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Faculty of Natural Resources and Agricultural Sciences

Polyandry and genetic diversity in populations of *Pholidoptera griseoaptera* along an environmental gradient

Polyandri och genetisk diversitet hos populationer av Pholidoptera griseoaptera längs en klimatgradient

Matilda Jutzeler

Photo: Peter Kaňuch 2018

Independent project • 15 hec

Biology and Environmental Science – Bachelor's Programme
Department of Ecology
Uppsala 2019

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Credits: 15 hec
Level: G2E
Course title: Independent project in Biology
Course code: EX0894
Programme/education: Biology and Environmental Science – Bachelor's programme
Course coordinating department: Department of Aquatic Sciences and Assessment

Place of publication: Uppsala
Year of publication: 2019
Cover picture: Peter Kaňuch
Online publication: https://stud.epsilon.slu.se

Keywords: polyandry, genetic diversity, multiple mating, microsatellites, nuptial gift, phenotype

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Abstract

Polyandry is a common mating pattern in different insect species and it leads to increased genetic diversity in the offspring and prevents inbreeding in populations. According to the theory of mate choice, mates should choose a partner that will increase heterozygosity in their offspring. In this way females will increase their reproductive success and offspring will have higher fitness and be good competitors. Phenotypic traits such as large body size is preferred by both females and males crickets according to earlier studies. The environment can also have an influence on mating pattern as sexual selection could vary depending on the environment. I used the dark bush-cricket, *Pholidoptera griseoaptera* in an experiment to examine the mechanisms behind polyandry and the correlation between phenotypic, genetic and environmental factors. My general aim was to broaden the knowledge about the mating system in this insect species. The study was placed at Slovak Academy of sciences in Slovakia. Bush-crickets from five different altitudes were collected, and mating experiments was performed using the offspring of these animals. Eleven mating groups were used with 5 females and 5 males in each group. The bush-crickets were kept in either warm or cold temperatures in open-air cages. Microsatellites was used to identify genetic diversity of populations to examine if there was a correlation with the frequency of copulations. Measurement of body-size were performed on each cricket and estimation of number of copulations were done for each female.I found that in warm temperature, females of *P. grisoptera* had the highest frequency of copulations and there males choose high quality females. In cold temperature there were less copulations, indicating that the environmental condition restrict the ability for mate choice. This study is only a snapshot of the pattern of polyandry and the behaviour needs to be studied further, but it has broadened the knowledge about the mating system in this nuptial gift-giving insect species.

Sammanfattning

Polyandri är ett vanligt parningsmönster hos olika insektsarter och det leder till ökad genetisk mångfald hos avkomman och förhindrar inavel i populationer. Enligt teorin om partnerval ska individer välja en partner som ökar heterozygositeten i sina avkommor. På detta sätt kommer honorna att öka sin reproduktiva framgång och avkommorna kommer att ha högre fitness och vara starka konkurrenter. Fenotypiska egenskaper som stor kroppsstorlek föredras av både honor och hanar hos buskvårtbitare enligt tidigare studier. Klimatet kan också påverka parningsmönstret eftersom sexuellt urval kan variera beroende på miljön. Jag använde den mörka buskvårtbitaren, *Pholidoptera griseoaptera* i ett experiment för att undersöka mekanismerna bakom polyandri och korrelationen mellan fenotypiska, genetiska och miljömässiga faktorer. Mitt syfte var att bredda kunskapen om parningssystemet i denna insektsart. Studien utfördes vid Slovak Academy of Sciences i Slovakien. Buskvårtbitare från fem olika altituder samlades in, och parningsexperiment utfördes på avkommorna från dessa insekter. Elva parningsgrupper användes med 5 honor och 5 hanar i varje grupp. Buskvårtbitare hölls i antingen varma eller kalla temperaturer i burar i fält. Mikrosatelliter användes för att identifiera den genetiska mångfalden hos populationer för att undersöka om det fanns en korrelation med frekvensen av parningar. Mätning av kroppsstorlek utfördes på varje buskvårtbitare och uppskattning av antal parningar gjordes för varje hona. Jag fann att vid varm temperatur hade honorna hos *P. grisoptera* den högsta frekvensen av parningar och där väljer hanarna även högkvalitativa honor. Vid kall temperatur var det mindre parningar, vilket indikerar att det aktuella miljöförhållandet begränsar möjligheten till partnerval. Denna studie är bara ett litet prov av mönstret för polyandri och behöver studeras ytterligare, men det har breddat kunskapen om parningssystemet hos denna näringsgåvo-givande insektsart.

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1 Introduction

1.1 Multiple mating - polyandry

There are many insect species where individuals copulate multiple times with different partners during a breeding season (Dorková et al. 2018). Multiple matings leads to an increased genetic diversity in the set of offspring (measured as allelic richness or heteorozygosity). It also reduces the risk of inbreeding in the population. This mating strategy leads to indirect benefits for the parents as they are likely to produce offspring with better genetic fitness. To increase fitness, both males and females try to mate with the best individuals and there are often many competitors for these high-quality individuals (Dorková et al. 2018).

