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Mating Behavior and Spermatophore Characteristics in Two Closely Related Bushcricket Species of the Genus *Phonochorion* (Orthoptera: Phaneropterinae)

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Abstract Bushcrickets comprise an important group of model organisms, which are intensively studied to understand the evolution of mating behavior. During the mating process, the male bushcricket transfers a costly and edible spermatophore consisting of two parts to the female. This unique behavior makes bushcricket species appropriate study organisms for investigating spermatophore size variations across entire bushcricket family (Tettigoniidae). The functions of the spermatophylax and the ampulla, which constitute the spermatophore, have been addressed in many studies. Although the costs and benefits of mating for male and female bushcrickets may vary depending on species, there are some common evolutionary traits. In the present study, mating behaviors and spermatophore characteristics of two endemic bushcricket species, *Phonochorion uvarovi* and *P. artvinensis*, distributed at the high altitudes of the Kaçkar Mountains, northeastern Turkey, were examined. The data on two closely related species belonging to the genus of *Phonochorion* show that they have different reproductive investments in terms of spermatophore and sperm numbers in spite of similar habitat and nutritional preferences. *P. artvinensis* had a larger spermatophore, spermatophylax and ampulla, but transferred fewer sperm. Male age and weight had a significant effect on spermatophore, spermatophylax and ampulla investments in *P. uvarovi* while only male weight was a significant factor in *P. artvinensis*. A positive relationship between the spermatophylax and the ampulla weights in both species supports the ejaculate protection hypothesis in the *Phonochorion* species. Even though further studies on *Phonochorion* should be conducted to provide a more clear comparison with other groups in the barbitistine, this study demonstrates that both *P. uvarovi* and *P. artvinensis* make little spermatophore investment relative to their body size in comparison to other barbitistines.

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Introduction

Sexual selection is one of the most important mechanisms driving evolution (Fisher 1958), based on variation in reproductive success among individuals of differing body size, appearance and mating behaviors. These different phenotypes between the sexes are major factors contributing to copulation preferences that affect male and female mating preferences, and can make significant contributions to reproductive success as well as offspring health (Andersson 1986 and 1994). In many species, this has led to the evolution of behavioral patterns that facilitate being chosen as a mate. For instance, in some species males find food, take care of offspring and provide females and their offspring with nutrient sources as gifts (Gwynne 2001). Females may prefer males with higher quality nutrient gifts because these gifts indicate better genetic quality or provision of direct benefits to the female (Vahed 1998; Møller and Jennions 2001).

In many insect species, males provide females with costly nuptial gifts as a source of nutrients during mating (Thornhill 1976; Thornhill and Alcock 1983; Gwynne 1990 and 2008; Vahed 1998). Courtship feeding is also widespread in the Orthoptera (Vahed 1998; Gwynne 2001). Male bushcrickets (Orthoptera: Tettigoniidae), for example, feed their mates with the nuptial gift, or spermatophylax, which is attached to the ejaculate and sperm-containing ampulla (Vahed 1998; Gwynne 2001). Bushcrickets are one of the groups that have drawn considerable attention in previous studies investigating the costs and benefits of mating (Wedell 1993; McCartney et al. 2008; Lehmann 2012). Both proximate and ultimate approaches have been used in these studies which a considerable interspecific variation in nuptial gift size has been reflected (review, Vahed 1998; Vahed and Gilbert 1996; McCartney et al. 2008).

Of the bushcrickets, the tribe Barbitistini species have been intensively examined in terms of mating behaviors and spermatophore investment strategies (e.g., Heller and Reinhold 1994; Vahed 1995; Sevgili and Reinhold 2007; McCartney et al. 2008; Lehmann and Lehmann 2009; Uma and Sevgili 2015). Typical mating behavior of the bushcrickets has been described by several authors (Gwynne 2001; Lehmann and Lehmann 2009). In most cases, male bushcrickets produce conspicuous mating signals by forewing stridulation to attract sexually receptive females (Gwynne 2001). Females use male calling songs to locate and choose mates. A sexually responsive female bushcricket often silently approaches a calling male, though in several bushcricket species the female also emits a simple acoustic signal in response to the male's calling song (Heller 1990; Sevgili et al. 2012). The spermatophore presented to the female during mating is highly nutritious (Heller et al. 1998), but also costly for the male (McCartney et al. 2008). After mating, the female consumes the spermatophylax and then the ampulla, which includes the ejaculate. An increased spermatophore investment can affect male refractory period and is likely to decrease the number of copulations during his lifetime (Sakaluk 1987; Simmons 1990; Heller and von Helversen 1991; Gwynne 2001).

Two different hypotheses regarding the role of the spermatophylax have been put forward and this issue has been studied from various angles (e.g., see Eberhard 1996;

Vahed 1998; Gwynne 2001). The first is the ejaculate protection hypothesis, and it assumes that spermatophylax and ampulla size or sperm number are positively related to each other (Gerhardt 1913 and 1914; Boldyrev 1915; Wedell 1993; Reinhold and Heller 1993; Heller and Reinhold 1994; Vahed and Gilbert 1996; McCartney et al. 2008). The other is the paternal investment hypothesis, which predicts a positive contribution of the male to its own offspring through the nutritional value of the spermatophylax (Gwynne 1988; Gwynne et al. 1984; but see Wedell and Arak 1989 and Reinhold and Heller 1993). Therefore there would be a positive relationship between the size of the spermatophylax and reproductive success (Thornhill 1976; Gwynne 1990; Reinhold 1999).

