ORIGINAL PAPER

Subsocial behaviour and brood adoption in mixed-species colonies of two theridiid spiders

Lena Grinsted · Ingi Agnarsson · Trine Bilde

Abstract Cooperation and group living often evolves through kin selection. However, associations between unrelated organisms, such as different species, can evolve if both parties benefit from the interaction. Group living is rare in spiders, but occurs in cooperative, permanently social spiders, as well as in territorial, colonial spiders. Mixed species spider colonies, involving closely related species, have rarely been documented. We examined social interactions in newly discovered mixed-species colonies of theridiid spiders on Bali, Indonesia. Our aim was to test the degree of intra- and interspecific tolerance, aggression and cooperation through behavioural experiments and examine the potential for adoption of foreign brood. Morphological and genetic analyses confirmed that colonies consisted of two related species *Chikunia nigra* (O.P. Cambridge, 1880) new combination (previously *Chrysso nigra*) and a yet undescribed *Chikunia* sp. Females defended territories and did not engage in cooperative prey capture, but interestingly, both species seemed to provide extended maternal care of young and indiscriminate care for foreign brood. Future studies may reveal whether these species adopt only intraspecific young, or also inter-specifically. We classify both *Chikunia* species subsocial and intra- and interspecifically colonial, and discuss the evolutionary significance of a system where one or both species may potentially benefit from mutual tolerance and brood adoption.

L. Grinsted (✉) · T. Bilde
Department of Bioscience, Aarhus University,
Ny Munkegade 114-116,
8000 Aarhus C, Denmark
e-mail: lena.grinsted@biology.au.dk

I. Agnarsson
Department of Biology, University of Vermont,
120A Marsh Life Science Building, 109 Carrican Drive,
Burlington, VT 05405-0086, USA

Introduction

The evolution of cooperation is often facilitated by high relatedness among social partners (Hamilton 1964; Boomsma 2009). However, cooperative behaviour can evolve between completely unrelated organisms such as two different species if both parties benefit from the relationship (Doebeli and Knowlton 1998). Mutualisms between pairs of phylogenetically separate species are widespread in nature, such as between plants and their mycorrhizal fungi, between mammals and their gut bacteria, or between protective ants and honeydew-producing aphids (Boucher et al. 1982). Mixed-species mutualistic associations between more closely related species, on the other hand, are less common. One example is the highly stable groups of two Amazonian tamarin species (*Saguinus fuscicollis avilapiresi* and *Saquinus mystax pileatus*) that both appear to benefit from the association due to a coordination of anti-predation tactics; while one species detects terrestrial threats, the other detects aerial and arboreal predators (Peres 1993). Another example is the parabiotic association (i.e. nest sharing) between two tropical ant species where *Camponotus rufifemur* provides nest defence whereas *Crematogaster modiglianii* provides nest initiation and more efficient foraging (Menzel and Bluthgen 2010). Such mutually beneficial mixed-species associations rarely include highly aggressive, carnivorous animals, since an association requires a high level of inter-individual tolerance. However, evidence suggests that mutualistic associations occur between some pairs of spider species; organisms that are usually both aggressive and cannibalistic. For example, the large tropical spider *Psechrus argentatus* has the benefit of higher growth rates from increased prey capture when its web is hosting the

smaller spider *Philoponella undulate* which in return gains structural support for its web (Elgar 1994). Other mixed-species associations of spiders that may represent mutualistic interactions include aggregations of colonial *Metepeira incrassata* and *Nephila clavipes* where the latter experience increased prey capture success in mixed-species colonies compared to single-species colonies (Hodge and Uetz 1996). Another example is the large inter-specific nest complexes of three species of jumping spiders (*Menemerus* sp. and two *Pseudicius* spp.) (Jackson 1986). Although intra- and inter-specific tolerance is a feature of all species in both of these latter examples, evidence for mutualistic benefits from inter-specific species interactions is lacking (Jackson et al. 2008). Hence, these examples could represent associations where one species benefits from the association whereas another species is unaffected (i.e. commensalism) or experiences costs associated with the interaction (i.e. parasitism), as seen in many kleptobiotic species associations amongst spiders (e.g. Grostal and Walter 1997).

In the spider world, a permanent high level of tolerance towards other individuals is rare, but does occur in group-living species. Intraspecific group living in spiders can take two very distinct forms that follow two different evolutionary pathways. These two types of group-living spiders are defined as social and colonial, respectively. Social spiders (i.e. non-territorial permanent–social spiders sensu Avilés 1997) live in kin groups and cooperate in prey capture and brood care, whereas colonial spiders (i.e. territorial permanent–social spiders sensu Avilés 1997) form aggregations wherein they defend their own territorial web and show limited or no cooperation. Common for the two types of communities is the strong tendency to aggregate and display permanent con-specific tolerance. However, the degree of cooperation and tolerance is by far greatest in the social spiders that have evolved via the subsocial route through extended maternal care, sibling interactions and the loss of costly dispersal (Kullmann 1972; Avilés 1997; Agnarsson et al. 2006; Lubin and Bilde 2007). High within-group relatedness suggests that kin selection has played a key role in the evolution of cooperation and allomaternal brood care (Lubin and Bilde 2007; Schneider and Bilde 2008). In contrast, relatedness may play a minor role in the evolution of colonial living in spiders, where aggregations of solitary webs provide direct benefits of increased prey capture rates and reduced cost of silk production (Uetz 1989; Uetz and Hieber 1997).

Group living in spiders is restricted to fewer than 25 social species (Agnarsson et al. 2006) and at least twice that number of colonial spiders (Bilde and Lubin 2011) out of about 42,500 spider species described to date (Platnick 2012). Interestingly, group living has evolved independently multiple times in phylogenetically distant families (Uetz and Hieber 1997; Agnarsson et al. 2006; Johannesen et al. 2007). Theridiidae (cobweb spiders) is the spider family with the largest number of social species and independent origins of permanent sociality. This family contains species expressing all levels of sociality including solitary, subsocial, social and colonial behaviour (Agnarsson 2004; Agnarsson 2006). Subsocial behaviour involves extended maternal care with feeding of young, and a temporary period of coexistence of the offspring in the maternal nest after the mother dies. During the stay in the maternal nest, spiderlings cooperate in prey capture, feeding, and web maintenance before they eventually disperse and live solitarily (Lubin and Bilde 2007). Understanding the level of sociality in group living species, as well as interactions among group members in mixed-species associations considerably adds to our general understanding of evolution of sociality and cooperation.