The female bush-cricket is polyandrous and copulates with more than one male during a breeding season. Multiple matings with several males may be advantageous if females copulate with a male of high genetic quality (‘good genes’) which will fertilize her eggs (Shuker & Simmons 2014). Except the indirect genetic benefit that the female may get from multiple matings, there are also direct benefits in form of the nuptial gift that males transfer during copulation (see Fig. 1)(Kaňuch et al. 2015). The male transfers a spermatophore containing a nutritious spermatophylax that is attached to the sperm-containing ampulla. The female consumes the spermatophylax after the copulation, and the sperm is stored in her spermatheca. These nuptial gifts have a direct positive effect on the female’s fitness such as increased offspring number, increased fecundity, assuring fertility and increased longevity (Shuker & Simmons 2014)

Figure 1. Female of dark bush-cricket Pholidoptera griseoaptera with nuptial gift. (Photo: Peter Kaňuch)

1.2 Genetic diversity and phenotype

According to the theory of mate choice, mates should choose a partner that will increase heterozygosity in their offspring (Brown 1997). This adaption is likely to produce offspring with higher fitness. The mate choice is done on a individual basis, and alleles that are good for one individual in one situation, may not be the optimal for other individuals in other settings. The female's strategy is expected to be to find a male that has alleles that best complement her own (Brown 1997). By using phenotypic traits to choose a male with good genes, females can increase their reproductive success. In a previous study by Dorková et al. (2018), they saw that body-size of the bush-cricket, *Pholidoptera griseoaptera*, is an important sexual trait and that both females and males prefer larger partners. Individual phenotypes may express 'good genes' in the form of trait size and this may be a reflection of heterozygosity at key loci or at many different loci. In choosing a partner with good genes the offspring will be likely be good competitors. Genetic variations of traits may also lead to a higher chance that adaptations to changing environment can occur (Brown 1997).

Neutral polymorphic genetic markers called microsatellites can be used to identify genetic diversity of populations. Analysis of microsatellites has become a popular

method to identify levels of genetic variability. Microsatellites are found throughout the chromosomes of most organisms and consist of arrays of up to several hundred simple sequence repeats. Microsatellite analysis results in pattern of DNA that shows an individual's genetic fingerprint. Individual genotypes based on the set of several microsatellites can identify individuals, their offspring, evaluate kinship and reveal differentiation among related populations (Marjorie A. Hoy 2003).

1.3 Environmental factors

The environment may also influence mating behavior and sexual selection in insects (Kaňuch et al. 2015). How well individuals can cope with the environment depends on both their genetic make-up and their phenotypic plasticity. Cold climate conditions can be difficult for individuals to cope with and for male bush-crickets it negatively effects the production of the spermatophore (Kaňuch et al. 2015). Reduced quality and quantity of sperms can have an influence on the mating pattern in the population. Males could be expected to be choosier in colder conditions and females then have to compete more for the limited supply of sperms. Tayler et al. (2014) used data from different taxonomic groups together in a plot and found that polyandry in colder environment is more common than in favorable conditions, but this finding needs to be verified (Kaňuch et al. 2015).

1.4 Study species

To study population genetic diversity and polyandry degree under different environmental conditions, the dark bush-cricket, *Pholidoptera griseoaptera,* (Orthoptera: tettigoniidae) was used. This species provides unique opportunities to examine hypotheses on mechanisms behind mating patterns. The ecology and behavior of this small bush-cricket is well studied and the mating system is known to be polyandrous. The species is easy to collect in the field, breed and handle in the lab.

The species is a common and has a wide distribution in central Europe. It can be found along an altitudinal gradient, from sea level to the tree line (Kaňuch et al. 2015). The species prefers habitats such as forest clearings, woodland edges and hedgerows and is strongly associated with tall herb, grass and shrub layers (Dieköt-ter et al. 2010)

The species is flightless but the male has short wings used for stridulation to attract females (Kaňuch et al. 2015). During copulation the male transfers a spermatophore to the female that contains ejaculate and a nuptial gift as a spermatophylax which the female consumes (Parker et al. 2017). After the fertilization during summer and

autumn the eggs are laid in rotten wood or wet soil (Diekötter et al. 2010). The eggs hatch either in the spring the following year or the next year (Parker et al., 2017). Nymphs then goes through six or seven nymphal instars until they reach adult stage (Diekötter et al. 2010)

1.5 Hypothesis

My hypotheses were: 1) that multiple copulations will relate to population genetic diversity that should facilitate adaptation in harsher environmental conditions and mating pattern will be influenced by 2) individual phenotype (body-size) and 3) actual treatment conditions (warm and cold).

1.6 Aim

The aim of this study was to examine the relationship between genetic diversity and degree of polyandry in *P. griseoaptera* populations originating from different climatic conditions. The general aim was to increase the knowledge about the mechanisms behind the mating pattern in this nuptial gift-giving insect species.

2 Materials and methods

2.1 Rearing of individuals and mating experiment

Bush-crickets (*P. griseoaptera*) were sampled at five different sites (see Fig. 2) with different altitudes in Slovakia in August 2017. They were transported to the lab and held in breeding containers were they were fed with leaves of European dewberries (*Rubus caesius*), dry cat food, oat-flakes and special foods for crickets which contains vitamins. Containers were cleaned every week and crickets were supplied with fresh drinking water. Females were then allowed to lay eggs in prepared Styrofoam bricks. The eggs were then held in climate-chambers under controlled conditions. The eggs first had a warm period of 3 months with 24°C, after that a embryonic diapause during 5 months with 5°C and then hatched during a period of 18°C. The nymphs were held separately to ensure that the adults were unmated. The adult individuals, which successfully developed, were randomly combined in mating groups of 5 males and 5 females and housed in open air cages (see Fig. 3) located in two different climatic conditions, one warmer and one colder (see Fig. 4). Six groups were treated in the warmer lowland experimental site (Fig. 2 - Mlyňany, red square) and five groups we treated in the colder mountain site Skalka (Fig. 2 - blue square). To be able to identify individuals throughout the study all were individually colour marked. Carbon monoxid anesthesia were used to calm the individuals down, then they were labeled on the top of the shield with different unique color combinations (Fig 5). After mating experiment that lasted for 14 days, bush-crickets were preserved in tubes with ethanol.