Large spermatophylax have been shown to decrease female mating frequency as well as increase the amount of sperm and ejaculate transferred to the female (Wedell and Arak 1989; Gwynne 2001). Thus, sexual selection has a strong effect on the size of the spermatophore produced by the male. On the other hand, the fact that the females feed on the spermatophylax and then the ampulla after mating could stimulate an increased mating frequency of females, because mating often would allow a female to obtain additional nutrients to increase her fecundity (Simmons and Parker 1989). A number of studies on insect species that consume seminal products have found a positive correlation between nuptial gift consumption and the refractory period of females, suggesting substantial sexual conflict over female remating rate (Simmons and Gwynne 1991; Wedell 1993; Engqvist 2007; but see Fleischman and Sakaluk 2004). Indeed, females from some species can survive by consuming only spermatophore during the mating period, without any extra food (*Isophya kraussi*: Voigt et al. 2005).

The size of the spermatophore in bushcrickets varies between 2 % (e.g., *Anonconotus alpinus*: Vahed 2002) and 40 % of the total male body weight (*Ephippiger ephippiger*: Busnel and Dumortier 1955; Wedell and Ritchie 2004). Sperm number is also quite variable ranging from 38,000 (*Phaneroptera nana*: Vahed and Gilbert 1996) to 37.3 million (*Poecilimon thessalicus*: McCartney et al. 2008).

The general trend in bushcrickets indicates that larger males produce larger spermatophores (Wedell 1993; Vahed 1995). This was supported in a study using 33 species within the genus *Poecilimon* in the barbitistine bushcrickets (McCartney et al. 2008) and by another study in a related genus, *Isophya* (*I. sikorai*: Uma and Sevgili 2015). Although a correlation has been found between body size and sperm number for some species, there is no relationship for other species (Vahed and Gilbert 1996; McCartney et al. 2008). On the other hand, it has been mentioned that there is a phylogenetic relationship between species to a certain degree in terms of spermatophore size (see Vahed and Gilbert 1996). However, some tettigoniids are comparatively very large and produce very small spermatophores (e.g., the spermatophore of *Meconema thalassinum* is barely 1 % of male body mass), while some other bushcrickets are relatively small in terms of body size, but produce relatively large spermatophores (McCartney et al. 2008). However, spermatophore size and spermatophore content are directly affected by food consumed, male age, mating season and mating history (e. g., Heller and von Helversen 1991; Reinhold 1994; Wedell and Ritchie 2004; McCartney et al. 2010).

Studies on the mating behaviors of Barbitistine bushcrickets have been mostly focused on the species of *Poecilimon* and *Leptophyes,* and data for the closely related genera are considerably limited. For example, there are few studies on the genus *Isophya,* which has more than 90 species in the Palearctic region and is the second largest genus after the genus *Poecilimon*, which contains about 150 species (see Can 1959; Sevgili 2004; Eades et al. 2014; Uma and Sevgili 2015). The genus *Phonochorion* is a little known group and only three species are described for the genus belonging to the barbitistine bushcrickets, representing a total of 15 genera in the world (Sevgili et al. 2010; Eades et al. 2014). Data on mating behavior of species of the genus *Phonochorion* may make contributions to evolutionary ecological approaches related to species within the barbitistine genera.

This study aimed to identify the mating behaviors and spermatophore characteristics for the species *Phonochorion uvarovi* Karabağ, 1956 and *P. artvinensis* Bei-Bienko, 1954. Both species have an attractive color pattern and are endemic to the Eastern Black Sea Region. Males produce calling songs during the mating period (Sevgili et al. 2010). The species are morphologically similar to each other, and phylogenetically is closely related to the genera *Poecilimon* and *Polysarcus* among the barbitistine genera (Heller 1990). In the study, spermatophore characteristics of these species, whose mating behaviors have not been studied previously, were investigated and compared with other barbitistine genera.

While a large variation in spermatophore characteristics appears to occur among barbitistine bushcrickets, comparisons between closely related species provide additional insights into the factors that influence gift investment and ultimate causes of mating behavior of species. Therefore, in the present study it was hypothesized that two closely related species occurring in similar habitats should have specific spermatophore contents. The ejaculate protection hypothesis was also evaluated by considering spermatophore weight, spermatophylax and ampulla weights, and sperm numbers. While these have been heavily studied on other bushcrickets, it is crucial to examine these mating behaviors across a diversity of species to understand the evolutionary history of these traits.

Material and Methods

The Insects

The genus *Phonochorion* Uvarov, 1916 (Orthoptera: Phaneropterinae) is a small genus consisting of three species: *P. satunini*, *P. artvinensis* and *P. uvarovi* (Sevgili et al. 2010). These species are distributed only on the northern slopes of the East Black Sea of Turkey and Lesser Caucasus Mountains in Georgia. Their limited vertical distribution (1200–2600 m) and preference for moist sub-alpine vegetation indicates this genus is an alpine endemic.

