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Abstract

In this study we compare the courtship behaviours of *Pardosa proxima* and *P. vlijmi*, two species of wolf spiders up to now regarded as "ethospecies", by means of motion analysis methodologies. In particular, we investigate the features of the signals, aiming at understanding the evolution of the courtship and its role in species delimitation and speciation processes. In our model, we highlight a modular structure of the behaviours and the presence of recurring units and phases. According to other similar cases concerning animal communication, we observed one highly variable and one stereotyped phase for both species. The stereotyped phase is here regarded as a signal related to species identity or an honest signal linked directly to the quality of the signaler. On the contrary, the variable phase aims to facilitate signal detection and assessment by the female reducing choice costs or errors. Variable phases include cues arisen from Fisherian runaway selection, female sensory exploitation and remaining of past selections.

Keywords : Display; *Pardosa vlijmi*; *Pardosa proxima*; motion analysis.

1. Introduction

Communication takes place when a signal, the "building blocks" of communication (Hebets and Papaj, 2005), is sent from one organism to another, altering the pattern of behaviour or the physiology in another organism. Communication in animals often consists of complex behavioural routines, incorporating several sensory modalities that could be used simultaneously or in sequence (Uetz and Roberts, 2002; Candolin, 2003; Hebets and Papaj, 2005). Moreover, behaviour, like morphology and genes, can be considered a mosaic composed of semi–independent modules evolving along relatively independent paths (Otte, 1972; West–Eberhard, 2003), with profound consequences on the directions and the speed of the evolutionary process (Wagner, 1996; Raff and Sly, 2000; West–Eberhard, 2003; Schlosser and Wagner, 2004; Emlen et al., 2005).

Male–female interactions is one of the most investigated fields in spider communication system (reviewed by Uhl and Elias, 2011). The complexity of the courtship, shaped by several selective forces, can be seen at different levels due to its hierarchical structure and the number of signals and communicative channels involved. Each signal or channel may transfer more than one type of information according to the "multiple message" hypothesis (Möller and Pomiankovski, 1993; Johnstone, 1996) or, on the other hand, it may convey the same type of information according to the "redundant signal hypothesis" (Möller and Pomiankovski, 1993). In the first case we may have different signals that can (i) convey information on different aspects of the signaler's quality; (ii) give information both about the signaler's quality and species identity; (iii) give information both about the signaler's quality and signaler's location; (iv) be addressed to different receivers (reviewed by Hebets and Papaj, 2005). The redundant signal hypothesis or "backup signal" hypothesis (Johnstone, 1996) states that different signals provide the same information increasing the accuracy in the assessment of mate quality by the receiver (Candolin, 2003). Since signalers could be imperfect in coding or accuracy, the total amount of information may thus increase if the signaler combines multiple signals encoding for similar or identical information. However, signals do not always indicate quality or direct benefits. Signals may also facilitate detection and evaluation of other signals (Möller and Pomiankovski, 1993). Fisherian runaway process (Fisher, 1930; Pomiankowski and Iwasa, 1993;

Pomiankowski and Iwasa, 1998), sensory bias exploitation (Ryan and Rand, 1993) and remnants of past selection (Holland and Rice, 1998) play a role in the evolution of this kind of signals. Several hypothesis on the function of the signals were proposed by Candolin (2003) and Hebets and Papaj (2005) such as (i) the ability to generate one signal is tightly correlated with the ability to generate a second signal and thus potentially informative about overall signaler quality, (ii) the presence of one signal increases the speed, the probability of detection and the accuracy in detection of a second signal; (iii) the presence of a signal provides the context in which a receiver can interpret a respond to a second signal; (iv) more signals combined together form a new and unique signal due to emergent proprieties.

Males of several spider families perform species–specific complex courtship displays characterized by sequences of movements that concur to form visual and vibratory signals. Such displays may resemble "dances", composed by a stunning range of behavioural elements and sequences among different signaling modalities (Schneider and Andrade, 2011). The most complex are performed by families Oxyopidae, Salticidae and Lycosidae, characterized by visual/seismic displays performed by the male facing the female (Uhl and Elias, 2011). Within lycosid family, *Hygrolycosa rubrofasciata* is one of the most investigated model regarding substrate-borne vibrations and drumming behaviour and their role in male quality assessment by the female (Mappes et al., 1996; Alatalo et al., 1998; Kotiaho et al., 1996, 2000; Ahtiainen et al., 2005, 2006). Concerning multimodal communication, behavioural complexity, sensory channels isolation and video/audio playback, the genus Schizocosa proved to be one of the most important model organisms for a multitude of experimental studies (Uetz and Roberts, 2002, Hebets, 2005; Framenau and Hebets, 2007; Gibson and Uetz, 2008).

Similarly, spiders of genus *Pardosa* (Lycosidae) show complex and ritualized courtship behaviour, however the majority of the studies are mainly descriptive and only few studies deal with the features of the signal (Rypstra, 2003; Chiarle et al., 2010). Moreover, most of the works (published between late 50's and early 80's) lack quantitative data and, in some cases (Schmidt, 1957; Kronestedt, 1979a) are not available to international readers.