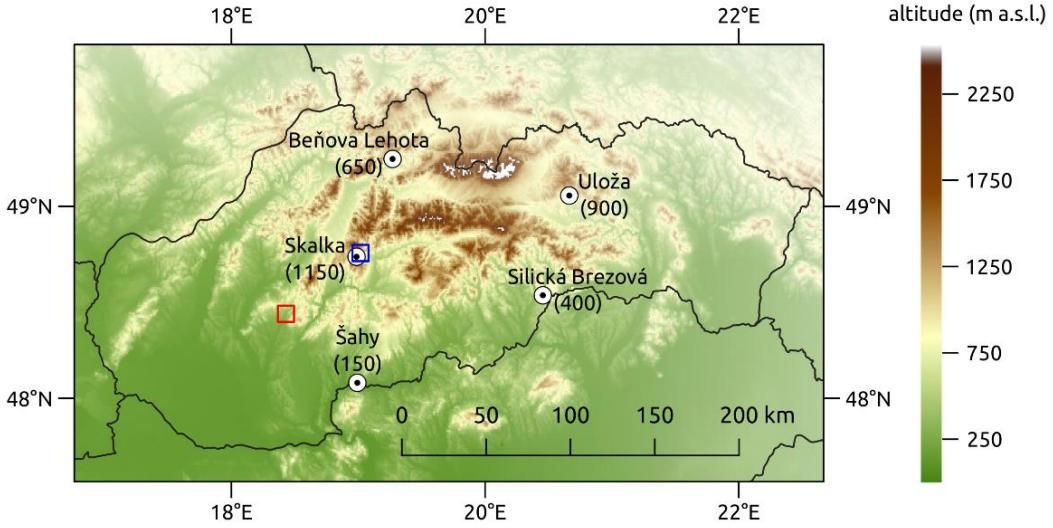

Figure 2. Sites were bush-crickets were sampled. Blue and red squares were the open air cages were placed during the trials.

Figure 3. Open-air cages used in the study. (Photo: Peter Kaňuch 2018)

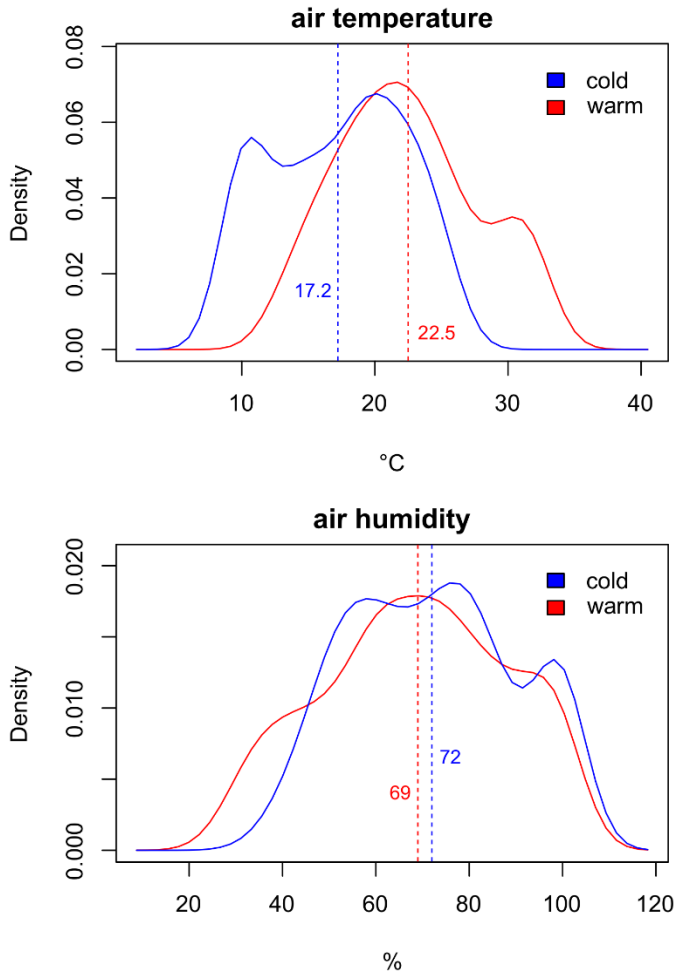

Figure 4. Temperature and humidity during the 2 week experiment. Blue - cold treatment (Skalka). Red - warm treatment (Mlyňany). Dotted lines show mean air humidity and mean air temperature

Figure 5. Id marked bush-crickets. (Photo: Peter Kaňuch 2018)

Table 1. *Number of bush-crickets from the five different populations (for locations see Fig. 2)*

population	altitude (m a.s.l.)	males	females	total
Šahy	150	4	2	6
Silicka Brezová	400	3	7	10
Beňova Lehota	650	25	23	48
Uloža	900	7	6	13
Skalka	1150	13	16	29
total		*52*	*54*	*106*

2.2 Measurement of body size

Total body length, length of the shield and femur were measured in all bush-crickets in the lab using a digital caliper. The body length was measured without the ovipositor in females and without the cerci in males.