The species resemble each other in terms of their colors and patterns (Figs. 1 and 2). The most important characters for the species identification are the shape of the pronotum for both sexes, the cercus and subgenital plates of the males and their calling songs (Sevgili et al. 2010). While the males typically have similar colors and patterns, the females can sometimes be completely green. Both *P. uvarovi* and *P. artvinensis* live

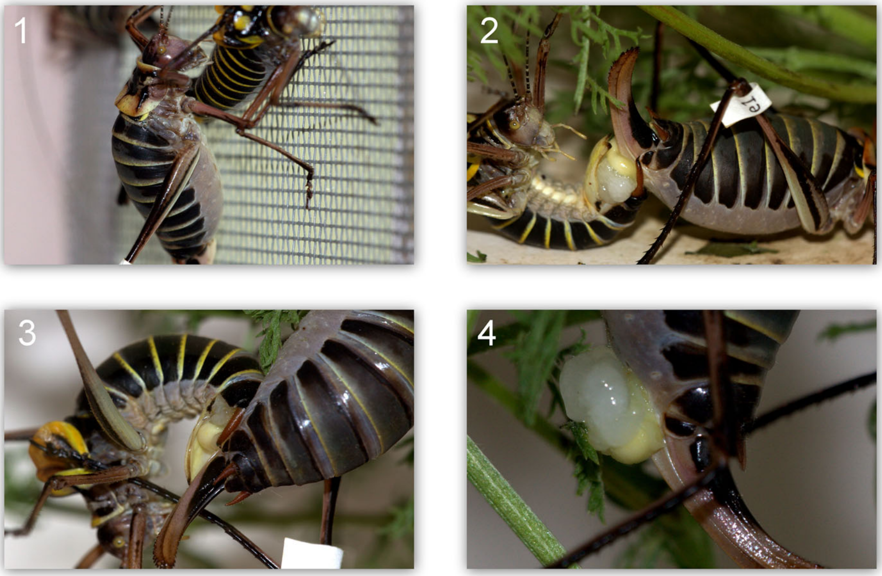

Fig. 1 Mating in *Phonochorion uvarovi*. *1*. A female mounts a male. *2–3*. A male accepts a female as a mate by curling her abdomen beneath her. *4*. A newly mated female

in similar habitats and feed on similar diets, but do not exhibit a perfect syntopic distribution. In the fields where they live syntopically, *P. artvinensis* has been observed to prefer lower altitudes. In our preliminary studies, although the females' wings were

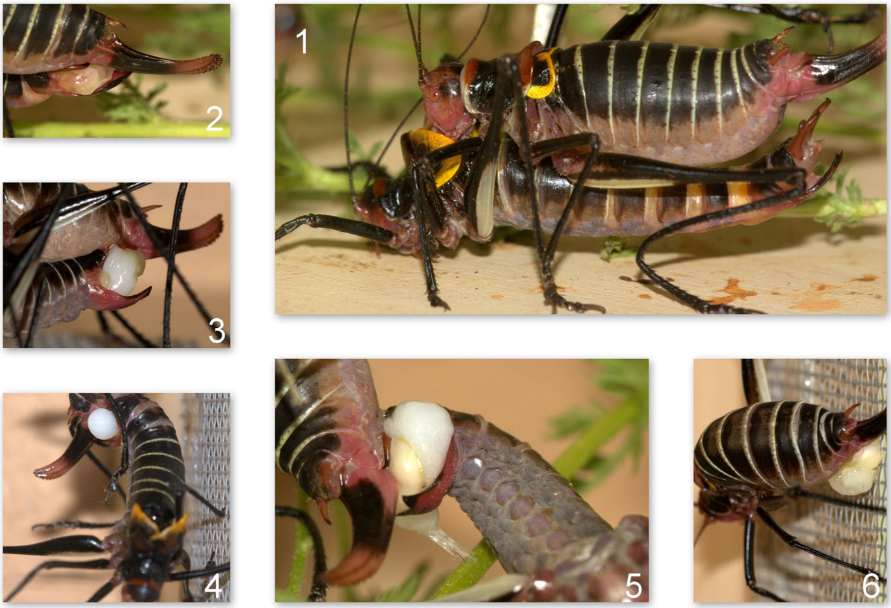

Fig. 2 Mating in *Phonochorion artvinensis*. *1*. A female mounts a male. *2–5*. A male accepts a female as a mate *6*. A newly mated female

in contact on the dorsum, they did not show any response to the male calling signals. However, the wing structures of the females appear to be appropriate for producing signals, so the possibility needs to be studied in further detail.

Field Studies

The samples for the species of *P. uvarovi* were collected as nymphs from Çamlıhemşin (Rize), high altitudes of Fırtına Valley and Çat Plateau (20–21 June 2012; 40°51′46.53″ N, 40°56′20.72″E, 1290 m). The samples for *P. artvinensis* were collected as nymphs from İkizdere (Rize) İspir Road, Ovit Mountains, Sivrikaya location (22 June 2011; 40°41′05.33″N, 40°42′13.10″E, 1790 m). We collected 150 females and 150 males from both species. After collection the samples were placed in cages (20×20×25 cm) and fed with the plants on which they were collected. Once collection was complete they were transferred to the laboratory.

Laboratory Studies

Bushcricket Maintenance

Once in the laboratory, the bushcrickets were transferred to four bigger cages (27×34×38 cm) and separated by sex. The numbers of the individuals in the cages were equal and the nymphs were reared until maturation. The laboratory was maintained between 25 and 26 °C with a 14 h:10 h light-dark cycle. Both species were fed with lettuce, cucumber and apple slices in addition to the plants of *Urtica* sp., *Anthemis* sp. and *Rubus* sp.. Their cages were cleaned and fresh food was provided daily. Mature individuals were removed daily and placed into smaller, single sex cages. In order to identify the ages (in days) of mature individuals, we recorded the date of their final molt. All individuals were tagged by attaching labels including individual numbers on hind femur. On average, each adult cage was stocked with eight individuals to avoid over crowded.