In the present study, we investigated interactions among group members in a newly discovered association of mixed-species colonies consisting of two theridiid spider species found on Bali, Indonesia. Our morphological and genetic analyses showed colonies to be comprised of two different, but closely related species; *Chikunia nigra* (O.P. Cambridge, 1880) new combination (prev. *Chrysso nigra*) and an undescribed *Chikunia* sp. Our aim was to estimate the degree sociality exhibited by the two species. Through a series of behavioural experiments we examined intra- and interspecific tolerance, aggression and cooperation, and tested the potential for adoption of foreign brood. Under colonial group living, adult females should defend territories where they exclusively care for own brood and engage in individual prey capture. If spiders engage in social behaviour, we would find evidence of allomaternal brood care or brood adoption, cooperative prey capture and potentially a female-biased sex ratio which is characteristic of the permanently social spiders (Lubin and Bilde 2007).

Methods

Study organisms and field site

Theridiid spiders that were later identified as two *Chikunia* spp. were found to live in high densities in mixed colonies on plants on Bali, Indonesia. While subsocial behaviour is known from many different theridiid genera (Agnarsson 2004), to our knowledge mixed-species colonies of *Chikunia* spp. have only been found at this one place on the border of Beratan Lake (8°16.45 S, 115°9.99 E). In October 2010, we identified and obtained GPS points of ten colonies from each of two different colony clusters located about 600-m apart. Henceforward, a colony of spiders will refer to all spiders of both species occupying a clearly defined vegetation unit such as a free standing bush or a small tree.

Behavioural assays

In order to determine the level of tolerance, cooperation and territoriality of the two *Chikunia* spp., an array of behavioural assays was performed in the field.

All behavioural tests were done before we discovered and confirmed that colonies consisted of two different species, and hence all assays were performed blind with respect to species, i.e. when using females or males from a colony, individuals from both species were used randomly. The two species are small (<5 mm) same-sized spiders with very similar appearances and were not distinguishable when performing behavioural tests.

Inter-individual distances

As a measure of tolerance levels and territoriality, the nearest neighbour distances were taken for all adult females, with or without brood, within a clearly defined section of each of four different colonies. The distance from a resting female to the nearest resting adult female in any direction, as well as to the nearest male if one was present in the vicinity, was measured in centimetres with a tape measure to nearest centimetres. The distance between a female and her nearest neighbour was used as a proxy for the size of her territory. Furthermore, brood area was estimated; young clustered together gregariously near their mother and occupied a small area under a leaf, and the diameter of this area was measured.

If adult females have territories suitable for prey capture and rearing of offspring which they defend against neighbouring colony members, the average distance between two adult females should be greater than the average diameter of the circle that a clutch of spiderlings occupies. If the spiders were social, the inter-individual distance from one spider to its nearest colony member should be minimal or non-existing when resting, and the position of females and clutches of young would overlap revealing no significant difference between clutch area and distance to nearest neighbour.

Sex ratio

Colonies of social spiders have female biased primary sex ratios (sex ratio at fertilization of eggs) on the order of up to ten females per male (Aviles and Maddison 1991; Lubin and Bilde 2007). All adult males and females of both species within clearly defined sections of each of four different colonies were counted in order to obtain within-colony tertiary (adult) sex ratios of the two species pooled.

Aggression tests

In order to assess tolerance levels towards intruders, we introduced adult male and female colony members into the assumed territory of females with brood in the field. If females defend territories they should show the highest levels of aggression towards intruders while they have brood, since a territory with offspring represents a high resource investment.

We introduced an intruder by catching an adult male or female and bringing the individual in close contact with a target female from the same colony on the underside of a leaf, holding the intruder gently with a pair of soft forceps. We ensured that the target female had detected the intruder by allowing gentle physical contact between the two spiders.

A target female was considered to act aggressively towards the intruder if she bit and chased away the intruder, whereas she was considered to behave non-aggressively if she either remained still on the spot or moved away from the intruder. Each target female was presented with one male and one female intruder in a random order. Target females with hatched young (N=19) and target females with egg sacs (N=20) from three different colonies were tested.

Acceptance of foreign brood and cooperative brood care

We tested whether females would accept foreign brood and whether two females would cooperate in brood care. We collected females from four different colonies together with the leaf on which they kept an egg sac or a clutch of hatched young and placed them in collection tubes (tubes with female and egg sac: height=2.7 cm, diameter=2.2 cm, tubes with female and young: height=6 cm, diameter=3.2 cm). Eighteen collected females with hatched young were removed gently with a pair of soft forceps from their own offspring. Half of these females were placed in a tube with the lone offspring of another female from the same colony (N=9) while the remaining females were returned to the tube they came from with their own offspring as a control (N=9). Similarly, 22 collected females were removed from their egg sac, and half of these were placed with a lone foreign egg sac from the same colony (N=11) while the rest (the control) were brought back to their own egg sac (N=11).

The manipulation took place in the afternoon between 3 and 5 p.m. at room temperature, and acceptance versus rejection of brood was recorded the following morning. We considered an egg sac accepted if the female had attached the egg sac with silk on the leaf and placed herself within 1 cm of the egg sac touching the silk on which the egg sac was attached, similarly to how females tend their eggs in the field. An egg sac was considered rejected if the female was found >1 cm from the egg sac without having attached it to silk.

Hatchlings were considered accepted if the female had placed herself within the circle that the clustering young occupied on the leaf, similarly to how females tend hatchlings in the field. When females rejected the hatchlings, the young had abandoned the cluster and were scattered randomly around the tube.