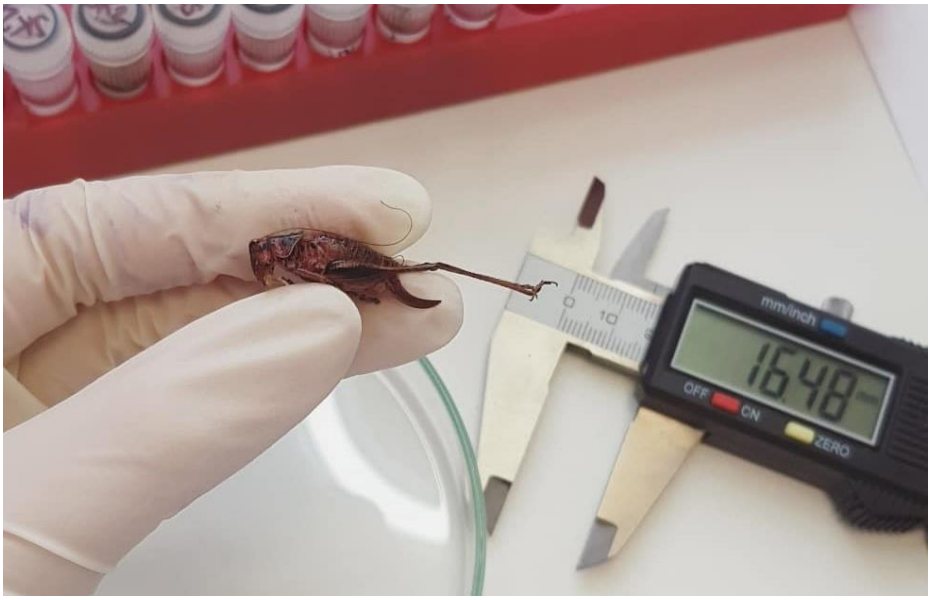

Figure 6. Measuring body-size with digital caliper. (Photo: Matilda Jutzeler 2019)

2.3 Dissection of spermatodoses

To count the number of copulations in each female the spermatodoses were dissected. After each copulation a spermatodose is formed and remains in the spermathaeca for the duration of the female's life (Kaňuch et al. 2015). The spermathaeca from the female was first dissected with needles under a magnifying glass and placed in a drop of Ringer's solution in a Petri dish. The number of spermatodoses were extracted and counted and placed in new tubes. Some females from the cold treatment were missing in the random mating groups or were either lost or dead during the experiment (see Appendix 1), so we were not able to measure number of copulations of these.

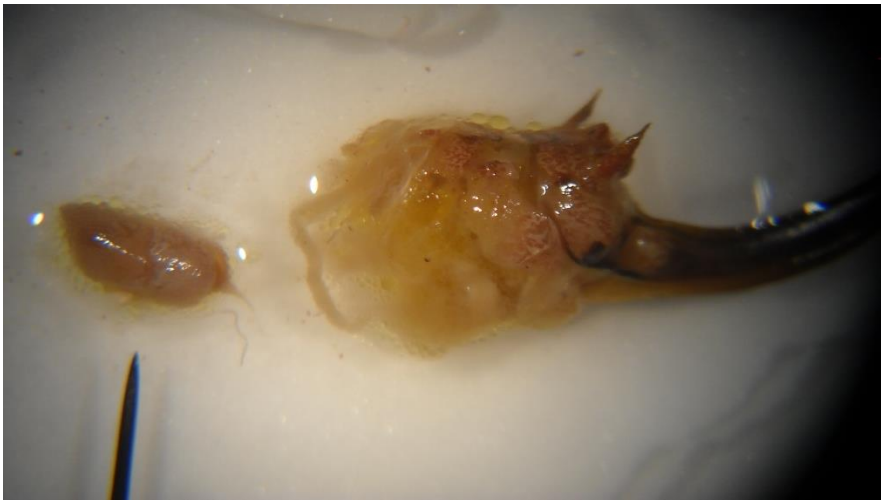

Figure 7. Spermathaeca. (Photo: Peter Kaňuch 2018)

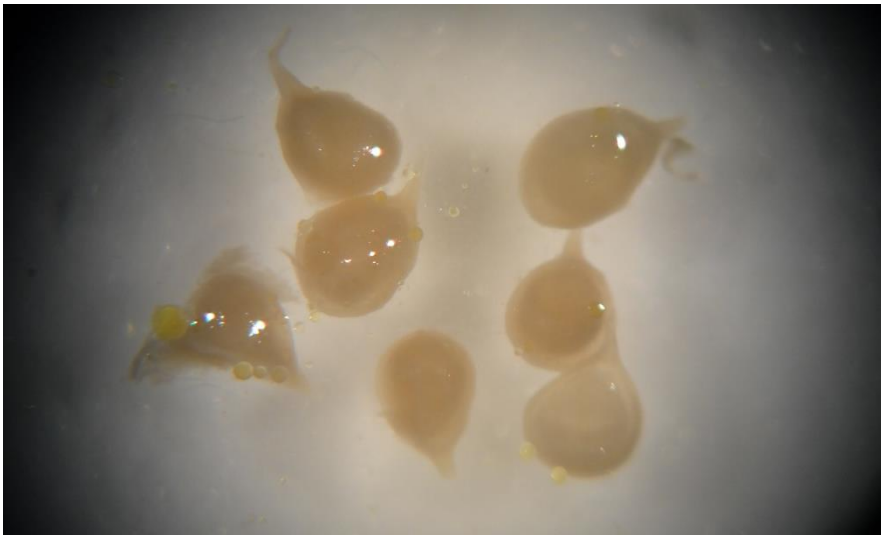

Figure 8. Spermatodoses. (Photo: Peter Kaňuch 2018)

2.4 Extracting DNA

DNA extraction was conducted using the Chelex 100 method (Walsh et al. 1991). This rapid and easy method extracts DNA of sufficient quality for amplification of microsatellite markers. Muscle tissue from femur was dissected with a scissor and pulled out with a tweezers. The tissue was dried for a few minutes and places in new tubes. The instruments used for dissection were sterilized by alcohol and fire between each cricket to avoid contamination.

In each tube with muscle tissue 4 µL of Proteinase K and 100 µL of 10% Chelex 100 was added. The samples were stirred for 10 seconds and then centrifuged. The samples were kept in a heating block in 56 °C for 3 hours to lysate the tissue. After this they were stirred and set in the heating block in 99 °C for 3 minutes to kill proteinase. Then the samples were stirred and centrifuged to collect the supernatant of 70 µL of each sample collected in new tubes.