Mating Experiment

It was observed that the males began to produce calling songs 3 days after the adult molt. Only males producing calling songs were used in mating trials. The typical calling songs of both species are different (Sevgili et al. 2010). In preliminary studies, it was found that the females were not sexually receptive until 7 days after adult eclosion. For this reason, and to allow the males to produce sperm and spermatophore properly, the mating trials were initiated when both bushcrickets were at least 7 days old. All the individuals were considered virgins at the first mating.

Body weights of the males and females were measured using a 0.1 mg precision balance (OHAUS Adventurer Pro) before the mating experiment. At each trial, ten randomly selected and marked males and ten females were put into the cages (50×50×45 cm) for an hour. The males and females that mated within an hour were taken out of the cage. The mated females were first weighed with the spermatophore, then the spermatophore was removed from the female by using a fine-tipped forceps and their weights were measured separately. The spermatophore is comprised of a large gelatinous spermatophylax and a smaller sperm-containing ampulla. The ampulla and spermatophylax were separated from each other quickly and carefully using a fine-tipped forceps and each was re-weighed. The ampulla was crushed with a small curved-tipped invert forceps in water (0.4 for *P. uvarovi* and 0.6 ml for *P. artvinensis*). After that, body weight of the mated male was measured once again to determine the loss of mass due to the spermatophore. The combination of ejaculate (sperm and seminal fluid) and water was mixed gently using a syringe 15 times. Sperm were counted with a Neubauer hemocytometer using a light microscope (an objective of LEICA DM500 X10). Five repeated counts were performed for each sample, and the mixture of ejaculate and water was homogenized at each time. If there was no mating in the cage, these individuals were taken away from the cage and kept for the next mating experiments. These individuals were housed in separate cages to avoid the confusion of their mating status. However, mating success level was quite high during the experiments.

Mated females and males were put into their cages and their mating dates were recorded. The mating protocol explained above was applied to all copulations. In order to make the males ready for the next copulations, a 3 day recovery period was allowed before the next copulations (Reinhold and von Helversen 1997; Sevgili and Reinhold 2007). Because many individuals died when they were young adults due to a maladaptation to the laboratory conditions, the females had to be used for repeated copulations with different males of *P. artvinensis*. For this reason, males and females of *P. artvinensis* had to be mated more than once to obtain more spermatophore data. Nevertheless, at each mating males were paired with different females. Although there were some males that mated as many as six times, data of only first three matings were considered in the analysis to reduce a possible bias due to repeated matings. Eventually, 52 successful matings of *P. artvinensis* were used in the analysis. At the end of the study, a total of 94 matings were performed for *P. uvarovi*. In the matings, 65 males and 90 females of *P. uvarovi* were used whereas 32 males and 25 females for *P. artvinensis*. All *P. uvarovi* females were mated once, with the exception of four who mated twice. Numbers of males mated once and twice were 65 and 29 respectively.

Pronotum, hind femur and ovipositor lengths in the females were measured using a caliper (Mitutoyo Digital Caliper, accuracy=0.01 mm). Statistical analyses were conducted using SPSS Statistics v22.0 (IBM, Armonk, NY, USA) and XLSTAT (Addinsoft, Version 2014.3.02). The means with standard errors (SE) for male and female body sizes and weights, and spermatophore characteristics were calculated. Normality of the data was checked with the Shapiro-Wilk's tests. Because the distributions of sperm number for both species and ampulla weight of *P. uvarovi* were not normal, the former was $\log_{10}$ transformed and ampulla weight was square root (sqrt) transformed. Relative sperm number was calculated as the absolute number of sperm over mean male body weight and expressed as sperm number × 10^4. Measurements on some body sizes and weights, spermatophore, spermatophylax and ampulla weights including their relative sizes, and sperm number were tested with an independent samples *t*-test between species. However, we used the data from only first matings for this test ($N=66$ to *P. uvarovi*; $N=33$ to *P. artvinensis*). The reason that we only used data from the first mating is that the samples were not independent within each group, because males and females were used multiple times within each group. Relative spermatophore weight was calculated as the percentage of male body weight before mating for each individual, and then the mean for all individuals taken to calculate a species average.

The mean weights of the spermatophore, spermatophylax and ampulla, absolute sperm numbers, relative spermatophore and relative sperm number for the species of *P. uvarovi* and *P. artvinensis* were compared with the mean values of the other barbitistine genera taken from the literature (Table 1).