To investigate whether the spiders may engage in cooperative brood care, 14 females with hatched young were collected. Two females were placed in a collection tube together with a single clutch of young ($N=7$). If the spiders have a tendency for cooperative brood care, two females restricted in space presented with only one clutch of young should tolerate each other and the spiderlings and settle together by the young. If females did not tolerate a second female close to their brood, continuous aggression would be observed until one female would settle at a distance to the clutch. Due to high levels of aggression between the pairs, the tests were terminated after 1 h in order to prevent females from extensive injury.

Cooperative foraging

To test whether spiders cooperate in prey attack or engage in cooperative feeding, a prey item (winged aphid, Hemiptera) was introduced into the silk threads extending between two leaves each occupied by an adult female (distance between pair of females <10 cm). Capture web between the retreats of two females from the same colony could potentially be used by both females and allow for cooperative foraging. Prey was introduced once between each pair of females ($N=30$) from three different colonies and response to the prey item was recorded. If both females approached the prey, attacked the prey or fed on the prey without aggression towards each other, capture webs would not be considered as strictly defended territories and cooperative foraging in colonies would be likely. If only one of the females in each trial would approach the prey with no reaction from the second female, each capture web might be considered a personal territory with little possibility for cooperative foraging.

Species identification and molecular methods

Colonies were initially found to consist of males and females of two different 'morphs'. To determine whether these two morphs belonged to the same species or to two separate species, males and females of both morphs were collected for examination in the laboratory. The morphs were found to putatively represent two morphologically different species based on conspicuous differences between male genitalia structures. Morphological species identification was subsequently confirmed by genetic analyses.

We sequenced five individuals of *Chrysso nigra* from India and Sri Lanka, and three individuals of the Bali specimens putatively assigned to this species. We sequenced seven individuals of the other 'morph'. Given the low within and high between species sequence divergence in all three genes and full congruence of DNA results with morphology, further sampling of specimens was deemed unnecessary to simply establish that these are two different species. We isolated DNA from

abdomens of males and carapace of females using the Qiagen DNAeasy Tissue Kit (Qiagen, Inc., Valencia, CA), and amplified fragments of the mtDNA COI (primers: LCOI1490 GGTCAACAAATCATAAAGATATTGG (Folmer et al. 1994) and C1-N-2776 GGATAATCAGAATATCGTCGAGG (Hedin and Maddison 2001) and the rapidly evolving nuclear DNA ITS2 (primers: ITS-4/ITS-28S TCCTCCGCTTATTG ATATGC and ITS5.8 GGGACGATGAAGAACGCAGC (White et al. 1990). We also amplified nuclear DNA 28S (primers: 28SO TCGGAAGGAACCAGCTACTA and 28SC GAAACTGCTCAAAGGTAAACGG (Whiting et al. 1997)) from two individuals of each species to verify nuDNA distinctiveness in more slowly evolving loci. These represent both mitochondrial and nuclear genomes and both COI (Hebert et al. 2003) and ITS2 (Agnarsson 2010) are readily amplifiable markers that are variable enough to be informative at the species level. Many closely related theridiid species, however, show very little differences in the more slowly evolving 28S (Agnarsson et al. 2007). We used standard protocols with 47 °C annealing temperature for 30 cycles. The PCR products were sequenced by the sequencing and genotyping facility of the University of Puerto Rico, and by MACROGEN, and sequences were submitted to GenBank with the accession numbers: [not yet available].

Sequences were inferred using Phred and Phrap (Green 1999; Green and Ewing 2002) through the Chromaseq package (Maddison and Maddison 2011a) in the evolutionary analysis program Mesquite 2.75 (Maddison and Maddison 2011b) with settings as described in (Agnarsson 2012). Subsequently, the sequences were proofread by comparing them with the chromatograms by eye. Sequences were aligned using ClustalW (Thompson et al. 1994) via Mesquite, with gap opening and extension costs set at 24/6. For all genes, the alignments were unambiguous and thus further exploration of alignment parameters was not necessary. Genetic distances were calculated in Mesquite. Neighbour joining analysis of DNA barcodes (COI fragment) was used to test species boundaries and identify the Bali species. A sequence each of COI and 28S from each species were then added to the phylogenetic matrix of Arnedo et al. (2009) to confirm the generic placement of these species. Phylogenetic analysis was done in MrBayes V3.1.2 (Huelsenbeck and Ronquist 2001) with substitution models chosen for each gene using jModeltest 0.1.1 (Posada 2008), using the AIC criterion (Posada and Buckley 2004) to select among the 24 models implemented in MrBayes. The best model for COI was GTR + I + Γ, and for 28S it was HKY + Γ. In the Bayesian analysis, the two loci combined ran the Markov chain Monte Carlo with four chains for 10,000,000 generations, sampling every 1,000 generations. Stationarity was reached within the first 1,000,000 generations while the sample points of the first 5,000,000 generations were discarded as "burnin".

Statistics

To compare nearest neighbour distances, and to compare counts of females and males within colonies in order to test for a sex ratio bias, we used the linear mixed model function lmer from the lme4 (Bates et al. 2011) package in R (R Development Core Team 2011). In all models constructed, we used colony ID as a random factor to control for colony effects. We obtained p values of the fixed effects by comparing the full model with a null model identical to the full model except with the fixed effect removed, using likelihood ratio tests (χ^2).

For the neighbour distances, we constructed three models, one for testing whether females kept a different distance to nearest male compared to female using gender of the neighbour as the fixed effect, and two for testing whether either nearest female or male distance differed from the diameter of the clutch area, using neighbour vs. clutch as fixed effect (one model for each neighbour gender). These models had Gaussian error, hence we checked whether the assumptions of normally distributed and homogenous residuals were fulfilled by inspecting qq-plots and the residuals plotted against fitted values. Due to non-homogenous residuals, we square root-transformed the nearest-neighbour distances.

To compare counts of males and females within colonies, we constructed a model with gender as a fixed effect. In this model, Poisson error with a log link function was used. We used an offset function of log of total number of counted spiders in each colony to account for differences in sampling among colonies.

We used Fisher's exact test to compare acceptance of own versus foreign brood as well as aggression towards males versus females in 2×2 contingency tables.