A spectrophotometer (NanoDrop) was used to measure the purity and concentration of DNA in each sample. One microliter of each sample was loaded to the spectrophotometer and then measured, and the concentration of DNA was measured in ng/µL.

2.5 PCR and microsatellite loci

To optimize the PCR two tests with two random samples were conducted with different concentrations of primers and annealing temperature (a gradient PCR was ran). When the PCR was optimized all samples were run in the same concentration and temperature cycle.

Six pairs of microsatellite primers (WPG 10_1, WPG 1_28, WPG 2_30, WPG 8_2, WPG, 2_15 and WPG 1_27) were used (Arens et al. 2005) according to reported polymorphism and fragment size of amplified loci (Parker et al. 2017). The primer pair WPG 1_27 amplifies two microsatellite loci, so the samples were genotyped in seven microsatellite loci. Forward primers were 5-end labelled with different fluorescent dyes for simultaneous fragment analyses relative to the GeneScan 600 LIZ dye Size Standard.

To amplify microsatellites in multiplex PCR a mastermix was mixed that contained purified water, primers and HS Taq Mix (Table 2). Finally 18 µL of mastermix and 2 µL of DNA were used for one sample. The samples were centrifuged and run in the PCR thermocycler with the following program. PCR amplifications started with one initial activation step at 95°C for 15 min, followed by 30 cycles of denaturation at 94°C for 120 s, annealing at 60°C for 90 s and extension at 72°C for 60 s and finally an extension at 60°C for 30 min.

Table 2. *Mastermix of purified water, primers, HS Taq mix and DNA*

Ingredient		Final concentration	Volume (µL)
ddH2O			3.10
WPG 10_1	forward	0.10 µM	0.20
	reverse	0.10 µM	0.20
WPG 1_28	forward	0.15 µM	0.30
	reverse	0.15 µM	0.30
WPG 2_30	forward	0.23 µM	0.45
	reverse	0.23 µM	0.45
WPG 8_2	forward	0.45 µM	0.90
	reverse	0.45 µM	0.90
WPG 2_15	forward	0.15 µM	0.30
	reverse	0.15 µM	0.30
WPG 1_27	forward	0.15 µM	0.30
	reverse	0.15 µM	0.30
HS Taq Mix		1×	10.00
DNA			2.00

2.6 Gel electrophoresis and fragment analysis

To control that the amplifying of the microsatellites in the PCR was successful, gel electrophoresis were conducted. A 2% agarose gel was prepared and placed in a TAE buffer-filled bath. Then 5 µL of each sample and a ladder were loaded into the gel, and then the electric field were set on 50 Volts for 90 minutes. To visualize multiple bands of PCR products, the gel was illuminated by UV- light and photographed.

Fluorescent labelled PCR products of samples suspended in formamide and separated by capillary electrophoresis in an ABI3730XL Genetic Analyser (SEQme company). Electropherograms which showed alleles of each microsatellite locus were edited using Geneious 7.1.9 software (Biomatters, Auckland, New Zealand).

2.7 Statistical analysis

To examine if there was a relationship between the body-length (males and females separately), number of copulations (in different treatments), genetic diversity (allelic richness and heterozygosity) and altitude we used simple linear regressions.
For modeling the number of copulations of females we employed also a generalized linear model (GLM) with a Poisson error distribution, log-link function, and type II SS. The female body length, its homozygosity by loci (measure to estimate inbreeding level of a individual), experimental treatment (cold vs. warm) and altitude (i.e. origin of population) were used as fixed factors with full interactions. Data were analyzed by default packages of the R 3.4.4 environment for statistical computing (R Core Team, 2018). Interaction plots of the GLM were constructed by the R-package ‘effects’ (Fox et al., 2015).

3 Results

3.1 Differences between populations

Females had between 0 to 4 spermatodoses in their spermatheca. This means that some females had zero copulations and some had copulated up to four times. Under the cold treatment, there was a trend in that females from the population from the highest altitude (1150 m.a.s.l) copulated more than females from the lower altitudes (Fig. 9). Under the warm treatment there were more copulations than in the cold treatment, but there was no difference in the frequency of copulations in individuals from the different populations.

The body size of females increased while males' body size decreased towards higher altitudes (Fig. 9).

Genetic diversity showed a trend to increase with higher altitudes. Expected heterozygosity showed a similar pattern but was not significantly correlated with altitude (Fig. 9).

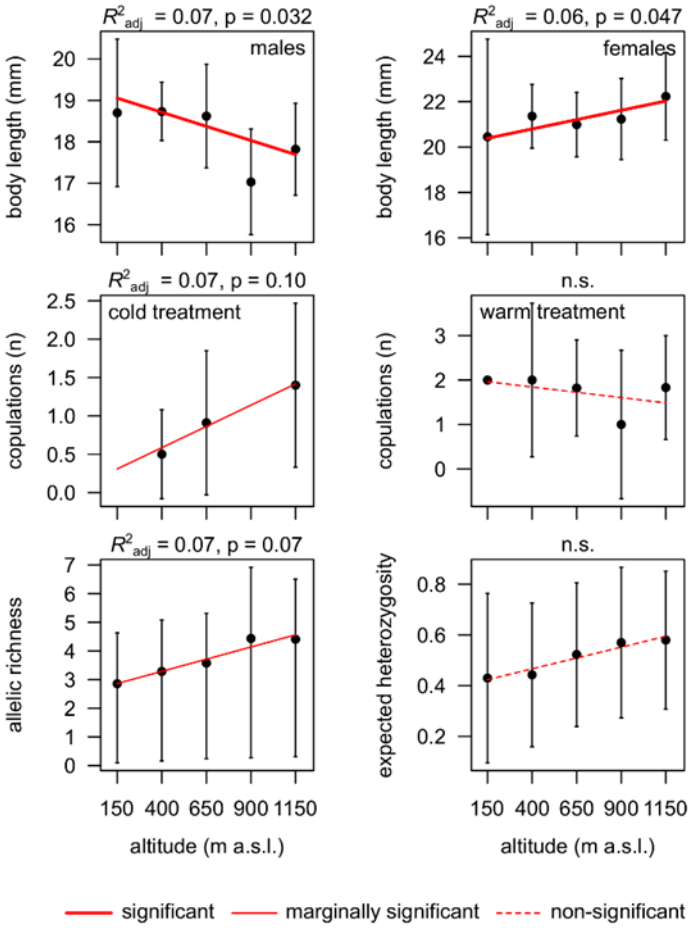

Figure 9. Body size, number of copulations and genetic diversity indices in relationshion to population origin altitude. Plots show means with ± standard deviations. Trend lines are derived form single linear regressions.