We used the following statistical approaches to test several predictions: (1) heavier males produce bigger spermatophore and ampulla than lighter males (e. g., McCartney et al. 2008); (2) ampulla and spermatophylax weight generally covary within species (e. g., Wedell 1994); (3) sperm number is usually independent of male body size, but a strong correlation between spermatophylax and ampulla weight in bushcrickets would suggest an ejaculate protection function (e. g., Reinhold and von Helversen 1997; McCartney et al. 2008). To determine the factors that may have effects on spermatophore characteristics of two *Phonochorion* species, we used a generalized linear mixed model (GLMM) with restricted maximum likelihood estimation (REML) (Bolker et al. 2008). In this analysis, we included a random effect (male identity) and fixed-covariate effects (male mating number, male age, male body weight, female body weight and ampulla weight). The basis for including the latter effects was that males

Table 1 Mean male weight, spermatophore (sp'phore), spermatophylax (sp'lax), ampulla weight, sperm number, and relative spermatophore weight for some the genera of Barbitistini (Orthoptera; Tettigoniidae, Phaneropterinae)

The Genera (*)	Male weight (mg)	Sp'phore weight (mg)	Sp'lax weight (mg)	Ampulla weight (mg)	Sperm number x10^5	Relative sp'phore (%)	Relative sperm number (#)
Leptophyes (4)[a]	250.00	39.02	32.48	6.55	69.43	15.61	27.77
Metaplastes (1)[b]	450.00	129.70	120.80	9.00	149.00	28.82	33.11
Ancistrura (1)[c]	511.33	34.60	31.50	3.10	no data	6.77	no data
Barbitistes(2)[d]	721.00	297.55	227.65	69.90	369.00	41.27	51.18
Isophya(5)[e]	766.66	161.58	123.71	36.49	1013.95	21.08	132.26
Poecilimon(37)[f]	794.12	140.75	106.67	22.42	56.00	17.72	7.05
Phonochorion (2)[g]	1074.82	125.75	98.13	23.73	211.97	11.70	19.72
Polysarcus (1)[h]	1688.00	239.90	221.00	48.60	362.00	14.21	21.45

Letters indicate source of reference

(*) Number of studied species

[a] Vahed and Gilbert 1996

[b] von Helversen and von Helversen 1991; Vahed and Gilbert 1996; Heller et al. 1998

[c] Heller et al. 1998 and data by D. von Helversen, K. G. Heller, personel communication

[d] Vahed and Gilbert 1996; Heller et al. 1998

[e] Can 1959; Uma and Sevgili 2015; unpublished data by A. Yiğit, H. Önal and H. Sevgili

[f] McCartney et al. 2008

[g] This study

[h] Vahed and Gilbert 1996

can make adjustments in their spermatophore components in relation to eclosion (age as days) and body conditions and mating status (Lehmann and Lehmann 2009; Uma and Sevgili 2015). There was no correlation between male body size (measured as pronotum length and hind femur length) and spermatophore and its contents (Pearson Correlation, $p>0.05$) in either species. Thus, these measurements were not used in the analysis.

Results

Body Size of Female and Male

Although there was no relationship between the male weight and pronotum length, the relationship of body weight with the hind femur was significant (Pearson Correlation, $p<0.01$, $N=83$) for *P. uvarovi.* In females, the relationship between body weight and both hind femur ($N=84$) and ovipositor length was significant (Pearson Corr., $p<0.05$, $N=80$), but the relationship with the pronotum length ($N=80$) was not. For *P. artvinensis,* both the relationship between male weight and hind femur ($N=47$) and pronotum length ($N=44$) were significant (Pearson Corr., $p<0.05$). Females displayed a different pattern, with larger females having longer ovipositors (Pearson Corr., $p<0.01$, $N=46$).

Male hind femur length as well as most of the morphological and reproductive variables analyzed in this study differed between the two species (Table 2).

Table 2 Means and standard errors for morphometric traits and spermatophore of the species *Phonochorion uvarovi* and *P. artvinensis* and results of independent *t*-tests between the two species (SE=Standard Errors)

	P. uvarovi			*P. artvinensis*				
Variables	N	Mean	SE	N	Mean	SE	*t*	*Sig.*
Female hindfemur length (mm)	59	19.24	0.12	32	20.46	0.15	−6.05	0.001
Female pronotum length (mm)	58	5.96	0.05	26	6.19	0.05	−3.04	0.003
Ovipositor length (mm)	58	11.27	0.09	32	12.60	0.08	−9.30	0.001
Male hindfemur length (mm)	60	18.48	0.13	24	18.95	0.26	−1.81	0.074
Male pronotum length (mm)	60	4.65	0.05	22	5.60	0.08	−9.06	0.001
Male body weight (mg)	66	987	16.49	33	1195	25.78	−7.04	0.001
Female body weight (mg)	66	1384	25.43	33	1738	37.24	−7.94	0.001
Spermatophore weight(mg)	66	118.45	4.39	33	142.61	6.23	−3.17	0.002
Spermatophylax weight (mg)	66	94.83	3.86	33	113.94	5.63	−2.83	0.006
Ampulla weight (mg)	66	23.62	0.72	33	28.66	0.98	−4.09	0.001
Rel. Spermatophore (%)	66	12.28	0.55	33	11.93	0.46	0.42	0.672
Rel. Spermatophylax (%)	66	9.85	0.47	33	9.52	0.42	0.45	0.654
Rel. Ampulla (%)	66	2.44	0.09	33	2.41	0.07	0.21	0.836
Sperm Number × 10^3	66	2459	133.97	33	1884	117.76	2.19	0.032
Rel. Sperm number ×10^4	66	25.44	1.87	33	16.37	1.55	9.06	0.002

Spermatophore Characteristics

The spermatophore, spermatophylax and ampulla weights, sperm numbers and other major parameters for the species of *P. uvarovi* and *P. artvinensis* are shown in Table 2. While the average values for spermatophore, spermatophylax and ampulla weights were greater in *P. artvinensis* than *P. uvarovi*, none of relative values of those parameters were significantly different (Table 2). However, both absolute sperm number and relative sperm number were significantly different between two species.