Results

General observations

Mixed-species colonies of two *Chikunia* spp. were found in two open, landscaped areas with managed bushes, plants and trees. In each of these areas, the spiders were abundant and occupied about half of the plants in the area. When they occurred on a bush or on a tree, they could be found on most leaves and would occupy the entire plant, with colony sizes of about 30 to >100 adult individuals. Both species were present in each colony examined; however, the proportion of each species within colonies was unknown. Single males of at least one of the two species could be found on otherwise unoccupied surrounding plants.

As many other theridiids, these spiders live on the underside of leaves from which they extend single sticky silk threads to nearby vegetation (Eberhard et al. 2008). These threads act as capture webs and are extended between leaves occupied by different individuals. Adult spiders were often surrounded by multiple juveniles of different instars and sometimes by sub-adults of both sexes. On several occasions, a number of adult males were observed sharing a single leaf, and adult females could be found in close proximity of each other.

Females performed brood care for egg sacs and hatched young. A female attached her egg sac to the underside of a leaf and stayed near it until the spiderlings emerged, after which she remained near her gregarious young. The young stayed in the clutch as a group through a number of molts, estimated up to instar IV juveniles (three molts away from adulthood) in some cases. Females were observed to actively feed young by catching prey and sharing it with their young.

Behavioural assays

Inter-individual distances

Females kept an average of 7.5 cm and a median of 7.0 cm distance to nearest female across the four colonies, ranging from 0 to 22 cm (Fig. 1). The distance from female to nearest female was consistently larger than the diameter of the area that brood occupied ($\chi^2=106.1$, d.f.=1, $P<0.001$). The average brood diameter was 2.1 cm (median=2.0 cm) ranging from 1.0 to 4.5 cm. Similarly, distance from female to nearest male was larger than the diameter of the brood ($\chi^2=9.2$, d.f.=1, $P<0.01$). This indicates that females defended territories large enough to keep other females and males at a distance from their brood. The distance from a female to nearest male (average of 5.0 cm, median of 3.5 cm, and a range from 0 to 14 cm) was consistently shorter than the distance to the nearest female within colonies ($\chi^2=45.1$, d.f.=1, $P<0.001$), suggesting that females had a higher tolerance towards males than females.

Sex ratio

The tertiary (adult) sex ratios within colonies were female biased in the order of almost three adult females per adult male. The ratios of males in four different colonies were 0.17, 0.24, 0.36 and 0.28, respectively which was significantly skewed ($\chi^2=66.4$, d.f.=1, $P<0.001$).

Aggression tests

When introducing a male or a female into the close vicinity of a female, aggression towards the intruder was observed in less than 40 % of the occasions (Fig. 2). Females with young were aggressive towards intruding females on eight out of 19 occasions and towards intruding males in six out of 19

Fig. 1 Box plot of inter-individual adult distances and diameter of clutch size. For each of four different colonies, *A* through *D*, the diameter of the area that a clutch occupied is given, as well as the distance from an adult female to the nearest other female and to the nearest male. Sample sizes are provided above each box
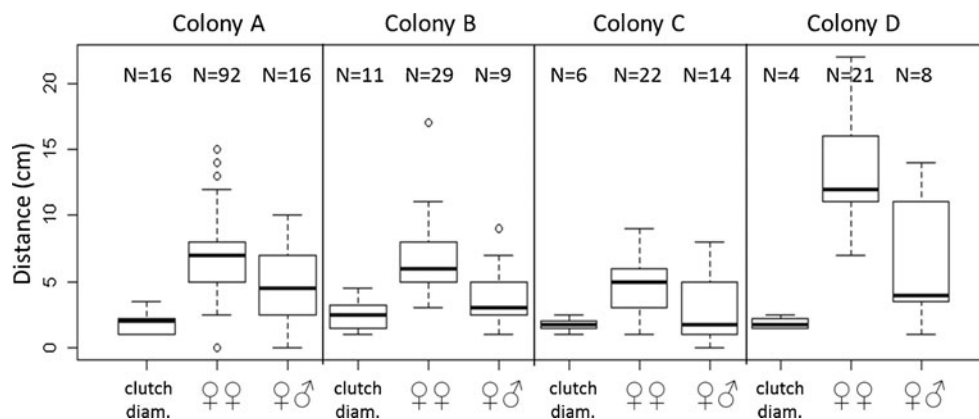
occasions. Females with egg sacs were aggressive towards a foreign female in ten out of 20 occasions, and in five out of 20 occasions towards a male intruder. No difference in aggressiveness towards males and females was detected ($P=0.74$ for females with hatched young, $P=0.19$ for females with egg sacs and $P=0.16$ for all females with brood combined, Fisher's exact test).

Acceptance of foreign brood and cooperative brood care

Females readily accepted both egg sacs and hatched young of other females. All nine females accepted own hatched young, eight out of nine accepted foreign young, ten out of 11 accepted their own egg sac and nine out of 11 accepted a foreign egg sac (Fig. 3). There was no difference between acceptance of own and foreign brood ($P=1$ for hatched young and $P=1$ for egg sacs, Fisher's exact test), suggesting that adoption of intra-specific foreign offspring is possible in these spiders and indicating relaxed selection for brood recognition. While inter-specific brood adoption may have occurred in our study as well, we cannot conclude from this study that it occurs in this system, as species was not controlled.

In none of the seven cases where female pairs were placed with one clutch of young did the females settle next to one another and close by the young. On the contrary,

continuous aggression was observed between most of the female pairs. Hence, cooperative brood care in these spiders seems unlikely.

Cooperative foraging

No evidence of cooperative prey attack was found. A total of 20 prey items (out of 30 prey items introduced) was accepted and attacked. In each of the 20 trials, a single female attacked the prey while the nearby female showed no visible reaction to the prey. In three out of the 20 occasions of prey attack, the female dragged the prey back to her clutch of young, after which mother and young fed communally on the prey, as is typical in species showing extended maternal care.