3.2 Number of copulations of a female

The degree of polyandry (number of copulations) was correlated to body-size (individual phenotype), treatment (environment quality – hot vs cold - during the mating experiment) and homozygosity by loci (genetic quality) (p = 0.038). The main effects of these three factors alone were not significant (Table 3).

Table 3. *Results of the generalized linear model examining the degree of polyandry. The number of copulations of females of Pholidoptera griseoaptera (dependent variable) is modelled by four fixed factors (independent variables) and their interactions (HL = homozygosity by loci – measure to estimate inbreeding level of individuals, body = body length of females, treatment = cold vs. warm treatment of mating experiment, altitude = origin of population). Significant interaction is in bold.*

Effect	χ^2	df	p
HL	0.03	1	0.87
Body	1.34	1	0.25
Treatment	3.12	1	0.08
Altitude	1.30	1	0.25
HL × body	0.03	1	0.87
HL × treatment	1.85	1	0.17
body × treatment	0.20	1	0.66
HL × altitude	0.27	1	0.61
body × altitude	2.58	1	0.11
treatment × altitude	1.15	1	0.28
HL × body × treatment	**4.32**	**1**	**0.038**
HL × body × altitude	0.66	1	0.42
HL × treatment × altitude	1.64	1	0.20
body × treatment × altitude	0.70	1	0.40
HL × body × treatment × altitude	0.01	1	0.94

In the warm treatment females with large body-size and the small females with higher genetic diversity (lower homozygosity by loci) copulated the most. In cold condition there were fewer copulations and the pattern was not so visible. There small females with low genetic diversity (higher homozygosity by loci) copulated most (Fig. 10).

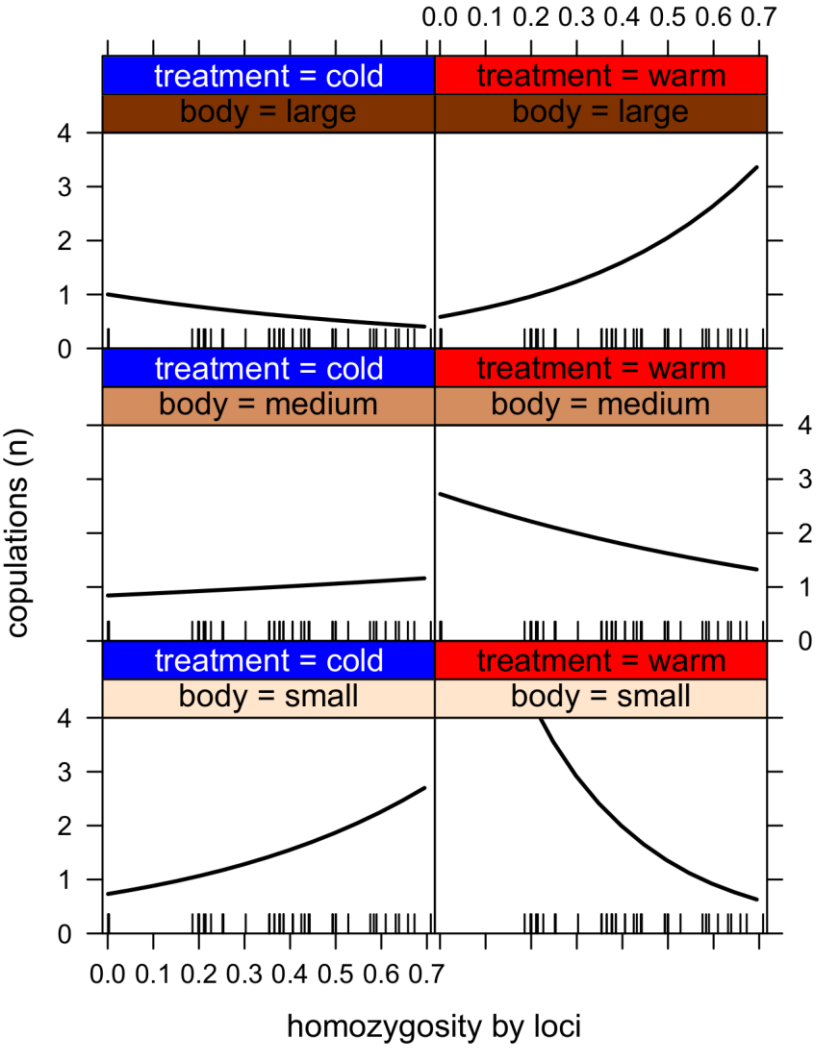

Figure 10. Interaction plot of the three factors that had significant effect on the number of copulations in females.

4 Discussion

Our experiment shows that the factors body-size, climate-condition and genetic quality influence the number of copulations in females (Fig. 10). In warm temperature, females of *P. grisoptera* had a higher degree of polyandry than females in cold temperature. There, also females with large body size and small females with high genetic diversity (lower lever of homozygosity) copulated the most. There it seems like males choose females with higher quality.