Male body weight had a significant positive effect on absolute spermatophore weight and ampulla weight for both species, but the effect was weaker in *P. artvinensis* (Table 3). While male body weight had a significant positive effect on spermatophylax weight of *P. uvarovi*, this was not the case in *P. artvinensis* (Table 3). Male age had a significant impact on spermatophylax and ampulla weights as well as absolute sperm number for *P. uvarovi*. However, there was only a significant interaction between male age and ampulla weight in *P. artvinensis* (Table 3). Male mating number had a significant positive effect on spermatophore and ampulla weights for only *P. uvarovi*, which was not the case in *P. artvinensis* (Table 3). For both species, there was a significant interaction between ampulla weight and spermatophylax weights (Table 3). Sperm number was independent of all factors and covariates, exluding male age in *P. uvarovi* (Table 3).

Although male body weight of *Phonochorion* was larger than other barbitistine genera, except *Polysarcus,* they had comparatively smaller spermatophores and fewer sperm (Table 1).

Discussion

Spermatophore Characteristics and Sperm Number

The lack of a relationship between the body sizes (protonum, hind femur lengths) and the spermatophore and its contents is a common situation among many crickets and bushcrickets (unpublished data, H. Sevgili). In the present study, although the male weight was useful predictor and affected the spermatophore and ampulla weights for both species, male mating number had an interaction with spermatophore weight and ampulla weight for only *P. uvarovi*. Spermatophore weight has been widely shown to be related to body weight (Gwynne et al. 1984; Wedell and Arak 1989; Heller and Reinhold 1994; Uma and Sevgili 2015) because body weight is a sex-linked trait and is closely associated with the spermatophore weight (Reinhold 1994) or, alternatively, can be a result of pleiotropy (Fedorka and Mousseau 2002). When body weights were compared, it was seen that *P. artvinensis* was larger than *P. uvarovi,* and accordingly their average of spermatophore, spermatophylax and ampulla weights were higher, but their relative weights were not significantly different. This may be despite the likely benefits to males, due to the fact that an allocation of a large spermatophore to the female is costly to the males (Simmons 1990; Vahed 2007).

Although relatively larger nuptial gifts are associated with relatively larger ampulla and relatively higher sperm counts in many bushcrickets (Vahed and Gilbert 1996), our data suggested that this may not always be a reliable sign. Male body weight did not

Table 3 Results of a general linear mixed model (GLMM with REML estimation) testing the effects of male mating number, male age, male and female body weights on spermatophore (Sp. weight), spermatophylax (Spx. weight) and ampulla weights and absolute sperm number

Random effects	*P. artvinensis*				*P. uvarovi*			
	SE	Estimate	Z	p	SE	Estimate	Z	p
[a]Male ID	361.09	649.25	1.80	0.072	136.52	483.56	3.54	<0.001
Residual	262.25	580.33	2.21	0.027	55.51	159.62	2.88	0.004
[b]Male ID	253.55	222.87	0.90	0.379	74.45	291.63	3.92	<0.001
Residual	242.09	544.96	2.25	0.024	26.62	79.85	3.00	0.003
[c]Male ID	8.45	23.10	2.70	0.006	0.04	0.03	0.71	0.003
Residual	2.44	3.42	1.40	0.160	0.04	0.13	3.46	0.001
[d]Male ID	–	–	–	–	0.01	0.02	3.12	0.002
Residual	0.01	0.05	3.74	<0.001	0.00	0.01	3.49	0.001
Effects	d.f.	den d. f.	F	p	d.f.	den d. f.	F	p
[a]Sp. weight								
Male Mating number	2	37	0.16	0.857	1	86	61.83	<0.001
Male age	9	37	0.42	0.917	4	86	2.74	0.034
Male body weight	1	37	5.16	0.029	1	86	53.16	<0.001
Female body weight	1	37	0.67	0.418	1	86	0.19	0.666
[b]Spx. weight								
Male Mating number	2	36	1.38	0.264	1	85	0.28	0.597
Male age	9	36	0.50	0.867	4	85	3.35	0.014
Male body weight	1	36	0.34	0.563	1	85	29.89	<0.001
Female body weight	1	36	2.96	0.094	1	85	0.51	0.475
Ampulla weight	1	36	11.93	0.001	*1	85	36.28	<0.001
[c]Ampulla weight					*			
Male Mating number	2	29	2.87	0.073	1	86	126.89	<0.001
Male age	9	29	3.25	0.008	4	86	17.45	<0.001
Male body weight	1	29	11.81	0.002	1	86	29.60	<0.001
Female body weight	1	29	3.73	0.063	1	86	0.35	0.556
Female mating number	8	29	1.52	0.195	–	–	–	–
[d]**Sperm number					**			
Male Mating number	2	28	2.18	0.131	1	85	3.93	0.051
Male age	9	28	1.01	0.457	4	85	10.39	<0.001
Male body weight	1	28	0.16	0.697	1	85	0.76	0.359
Female body weight	1	28	0.70	0.410	1	85	0.61	0.451
Ampulla weight	1	28	1.49	0.232	*1	85	2.03	0.158
Female mating number	8	28	0.14	0.997	–	–	–	–