Species identification and phylogeny

The two spider morphs were confirmed to belong to two different species by observing differences in genitalia and by using a DNA barcoding approach (Figs. 4 and 5) and are supported by genetic distances between the species. While females have similar genitalia, the male habitus (Fig. 4) and male genitalia differ dramatically, especially in the length and shape of the embolus. The two morphs belonged to two
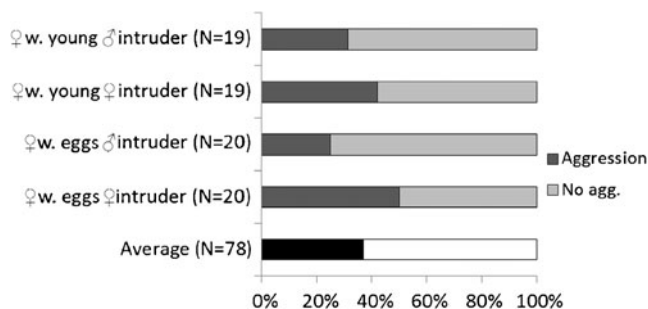

Fig. 2 Aggression towards intruders. A bar graph showing the proportion of occasions in which females with hatched young or egg sacs acted aggressively towards a male or female intruder, as well as the overall proportion of aggressive acts. Sample sizes are provided for each treatment
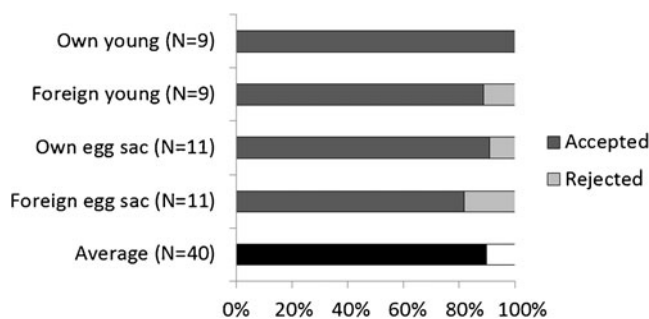

Fig. 3 Acceptance of foreign brood. A bar graph showing the proportion of occasions in which females accepted their own or foreign egg sacs or hatched young, as well as the overall proportion of accepted brood. The sample sizes are provided for each treatment

Fig. 4 Photographs of males and females of the two species. **a** Male *Chikunia nigra*. **b** Female *Chikunia nigra* performing brood care by sharing prey with her young. **c** Male undescribed *Chikunia* sp. **d** Female undescribed *Chikunia* sp. with her hatchlings
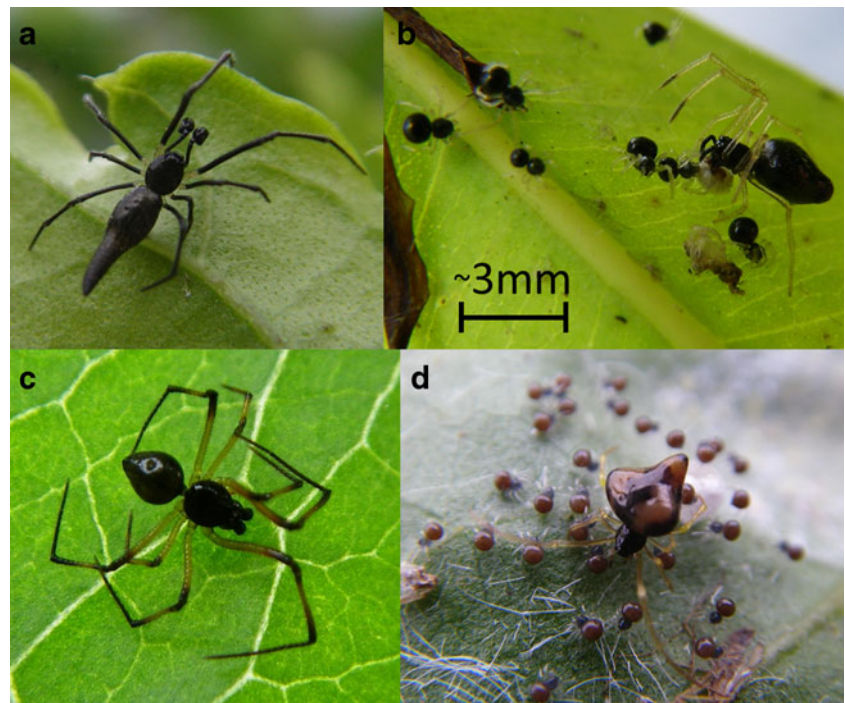
distinct genetic clusters. For the mtDNA COI, variation within morphs was <2 % while variation among morphs was 7–8 % (typical differences between sister species in the theridiid genus *Anelosimus* is 3–7 %). In nuclear DNA, variation within morphs was <1 % for both genes, and about 3.8–5.5 % between morphs in ITS2 and 2.9–3.8 % in the relatively slowly evolving 28S. By morphological examination of genitalia structure of both males and females of one species, it was identified as likely *Chrysso nigra*, and the identification was subsequently confirmed through barcoding analysis including *Chrysso nigra* individuals from India

and Sri Lanka, the type locality. However, phylogenetic analyses did not place this species close to the genus *Chrysso*, but as close relatives of the morphologically similar *Chikunia albipes* the sole member of the currently monotypic *Chikunia*. This confirms the findings of Deeleman-Reinhold (2009) who concluded that *Chrysso nigra* did not belong to *Chrysso*, although she was unable to place the species. We therefore transfer *Chrysso nigra* to *Chikunia* (new combination), a genus now containing two described and one undescribed species that all share similar body shape and coloration pattern (Fig. 4).