As my hypothesis 1 suggest, that multiple copulations will relate to population genetic diversity, the experiment show some relationship between the genetic diversity and frequency of copulations. Also, hypothesis 2 and 3 that mating pattern will be influenced by individual phenotype and actual treatment is verified in this experiment. But results show that the pattern differs according to population origin and actual condition.

In cold treatment (Fig 10) it seems like it's the environmental factor that dominates other factors and has the main-effect on the mating pattern. Maybe males do not copulate as much in cold temperature because they have to save nutrients and energy for the productions of nuptial gifts (Kaňuch et al. 2015). In warm conditions they can copulate more because they do not have the same energy limitation.
Choosing a female with larger body size confirms earlier experiments (Fig. 10 top right panel) which shows that body-size is an important sexual trait and that larger body-size is preferred (Dorková et al. 2018). A larger female is more fecund and may contain more fertile eggs which make these females more favorable for males (Kaňuch et al. 2014). Choosing a female with higher genetic fitness (and higher heterozygosity) also supports the theory that mates should choose a partner that will increase heterozygosity in their offspring (Fig 10. down right panel) (Brown 1997).

In mating groups in the cold treatment, there were less copulations and the mating pattern opposite of the warm treatment. Here, smaller females with low genetic

diversity had copulated the most (Fig 10. down left panel). Possibly individuals that originated from lower altitudes (warmer areas), could not adapt to the colder environment for these two weeks experiment. The cold-condition seems to have a large effect for individuals not adapted to such environment. That could explain why bush-crickets here don't copulate with best quality females.

In cold temperature, bush-crickets origin from low altitudes were not that active due to the changing environment and mating behavior changed (Jaworski & Hilszczański 2013). Possibly males did not have time or could not spend energy to find the "best" female. Maybe it was not a question about finding the best quality female, it was instead a question about just finding the opportunity to copulate.
In earlier study Kaňuch et al. (2013) show that with colder environment, there is more copulations due to smaller ejaculate volume. This is because females cannot remate if they receive too large ejaculates and this will be a fitness cost for them. Also, earlier study by Tayler et al. (2014) showed that polyandry is more common in northern latitudes. We also expected that the degree of polyandry would be higher in colder condition, because that should facilitate adaptation of the offspring in harsher environment.

Our results suggest that individuals mating behavior may be strongly affected if the environment has changed to be colder. Because we found a marginally significant trend that populations from higher altitude have more allelic richness and females are larger, it could indicate that there are more copulations in colder condition at higher altitudes (Kaňuch et al. 2013, Tayler et al. 2014). This also confirms that individuals that are not adapted to cold environment, change their mating behavior due a climate-condition factor.
Insects are highly dependent on the climate of their environment and it influences how active they are, and how they behave. Changing temperature with climate change will also affect insects' range, natural enemies and winter survival. Warmer climate will likely also lead to that many insect species moving towards higher altitudes (Jaworski & Hilszczański 2013). Adaption and behavioral plasticity of the insects therefore plays a big role during the ongoing environmental change.

5 Conclusion

I found some relationships between genetic diversity and frequency of copulation, but the pattern differs according to the population origin and the environment. There were indications that populations that origin from warm environment (low altitudes), could not quickly adapt to colder temperatures. In warm temperatures crickets could adapt regardless of their origin. There males choose best quality females with large body-size and high genetic quality.

This preliminary experimental evidence is only a snapshot of the pattern in the degree of polyandry of P. griseoaptera. Further examinations and experiments are required to disentangle this complex mechanism of interactions between factors of phenotype, genetic diversity and climate-condition.
There are limitations in this study and some of these could be addressed in future studies. The main changes should be done in the experimental set-up. The mating groups should be selected differently and not by random. Instead crickets from lower altitudes should be placed in the cold treatment and vice versa (a full treatment set-up), to further examine the environmental-factor impact on polyandry. The treatments in this experiment could also be replicated in more field sites. What also would be suitable is an experiment performed in climate-chambers using different mating groups. A more advanced modelling approach would also account for non-independence i.e by including a cage ID as a variable in the analysis. A final suggestion for future studies is to broaden the study to examine crickets in several altitudes, keep the individuals for a longer period in open-air cages to give them more opportunities to copulate.

Nevertheless, this experiment broadens our knowledge about interesting mating system in this nuptial gift-giving insect species.

Acknowledgement

I would like to thank my supervisor Dr. Åsa Berggren for giving me the opportunity to carry out this bachelor thesis at Slovak Academy of Sciences in Slovakia. I want to thank you for your support and feedback when writing my bachelor thesis! I also want to give a big thank to my supervisor in Slovakia Dr. Peter Kaňuch. Thank you for your guidance in the laboratory and helping me while writing the report. Without your help I could not have complete this thesis!

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Appendix 1

Table 4. *Random mating groups, population origin and measurement of body-size and number of spermatodoses.*