*$\text{Sqrt}_{\text{ampulla weight}}$; a, b, c, d refer to variables

**Log sperm number

Z, statistic value for random effect

(den) d. f., (denominator) degrees of freedom affect the sperm number in either species, which is consistent with the findings of many previous studies. For instance, no relationship has been shown between sperm number and body size for *Poecilimon veluchianus* and *P. jonicus jonicus* (respectively Reinhold and von Helversen 1997; Sevgili and Reinhold 2007; for *Pieris rapae*: Cook and Wedell 1996). Although *P. artvinensis* had a bigger body, larger spermatophore and ampulla, its mean sperm number was lower than in *P. uvarovi.* Absence or presence of a significant relationship between body size and sperm number among species even within the same genus may not be a common feature (e.g., *Gryllodes sigillatus* and *Gryllus texensis*: Schaus and Sakaluk 2001; the genus *Poecilimon*: McCartney et al. 2008; but see European bushcrickets: Vahed and Gilbert 1996 and 74 butterfly species: Gage 1994). It can be concluded that sperm number is rather variable, more or less independent from body size / weight in many species, and influenced by various physiological factors (such as nutritional status, Roth and Reinhardt 2003; Gage and Cook 1994; *T. castaneum*: Arnoud et al. 2001).

A positive relationship between spermatophore, spermatophylax and ampulla weights and the male body weight has been reported for many bushcrickets (Wedell 1993; Wedell and Sandberg 1995; Vahed and Gilbert 1996; McCartney et al. 2008; Lehmann and Lehmann 2009; Uma and Sevgili 2015). Sexual characteristics influence partner choice to a great extent. For instance, male calling song characteristics, body size, and spermatophore (nuptial gift) size are important factors for female choice (Wedell and Sandberg 1995). This is because large nuptial gifts have been shown to increase fertilization success and/or reproductive success of the female in many related species (Gwynne 1984; Simmons 1990; Wedell 1997; Vahed 1998; but not Wedell 1993). In the current investigation, it is remarkable that the difference for relative spermatophore, spermatophylax and ampulla weights were not significantly different despite the fact that *P. uvarovi* had a smaller spermatophore than *P. artvinensis.* The same relationship has also been reported for other species within the genus *Poecilimon* (McCartney et al. 2008). A heavier body is the best predictor of larger spermatophore production (e. g., Lehmann and Lehmann 2009), but the relative spermatophore size is comparable among the species due to its high cost (e. g., immunological cost, Kerr et al. 2010) and the trade-off between body size/mass and relative spermatophore, spermatophylax and ampulla. On the other hand, it has been stated that the relative spermatophore sizes are significantly different in the subspecies of *P. v. veluchianus* and *P. v. minor* (Heller and Reinhold 1994). While strong sexual selection has a strong influence on body size and absolute spermatophore content (e.g., Gwynne 1981), it has no effect on the relative spermatophore size. The size of the spermatophore and its content are features that are evolutionarily unstable and responsive to selection (McCartney et al. 2011). Given that the two species studied here are closely related, such differences show that body sizes and spermatophore components (but not their relative sizes, excluding sperm number and its relative) are evolutionary labile and that the sexual selection pressure seems to follow a species-specific pattern. At least, selection strongly acts on body weight and absolute spermatophore contents.

A positive correlation between male body weight and ampulla weight has been reported for many species (e.g., some Gryllid species: Schaus and Sakaluk 2001; *Poecilimon*: McCartney et al. 2008). However this relationship does not hold for all species and could change depending on age (McCartney et al. 2010; Uma and Sevgili 2015). Male age had a positive effect on ampulla weight in both species. This suggests that investment during a period of active reproduction in both species (about 10–30 age/days) leads to a more positive selection of ampulla weight. While this selection could be effective on absolute sperm number within the ampulla of *P. uvarovi*, it was not shown in *P. artvinensis*. One possible reason for this contradiction between the two species could be cost variations (e.g., whether metanotal feeding occurs) during mating. Still, it is difficult to explain the difference using the present data set. The fact that there is an effect of male age on sperm number in *P. uvarovi*, but not in *P. artvinensis,* may indicate that both species have different strategies for sperm allocation. It is noteworthy that male mating number had no significant impact on spermatophore contents in *P. artvinensis*. Despite a limited number of matings in *P. uvarovi* (maximum twice), mating status significantly affected ampulla and spermatophore weights, which could imply that mating status (virgin-nonvirgin) rather than mating number is more important in investment on spermatophore components.

All tested factors had no affect on sperm number in both species, suggesting that sperm number may be highly comparable for each male, except age which was a significant factor (e. g., *Poecilimon veluchianus*, Reinhold and von Helversen 1997). In addition, there is no relationship between ampulla weight and sperm number in some species (Simmons et al. 1993; Gao and Kang 2006). Selection acts on spermatophylax weight rather than the ampulla weight both because spermatophylax comprises the most costly part of the spermatophore (McCartney et al. 2011) and also because the ejaculate volume can change independently from sperm number in response to sexual selection (McCartney et al. 2010).