Fig. 5 A neighbour joining analysis of DNA barcodes, along with genitalia differences, confirms the presence of two species in the colonies and suggests a placement of these species within the genus *Chikunia*. Only the small portion containing the species of interest is shown. A Bayesian phylogenetic analysis based on COI and 28S loci corroborates these results (full phylogenetic tree available from authors). *Numbers* above branches are posterior probability values and percentage values illustrate mtDNA divergences between the study species
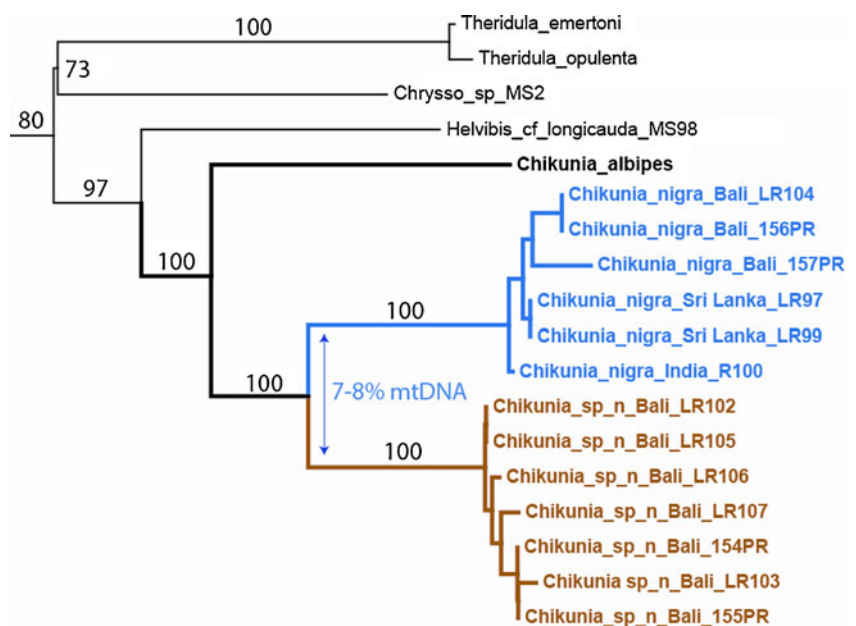
Discussion

We report field data on mixed-species colonies consisting of two separate, yet closely related, species: *Chikunia nigra* (O.P. Cambridge 1880) new combination, previously not reported to be either colonial or subsocial (Sebastian and Peter 2009), and a yet undescribed *Chikunia* sp. Our behavioural experiments suggest that both of these group living spider species have strong aggregation tendencies, high levels of tolerance towards neighbouring conspecifics and hetero-specifics, as well as extended maternal care. Direct cooperation in prey capture and brood care, however, appeared to be absent. We found that spiders of both sexes and of all instars lived interspecifically in close proximity of each other, often within a few centimetres to the nearest neighbour, and sometimes even immediately beside one another with no signs of aggression. Aggression towards intruders was observed in less than half of our aggression trials, indicating relatively low levels of aggression towards neighbours. As a comparison, adult females of the subsocial spider *Delena cancerides* killed 100 % of intruding large juveniles in a study by Beavis et al. (2007). However, we found that adult females occupied distinct territories and kept a larger distance to other adult females than they did to males. Territorial behaviour within groups is consistent with that exhibited in colonial spiders (Bilde and Lubin 2011). Hence, we classify both species as subsocial as well as colonial, forming mixed-species colonies.

Adult females actively fed their offspring by catching prey and sharing it with them, and juveniles of a range of different instars were found in clutches in close proximity of each other. These results are consistent with the definition of subsocial behaviour with extended maternal care and an extended period of maternal tolerance of young. Furthermore, females did not discriminate between their own and foreign brood, whether the brood was in an egg sac or had hatched; they readily adopted foreign brood when presented with that rather than their own, a feature similar to other subsocial spiders (Furey 1998; Schneider 2002). Given that both species were present in all colonies, brood adoption may have occurred in both species and potentially even between species, although future studies controlling for species are needed to confirm hetero-specific brood adoption. Adoption of foreign young in the field could happen by juveniles dispersing out to nearby neighbouring females if their own mother dies. The potential for brood adoption opens up for several possible consequences of interspecific coloniality:

First, it allows for the potential benefit of increased survival rate of offspring due to adoption by neighbouring females, in the case their mother dies. This bet-hedging strategy for females to stay in groups rather than to breed solitarily is thought to be a strong selective advantage in the fostering model presented by Jones et al. (2007). This model was developed to explain facultative group living in the subsocial theridiid *Anelosimus studiosus*. This model shows that group living can be beneficial when the altricial period is long, i.e. the period in which the juveniles depend on maternal care for survival. With the increasing length of the altricial period, the risk that the mother dies before her offspring reach independence increases. The model assumes that females will adopt foreign young of deceased females, an assumption supported by the fact that subsocial females do not seem to discriminate between own and foreign brood (Furey 1998). A long altricial period seems to be prevalent in our study system where juveniles stay in clutches with extended maternal care through a number of different instars. Furthermore, the fostering model assumes no relatedness among colony members. Hence, perhaps group living based on potential benefits of adoption of broods from deceased females can be adaptive even for spiders of mixed species.

Second, if these spiders do indeed adopt young from the other species, it would allow for exploitation of one species by the other. Perhaps slight behavioural differences between the species do exist in propensity to adopt foreign young, or even in propensity to leave an egg sac or a clutch of young if chances are high that the brood will be adopted by a neighbouring female. In this case, selection could favour brood parasitism in one species while selecting for brood recognition or increased aggressiveness towards intruders in the other (Rothstein and Robinson 1998). In fact, differences in tolerance and aggressiveness could occur in the two species. In our behavioural assay, about 40 % of females were aggressive towards intruders while the rest were passive. This result could reflect differences in the level of tolerance between species, or different levels of tolerance towards own versus foreign species. Mixed species associations in group living animals involving related species may often be unbalanced. Although a mutualistic associations of two species of Amazonian tamarins benefits both species with regards to predator detection and avoidance (Peres 1993), *Saguinus fuscicollis avilapiresi* appear to have a large advantage of increased food uptake when foraging in groups with *Saguinus mystax pileatus*, whereas the latter appear to gain nothing with regard to food uptake when foraging in mixed-species groups (Peres 1992). Also, in mutualistic mixed-species groups of two ant species, the relative benefit of the association seems to be asymmetric, leaving *Camponotus rufifemur* obligate dependent upon the nest initiating *Crematogaster modiglianii*, whereas for the latter ant species, the mutualism is merely facultative (Menzel and Bluthgen 2010). Strictly parasitic associations between closely related species also occur in group living animals. For example, at least 200 species of ants are parasites of other ant species, and sometimes use same-genera species as hosts (e.g. parasites and their host species within the genus *Lasius*) (Buschinger 1986). Studies controlling for species in

behavioural assays are needed to reveal the specific association between the two mixed species studied here. Only by investigating the particular interactions between the species, we will know whether this occurrence of interspecific coloniality reflects a mutualistic relationship or a more unbalanced association where one benefits more than the other, approaching commensalism or even parasitism.

