. **274**(5292), 1527.

Lerch-Haner, J. K., Frierson, D., Crawford, L. K., Beck, S. G., and Deneris, E. S. (2008). Serotonergic transcriptional programming determines maternal behavior and offspring survival. *Nat Neurosci*, **11**(9), 1001-1003.

Librado, P., and Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics.* **25**: 1451-1452.

Magalhães, C.P., de Freitas, M.F., Nogueira, M.I., Campina, R.C., Takase, L.F., de Souza, S.L., de Castro, R.M. (2010). Modulatory role of serotonin on feeding behaviour. *Nutr. Neurosci.* **13**: 246-255.

Murphy, D.L., Fox, M.A., Timpano, K.R., Moya, P.R., Ren-Patterson, R., Andrews, A.M., Holmes, A., Lesch, K.P. and Wendland, J.R. (2008). How the serotonin story is being rewritten by new gene-based discoveries principally related to SLC6A4, the serotonin transporter gene, which functions to influence all cellular serotonin systems. *Neuropharmacology.* **55**(6), 932-960.

Nettle, D. (2006). The evolution of personality variation in humans and other animals. *American Psychologist*, ***61***(6), 622.

van Oers, K., de Jong, G., Drent, P. J., and van Noordwijk, A. J. (2004a). A genetic analysis of avian personality traits: correlated, response to artificial selection. *Behav. Genet*. **34**(6), 611-619.

van Oers, K., De Jong, G., Van Noordwijk, A. J., Kempenaers, B., and Drent, P. J. (2005). Contribution of genetics to the study of animal personalities: a review of case studies. *Behaviour.* **142**(9-10), 1185-1206.

O'Leary, O. F., and Cryan, J. F. (2010). The Behavioural Genetics of Serotonin: Relevance to Anxiety and Depression. Handbook of Behavioural Neuroscience, **21**, 749-789.

Pagani-Núñez, E., and Senar, J. C. (2013). One hour of sampling is enough: Great Tit Parus major parents feed their nestlings consistently across time. *Acta Ornithol*. **48**(2), 194-200.

R Development Core Team (2014) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Ridley, A. R. (2008). Handbook of the Birds of the World. Volume 12: Picathartes to Tits and Chickadees.

Riyahi, S., Sánchez-Delgado, M., Calafell, F., Monk, D., and Senar, J.C. (2015). Combined epigenetic and intraspecific variation of the *DRD4* and *SERT* genes influence novelty seeking behaviour in great tit Parus major. *Epigenetics*, **10**, 516-525. doi:10.1080/15592294.2015.1046027

Savitz, J. B., and Ramesar, R. S. (2004). Genetic variants implicated in personality: a review of the more promising candidates. *Am. J. Med. Genet. B*. **131**(1), 20-32.

Serretti, A., Calati, R., Mandelli, L., and De Ronchi, D. (2006). Serotonin transporter gene variants and behaviour: a comprehensive review. *Curr. Drug Targets.* **7**(12), 1659-1669.

Smith, B. R. and D. T. Blumstein (2008). "Fitness consequences of personality: a meta-analysis." *Behav. Ecol.* **19**(2): 448-455.

Søeby, K., Larsen, S. A., Olsen, L., Rasmussen, H. B., and Werge, T. (2005). Serotonin transporter: evolution and impact of polymorphic transcriptional regulation. *Am. J.Med. l Genet. B*. **136**(1), 53-57.

Tilgar, V., Moks, K., and Saag, P. (2011). Predator-induced stress changes parental feeding behaviour in pied flycatchers. *Beh. Ecol*. **22**(1), 23-28.

Timm, K., Tilgar V and Saag, P. (2015). *DRD4* gene polymorphism in great tits: gender-specific association with behavioural variation in the wild. *Beh. Ecol. Sociobiol*. **69**: 729-735.

Van der Veen, I. T., and Sivars, L. E. (2000). Causes and consequences of mass loss upon predator encounter: feeding interruption, stress or fit-for-flight? *Func. Ecol*. **14**(5), 638-644.

Venables, W. N. and Ripley, B. D. (2002) Modern Applied Statistics with S. Fourth Edition. Springer, New York.

Visser, M. E., Van Noordwijk, A. J., Tinbergen, J. M., and Lessells, C. M. (1998). Warmer springs lead to mistimed reproduction in great tits (Parus major). *Proc. Roy. Soc. B.* **265**(1408), 1867-1870.

Vrublevska, J., Krama, T., Rantala, M. J., Mierauskas, P., Freeberg, T. M., and Krams, I. A. (2015). Personality and density affect nest defence and nest survival in the great tit. *Acta Ethol*. **18**(2), 111-120.

Figure legends

Figure 1

Feeding delay (over 2 years; 2012 and 2014) of female (empty circles) and male (filled circles) great tits (averaged over control and novelty phases) and the effect of *SERT* gene (exon 3 SNP253). Vertical bars denote SE, numbers are sample sizes. Individuals are wild-caught, released after measuring (see Methods). The experiment was conducted only in the wild population. P= 0.007 (see also Table 3). In order to take into account multiple testing, we calculated critical P-values by using Holm´s correction for the number of independent SNPs: $P_{critical} = 0.0125$. See detailed statistical methods in the Methods section.

Figure 2

Effect sizes with 95% confidence intervals for the feeding delay in relation to *SERT* genotypes (averaged over control and novelty seeking phases and over sexes) in 2012 and 2014 (combined data). Effect sizes were calculated between the genotypes with the largest difference in the latency behaviour. Individuals are wild-caught, released after measuring (see Methods). The experiment was conducted only in the wild population. P= 0.007 (see also Table 3). In order to take into account multiple testing, we calculated critical P-values by using Holm´s correction for the number of independent SNPs: $P_{critical} = 0.0125$. See detailed statistical methods in the Methods section.

Figure 3

The delay in nest visitation of great tits (averaged over sexes) in different treatment phases (trap/novel object/control) with respect to *SERT* SNP197 genotypes in exon 8 in 2014. Vertical bars denote SE, numbers are sample sizes. Individuals are wild-caught, released after measuring (see Methods). The experiment was conducted only in the wild population. P= 0.007 (see also Table 3). In order to take into account multiple testing, we calculated critical P-values by using Holm´s correction for the number of independent SNPs: $P_{critical} = 0.0125$. See detailed statistical methods in the Methods section.