Although the spermatophylax gift is maintained by natural selection (paternal investment) and sexual selection (ejaculate protection), a positive relationship between spermatophylax and ampulla size in both species is generally a signal for the ejaculate protection hypothesis (Gwynne 2001). The existence of a positive effect on spermatophylax weight for both species in the present study suggests that the spermatophylax serves as ejaculate protection for the *Phonochorion* species. Indeed, this is also the case for other closely related species (Vahed and Gilbert 1996; McCartney et al. 2008; Uma and Sevgili 2015). However, for the two examined *Phonochorion* species, no significant relationship was found between both spermatophore and spermatophylax size and absolute sperm number, which is consistent with the findings from the closely related species of *Poecilimon* and the Australian bushcricket *R. verticalis* (Simmons et al. 1993; Reinhold and von Helversen 1997; Gao and Kang 2006; McCartney et al. 2008). This can be a result of spermatophore investment and sperm production having evolved independently from each other (Lehmann 2012). On the other hand, some have reported that spermatophore size is larger than necessary for sperm transfer (*R. verticalis*: Gwynne et al. 1984; *Poecilimon*: McCartney et al. 2013). The main reason for non-existence of the relationship between the spermatophylax size and sperm number may be that it is related to sperm transfer duration rather than sperm number (McCartney et al .2010). The present study did not examine the paternal investment hypothesis for *Phonochorion*, and this issue remains to be examined.

The reason for this inconsistency between these two closely related species may result from either proximate causes (e. g., Reinhold 1994) or insufficient mating data for *P. artvinensis*. On the other hand, because it was observed that both *Phonochorion* species fed on similar diets (e.g., *Sambucus nigra, Urtica dioca, Rumex tuberosum*) in the field, similar diets were used in the laboratory. However, many nymphs of *P. artvinensis* did not survive under the laboratory conditions and high mortality rate was seen in young adults.

Spermatophore Components of the Genus Phonochorion and Related Genera

The two *Phonochorion* species always produced a spermatophylax that was larger than the ampulla as is typical in other genera of Barbitistini (but see, Heller et al. 2004). The mean spermatophore weight and relative spermatophore weight (11.6 %) for both species were lower than most other barbitistine genera studied so far. However, the relative spermatophore mass is notably variable among the species belonging to the same genus (e.g., this value ranges between 4 and 40 % for the genus *Poecilimon*, McCartney et al. 2008). Moreover, spermatophore weight and sperm number vary often among species (e.g., *Leptophyes laticauda*; Vahed 1995; Vahed and Gilbert 1996). It can be argued that relative spermatophore size will show a tendency to decrease in species that have larger bodies due to the high cost of producing spermatophores (Simmons 1990; Vahed 1998). The mean sperm number is the highest in the *Isophya* genus among the genera. Nevertheless, it is premature to make a decisive conclusion for the genus because data is currently only available for five of the roughly 90 species in the genus. The genera of *Polysarcus* and *Poecilimon* are phylogenetically close relatives of *Phonochorion* (see Heller 1990; Ullrich et al. 2010), but *Polysarcus* differs from the other two genera in terms of spermatophore content as they have much larger bodies than other barbitistine genera. Although the body size of *Phonochorion* species are bigger than most of *Poecilimon* species, they have comparable spermatophore and sperm number investment values to many species within the genus *Poecilimon*. The species of the genera *Leptophyes* and *Ancistrura* have smaller bodies in the tribes and smaller spermatophore investment. *Metaplastes* produces large spermatophores relative to its body size (von Helversen and von Helversen 1991). Yet, the relative spermatophore investment reaches nearly 41 % in the genus *Barbitistes* (see also for *Ephippiger*: Busnel and Dumortier 1956; *Lluciapomaresius stalii-Uromenus (Steropleurus) stali-*: Gwynne 2001). The fact that the relative spermatophore is about 7 % for *Ancistrura* suggests that spermatophore investment in the same phylogenetic group is quite variable (see also Gwynne 1995; Vahed and Gilbert 1996). The analyses conducted by Wedell (1993) and Vahed and Gilbert (1996) reveal that, in many bushcrickets spermatophylax size is linearly related to both ampulla and sperm number. This relationship may imply both the ejaculate protection and the parental investment hypothesis (Gwynne 2001). However, with the data available, it is premature to make a conclusion about the function of nuptial gifts in the barbitistine bushcrickets. There was no correlation between spermatophylax and sperm number for *Phonochorion,* but there was a positive correlation with ampulla weight, supporting the ejaculate protection hypothesis. On the other hand, an unbiased discussion about two the hypotheses remains to be explored because we do not know anything about the relationship between the duration of spermatophore consumption and sperm transfer for *Phonochorion*, and the effect of spermatophore consumption on female fitness (e.g., egg size) has not yet been studied in this genus.

Conclusions

The data on two species belonging to the genus of *Phonochorion* endemic to the Kaçkar Mountains show that they have different reproductive investments in terms of spermatophore and sperm numbers. It should be stated that bioacoustic characteristics (Sevgili et al. 2010) and sexual behaviors are exposed to stronger selection, which differentiates these two species which are morphologically quite similar. Generally, their distribution patterns do not overlap, but our observations indicate that both species eclose in similar periods and feed on similar diets. When the data obtained from the species of *Phonochorion* are assessed together with the other groups in the barbitistine bushcrickets, it is seen that the genera have not been studied adequately and thus the evolutionary shaping of spermatophore investment and mating behavior in the tribes is not clear. There is little data available, especially for the large genus *Isophya* which includes many species (Sevgili 2004). The results of this study will help to better understand the effects of mating status (virgin-nonvirgin), male age and mating number on investment in spermatophore and its contents in species of the genus *Phonochorion* in future studies.

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