We found female biased adult sex ratios within colonies; however, this does not necessarily imply that these spiders have a skewed primary sex ratio, i.e. at the time of fertilization of the eggs (Avilés 1997; Lubin and Bilde 2007). A strongly female-biased primary sex ratio is usually associated with permanent social spiders with a regular inbreeding mating system, whereas a biased adult sex ratio could be the result of differential mortality rates between sexes, different dispersal patterns between the sexes, or greater longevity of brood caring females, as is common in most spiders (Foelix 1996). Is it likely that dispersal rate is higher in males than in females of these *Chikunia* spp. since lone males sometimes were observed in otherwise unoccupied plants in the area. It would be necessary to investigate the primary sex ratio in these spiders in order to compare with the highly female-biased sex ratio in social spiders.

Understanding the evolutionary steps involved in this mixed-species association is intriguing. Colonial spiders evolved through mutual benefits whereby joining individual webs into aggregations increased prey capture success and reduced silk cost, and therefore do not per se require high relatedness between group members (Uetz 1989; Uetz and Hieber 1997). Furthermore, since group living in colonial spiders has evolved through aggregations of otherwise strictly solitary species with limited maternal care, permanent sociality including cooperative, allomaternal brood care is unlikely to arise from coloniality (PlateauxQuenu et al. 1997). Social spiders evolved via the subsocial route, where maternal tolerance of her offspring in the nest, feeding of young, and pre-dispersal cooperation among siblings in the maternal nest has evolved into life-long tolerance and cooperation (Avilés 1997; Lubin and Bilde 2007). Subsocial spider adults usually live solitarily (Avilés 1997; Lubin and Bilde 2007), and when they do live in groups, relatedness is most likely elevated since group living is then a result of refraining from dispersal from juvenile sibling groups (Furey 1998). Hence, if group living in these two subsocial *Chikunia* spp. evolved via the colonial route through aggregations of unrelated adults resulting in benefits of group living such as increased prey capture and decreased silk expenditure, it is unusual that they, at the same time, show subsocial behaviour. If, on the other hand, these species have evolved group living via the subsocial route through mother–offspring interactions, sibling tolerance and limited dispersal, it is highly unusual that this occurs among individuals of different species. Which factors have been involved in the selection for this system? Is cost of dispersal unusually high and survivability of solitary spiders low, similar to other subsocial spiders (Bilde et al. 2005)? Is prey capture or predator defence more efficient in aggregations as known for other colonial spiders (Bilde and Lubin 2011)? Further studies on this mixed-species colonial spider system are now called for, investigating how widespread the association is between the two species, as well as quantifying the costs and benefits of the relationship to each species and the potential for inter-specific brood adoption. Perhaps further studies of this system will reveal an additional case of the rare occurrences of mutualistic associations between two closely related species in the animal world.

References

Agnarsson I (2004) Morphological phylogeny of cobweb spiders and their relatives (Araneae, Araneoidea, Theridiidae). Zool J Linnean Soc 141(4):447–626

Agnarsson I (2006) A revision of the New World eximius lineage of *Anelosimus* (Araneae, Theridiidae) and a phylogenetic analysis using worldwide exemplars. Zool J Linnean Soc 146(4):453–593

Agnarsson I (2010) The utility of ITS2 in spider phylogenetics: notes on prior work and an example from *Anelosimus*. J Arachnol 38(2):377–382

Agnarsson I (2012) A new phylogeny of *Anelosimus* and the placement and behavior of *Anelosimus* vierae n. sp from Uruguay (Araneae: Theridiidae). J Arachnol 40(1):78–84

Agnarsson I, Aviles L, Coddington JA, Maddison WP (2006) Sociality in theridiid spiders: repeated origins of an evolutionary dead end. Evolution 60(11):2342–2351

Agnarsson I, Maddison WP, Aviles L (2007) The phylogeny of the social *Anelosimus* spiders (Araneae: Theridiidae) inferred from six molecular loci and morphology. Mol Phylogenet Evol 43(3):833–851

Arnedo MA, Hormiga G, Scharff N (2009) Higher-level phylogenetics of linyphiid spiders (Araneae, Linyphiidae) based on morphological and molecular evidence. Cladistics 25(3):231–262

Avilés L (1997) Causes and consequences of cooperation and permanent-sociality in spiders. In: Choe JC, Crespi BJ (eds) The evolution of social behavior in insects and arachnids. Cambridge University Press, Cambridge, pp 476–498

Aviles L, Maddison W (1991) When is the sex-ratio biased in social spiders?: chromosome studies of embryos and male meiosis in *Anelosimus* species (Araneae, Theridiidae). J Arachnol 19(2):126–135

Bates D, Maechler M, Bolker B (2011) lme4: Linear mixed-effects models using S4 classes, http://CRAN.R-project.org/package=lme4

Beavis AS, Rowell DM, Evans T (2007) Cannibalism and kin recognition in *Delena cancerides* (Araneae: Sparassidae), a social huntsman spider. J Zool 271(2):233–237

Bilde T, Lubin Y (2011) Group living in spiders: cooperative breeding and coloniality. In: Herberstein ME (ed) Spider behaviour, flexibility and versatility. Cambridge University Press, New York

Bilde T, Lubin Y, Smith D, Schneider JM, Maklakov AA (2005) The transition to social inbred mating systems in spiders: role of inbreeding tolerance in a subsocial predecessor. Evolution 59(1):160–174

Boomsma JJ (2009) Lifetime monogamy and the evolution of eusociality. Philos Trans R Soc B Biol Sci 364(1533):3191–3207