Pop	Box	ID	Body (mm)	Shield (mm)	Femur (mm)	Dead	Sperma-todoses
G	SK1	F1	22,8	5,4	16,7		2
W	SK1	F2	19,6	4,7	15,7		0
R	SK1	F3	21,2	5	15,1		1
W	SK1	F4	20,5	5,2	16,8		1
R	SK1	F5	24,7	5,6	16,2		1
R	SK1	M1	17,3	5	15	16.7.18	
G	SK1	M2	na	2	na	16.7.18	
G	SK1	M3	18,5	4,5	14,9		
G	SK1	M4	18	4,8	14,6		
G	Sk1	M5	19,5	5	15,8		
G	SK2	F1	20,2	4,9	15,2		0
W	SK2	F2	21,4	5,1	15,7		1
G	SK2	F3	21,9	5,2	16,5		2
G	SK2	F4	19,5	4,8	14,9		1
R	SK2	F5	26,8	5,5	16,5		0
G	SK2	M1	18	5,3	16,2		
R	SK2	M2	19,4	5	15,7		
Y	SK2	M3	17,5	5	14,9		
Y/R	SK2	M4	19	4,9	15		
Y/R	SK2	M5	19,2	4,6	14		
G	SK3	F1	19,8	5,3	16,4		0
R	SK3	F2	23,1	5	15,4		1
R	SK3	F3	22,3	5,1	15,3		0
G	SK3	F4	19,7	4,9	14,8		2
R	SK3	F5	21,3	5,3	16,6		3
W	SK3	M1	18,8	5,1	15,1		
R	SK3	M2	17,1	4,5	12,7		
R	SK3	M3	18,8	5,3	15,5		
G	SK3	M4	17,4	5	16,2		
G	SK3	M5	17,1	4,8	15		
R	SK4	F1	23,1	5,3	16,8		1

W	SK4	F2	21,6	5,2	16,9		0
G	SK4	F3	20,6	5,2	16,6		1
G	SK4	F4	23,4	5,4	17,2		0
G	SK4	F5	20,4	4,8	15,4		0
Y	SK4	M1	na	na	na	9.7.18	
Y	SK4	M2	15,1	4,9	na		
Y	SK4	M3	18,8	4,8	15,4		
G	SK4	M4	17,1	4,9	14,9		
G	SK4	M5	17,7	5	14,4		
R	SK5	F1	21,6	5,7	17,3		2
R	SK5	F2	21,3	4,8	15,2		3
G	SK5	F3	20,4	5,2	16,4		0
R	SK5	F4	18,2	5,2	na		2
G	SK5	F5	21,4	5	16		2
R	SK5	M1	16,4	4,9	na		
Y	SK5	M2	17	4,5	14,6		
G	SK5	M3	20,5	5,1	16,2		
G	SK5	M4	19,7	5,3	15,7		
Y	SK5	M5	16,2	4,7	14,6		
R	ML1	F1	23,6	5,1	15,9		1
R	ML1	F2	20	5	15,2		2
G	ML1	F3	20,2	5,5	15,7		2
G	ML1	F4	23,3	5,5	17,3		1
G	ML1	F5	20,3	5	14,8		2
R	ML1	M1	15,8	5,3	na	9.7.18	
W	ML1	M2	18	4,6	15,2	16.7.18	
G	ML1	M3	17,9	4,7	14,7		
R	ML1	M4	18,4	4,8	15,7		
G	ML2	F1	21,7	5,3	16		2
G	ML2	F2	21	5,1	15,4		1
Y	ML2	F3	18,9	5	14,4		0
G	ML2	F4	22	5,1	16,3		1
G	ML2	F5	20,8	5,2	16,1		0
W	ML2	M1	19,4	5	15,1		
G	ML2	M2	17	4,4	14,4		
R	ML2	M3	17,9	4,7	15,5		
R	ML2	M4	16,7	4,5	13,7		
G	ML2	M5	15,9	4,6	14,7		

G	ML3	F1	20	4,8	16,3		2
G	ML3	F2	20,8	5,2	17		4
Y	ML3	F3	20	5	15		0
G	ML3	F4	17,8	5,5	12,4		0
Y	ML3	F5	23,9	5,1	15,6		4
R	ML3	M1	18,3	5,1	16		
G	ML3	M2	19,6	4,7	15,1		
Y	ML3	M3	17,6	4,6	13,9		
G	ML3	M4	20,3	5,2	16,7		
G	ML3	M5	19,1	5,1	15,2		
G	ML4	F1	20,9	4,9	15,8		3
W	ML4	F2	22,1	5,3	17,3		4
R	ML4	F3	21,5	5,2	16,6		3
Y	ML4	F4	21,3	4,3	14,2		0
G	ML4	F5	23,9	5,4	18		2
R	ML4	M1	18	4,6	14,1		
G	ML4	M2	19,2	4,7	14,7		
G	ML4	M3	20,2	4,8	15,5		
G	ML4	M4	19	5,3	15,3		
R	ML4	M5	18,1	4,8	15		
Y	ML5	F1	22,5	5	16,6		0
R	ML5	F2	22	5,8	17,1		2
R	ML5	F3	23	5	15,9		0
W	ML5	F4	23,9	5,4	16,8		1
R	ML5	F5	22	5,1	15,3		3
G	ML5	M1	18,6	4,2	13,4		
G	ML5	M2	18,5	4,3	13,6		
G	ML5	M3	20,5	4,8	15,9		
Y/R	ML5	M4	20,4	4,6	14,7		
Y	ML6	F1	20,8	5,1	15,8		2
W	ML6	F2	20,4	5,2	16,3		1
G/R	ML6	F3	23,5	5,1	16,1		2
G/R	ML6	F4	17,4	4,9	16,8		0
G/R	ML6	M1	16,2	4,1	13,3		
R	ML6	M2	19,4	5,1	15,1		
G	ML6	M3	19	4	13,6		
G	ML6	M4	na	na	na		

Appendix 2

Table 5. *Genetic diversity of seven microsatellite markers. Number of alleles and size of base-pair that we found in all populations combined. Missing data are due to technical problem with the PCR*

locus	no. of alleles	size (bp)	Ho	He	missing data
WPG10-1	2	121–127	0.02	0.02	
WPG1-28	18	251–359	0.43	0.56	
WPG2-30	7	140–182	0.32	0.62	
WPG8-2	11	246–282	0.12	0.86	
WPG2-15	8	230–251	0.75	0.79	55%
WPG1-27a	2	189–198	0.88	0.49	9%
WPG1-27b	11	255–297	0.50	0.65	15%

Ho – observed heterozygosity

He – expected heterozygosity