Boucher DH, James S, Keeler KH (1982) The Ecology of Mutualism. Annu Rev Ecol Syst 13:315–347

Buschinger A (1986) Evolution of Social Parasitism in Ants. Trends Ecol Evol 1(6):155–160

Deeleman-Reinhold CL (2009) Spiny theridiids in the Asian tropics. Systematics, notes on behaviour and species richness (Araneae: Theridiidae: Chrysso, Meotipa). Contrib Nat Hist 12:403–436

R Development Core Team (2011) R: A language and environment for statistical computing, http://www.R-project.org/R Foundation for Statistical Computing. Vienna, Austria

Doebeli M, Knowlton N (1998) The evolution of interspecific mutualisms. Proc Natl Acad Sci U S A 95(15):8676–8680

Eberhard WG, Agnarsson I, Levi HW (2008) Web forms and the phylogeny and theridiid spiders (Araneae: Theridiidae): chaos from order. Syst Biodivers 6(4):415–475

Elgar MA (1994) Experimental evidence of a mutualistic association between 2 web-building spiders. J Anim Ecol 63(4):880–886

Foelix RF (1996) Biology of spiders. Oxford University Press, Oxford

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3(5):294–299

Furey FE (1998) Two cooperatively social populations of the theridiid spider Anelosimus studiosus in a temperate region. Anim Behav 55:727–735

Green P (1999) PHRAP, http://phrap.org/

Green P, Ewing B (2002). PHRED, http://phrap.org/

Grostal P, Walter DE (1997) Kleptoparasites or commensals? Effects of Argyrodes antipodianus (Araneae: Theridiidae) on Nephila plumipes (Araneae: Tetragnathidae). Oecologia 111(4):570–574

Hamilton WD (1964) Genetical evolution of social behaviour I. J Theor Biol 7(1):1–16

Hebert PDN, Ratnasingham S, deWaard JR (2003) Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proc R Soc London, Ser B 270:S96–S99

Hedin MC, Maddison WP (2001) A combined molecular approach to phylogeny of the lumping spider subfamily Dendryphantinae (Araneae: Salticidae). Mol Phylogenet Evol 18(3):386–403

Hodge MA, Uetz GW (1996) Foraging advantages of mixed-species association between solitary and colonial orb-weaving spiders. Oecologia 107(4):578–587

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17(8):754–755

Jackson RR (1986) Communal jumping spiders (Araneae, Salticidae) from Kenya—interspecific nest complexes, cohabitation with web-building spiders, and intraspecific interactions. N Z J Zool 13(1):13–26

Jackson RR, Nelson XJ, Salm K (2008) The natural history of Myrmarachne melanotarsa, a social ant-mimicking jumping spider. N Z J Zool 35(3):225–235

Johannesen J, Lubin Y, Smith DR, Bilde T, Schneider JM (2007) The age and evolution of sociality in Stegodyphus spiders: a molecular phylogenetic perspective. Proc R Soc Lond B Biol 274(1607):231–237

Jones TC, Riechert SE, Dalrymple SE, Parker PG (2007) Fostering model explains variation in levels of sociality in a spider system. Anim Behav 73:195–204

Kullmann EJ (1972) Evolution of social behavior in spiders (Araneae - Eresidae and Theridiidae). Am Zool 12(3):419–426

Lubin Y, Bilde T (2007) The evolution of sociality in spiders. Adv Study Behav 37:83–145

Maddison D, Maddison W (2011a) Chromaseq: a Mesquite module for analyzing sequence chromatograms. Version 0.99, http://mesquiteproject.org/packages/chromaseq

Maddison W, Maddison D (2011b) Mesquite: a modular system for evolutionary analysis. Version 2.75, http://mesquiteproject.org

Menzel F, Bluthgen N (2010) Parabiotic associations between tropical ants: equal partnership or parasitic exploitation? J Anim Ecol 79 (1):71–81

Peres CA (1992) Prey-capture benefits in a mixed-species group of Amazonian tamarins, Saguinus fuscicollis and Saguinus mystax. Behav Ecol Sociobiol 31(5):339–347

Peres CA (1993) Anti-predation benefits in a mixed-species group of Amazonian tamarins. Folia Primatol 61(2):61–76

PlateauxQuenu C, Horel A, Roland C (1997) A reflection on social evolution in two different groups of arthropods: halictine bees (Hymenoptera) and spiders (Arachnida). Ethol Ecol Evol 9 (2):183–196

Platnick NI (2012) The World Spider Catalog, Version 12.5, http://research.amnh.org/iz/spiders/catalog American Museum of Natural History

Posada D (2008) jModelTest: phylogenetic model averaging. Mol Biol Evol 25(7):1253–1256

Posada D, Buckley TR (2004) Model selection and model averaging in phylogenetics: advantages of akaike information criterion and Bayesian approaches over likelihood ratio tests. Syst Biol 53 (5):793–808

Rothstein SI, Robinson SK (1998) Parasitic birds and their hosts: studies in coevolution. Oxford University Press, New York

Schneider JM (2002) Reproductive state and care giving in Stegodyphus (Araneae: Eresidae) and the implications for the evolution of sociality. Anim Behav 63:649–658

Schneider JM, Bilde T (2008) Benefits of cooperation with genetic kin in a subsocial spider. Proc Natl Acad Sci U S A 105(31):10843–10846

Sebastian PA, Peter KV (2009) Spiders of India. Universities Press (India), Hyderabad

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22(22):4673–4680

Uetz GW (1989) The Ricochet effect and prey capture in colonial spiders. Oecologia 81(2):154–159

Uetz GW, Hieber CS (1997) Colonial web-building spiders: balancing costs and benefits of group-living. In: Choe JC, Crespi BJ (eds) The evolution of social behavior in insects and arachnids. Cambridge University Press, Cambridge, pp 458–475

White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gefland DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, Inc., San Diego, pp 315–322

Whiting MF, Carpenter JC, Wheeler QD, Wheeler WC (1997) The strepsiptera problem: phylogeny of the holometabolous insect orders inferred from 18S and 28S ribosomal DNA sequences and morphology. Syst Biol 46(1):1–68