In this work, we describe in detail the reproductive signaling behaviour of two sibling species: *P. proxima* (C. L. Koch, 1847) and *P. vlijmi* Den Hollander and Dijkstra, 1974 highlighting the features

of courtship signals. According to Tongiorgi (1966a), *P. proxima* is widely distributed in the Palearctic and can be found mainly in meadows, along exposed streambeds, on the edges of ponds, lakes and swamps. Though *P. vlijmi* had not yet been described, Tongiorgi (1966a) noticed that *P. proxima* showed high morphological variability regarding both the external pattern and the genital structures, even within specimens of the same population. *Pardosa vlijmi* was described a few years later by Den Hollander and Dijkstra (1974) as an "ethospecies", i. e. on the base of the observation of *P. vlijmi* 's "deviating courtship behaviour" compared to the one of *P. proxima*. We compared the courtship behaviour of these two sibling species investigating the features of the signal and providing some insight about the information sent by different signals. In particular we (i) analyzed both courtship behaviours identifying recurring courtship units and their hierarchical organization, (ii) evaluated the connectedness among courtship units; (iii) analyzed in detail the main units within the courtship and their features (vi) analyzed intra– and inter–specific variability of signals between species supplying preliminary indications of courtship signal function and evolution.

2. Methods

2.1. Animal collection and maintenance

Thirty–one males and seventeen females attributable to *P. proxima* were field collected in northwestern Italy, Piedmont, Province of Cuneo. Twenty–two males and eleven females in an uncultivated meadow surrounded by fields near a small irrigation ditch (loc. Sotteri, municipality of Guarene) and nine males and six females in a meadow surrounded by chestnut woods (loc. Valle Ermetta, municipality of Vicoforte Mondovì).

All specimens were reared in cylindrical plastic containers (6 cm diameter, 2.5 cm high) containing a hydrated piece of cotton for water and humidity. Spiders were maintained at $22 \pm 1°C$ with a 10:14 h light:dark photoperiod. Individuals were fed *ad libitum* with *Drosophila melanogaster*.

After testing the reproductive behaviour, all the specimens were stored in 95% alcohol for future genetic analysis. The material is kept at the Department of Life Sciences and Systems Biology (DBIOS) (Italy).

2.2. Display recording

On the basis of the observation of courtship behaviour and according to Den Hollander and Dijkstra (1974) we assigned seven males to *P. vlijmi* (thus a new record for the Italian fauna) and eleven males to *P. proxima* (for a total of eighteen filmed males) Specimens that showed low courtship activities were not recorded, moreover a few cases of cannibalism were observed (likely due to the impossibility to clearly discriminate specimens between the two species). Only two males (DRM19 and DRM27) of *P. vlijmi* from Vicoforte Mondovì were filmed. Specimens from this locality were infected with endoparasitic nematodes, which probably inhibited the reproductive behaviour of males. The courtship behaviour of males was studied by placing them in contact with a female in a glass arena. A sheet of absorbent paper was placed at the bottom of the arena in order to optimize the transmission of vibrations and the persistence of chemical traces (Rypstra et al., 2003; Chiarle et al., 2010). The female was first placed in the arena for 15 minutes followed by the male. Each specimen was tested for one hour. After each trial the arena was cleaned and new absorbent paper added. All behaviours were recorded with two Canon HV30 cameras at a recording speed of 50i (50 frame per second interlaced). The movies were acquired with Adobe Premiere Pro CS3 (Adobe Systems Incorporated) (HDV resolution: 1480x900 at 50i frames). All the videos were sliced with Avidemux 2.5.2 and edited with Virtual Dub 1.9.8 to obtain de–interlaced AVI files at 50 frames per second. Video sequences of interest were cropped and reduced to half size the original resolution.

2.3. Courtship description and pattern analysis

The pre–copulatory behaviour was hierarchically characterized in accordance with Lehner (1998) as follows (fig. 1):

- courtship element: the movement of the anatomical part directly involved in the behaviour (palps, abdomen, first pair of legs and general body movement);
- courtship unit: several courtship elements grouped together and repeated over time. Courtship elements recurring in cycles repeated over time represent a phase within the courtship unit. A courtship unit can be composed of more than one phase.
- courtship pattern: linkage of several consecutive courtship units showing predictable patterns;
- courtship session: a sequence of courtship patterns repeated over time.

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Videos were analyzed removing the pauses between courtship patterns.   First, we observed the courtship behaviour of *P. proxima* and *P. vlijmi* and then we listed the courtship elements and courtship units composing the courtship pattern. Consecutive courtship units were modeled as a Markov chain, i. e. sequences of behaviours that follow each other at some level of probability (Lehner, 1998). We developed a script in Matlab 7.0 (MathWorks, Natick, MA) in order to analyze the relationship and the linkages among courtship units within a defined courtship pattern.

The output of the script was a transition matrix (one for each specimen) which included the observed frequencies of all unit combinations (i. e. if the courtship display includes N courtship units, the transition matrix will have N rows and N columns; the cell $N(i, j)$ will contain the number of times that the unit i precedes the unit j).The final result was a flow–chart representing the mean courtship pattern for each species.

2.4. Motion analysis

Optical flow analysis is an image–based method for estimating motion. This method measures the change in pixel intensity between video frames as a proxy for motion (see Elias et al., 2006b for details).

This analysis was performed for both species on the main courtship unit, due to the abundance of repetitions and conspicuousness of the movements.

The optical flow results were analyzed for each specimen with univariate ANOVA in order to compare the duration and features of the two main units within and between the two species regarding their mean duration (M), standard deviation (SD) and coefficient of variation (CV).

Differences in mean and variations in the duration of the main unit of both species were analyzed by means of a linear mixed model (Proc mixed in SAS v. 9.1.3). As several measurements on the same individual were performed, we included the random factor "specimen" as a random effect to correct the non–independence of the data. This also allows estimating and testing of variance among individuals.

3. Results

3.1. Courtship description and pattern analysis

The courtship behaviour of *P. proxima* and *P. vlijmi*, constant during the one hour recording of each specimens, was subdivided in courtship elements (table 1, indicated in brackets in the following textual description). In both species, courtship elements were assigned to three main courtship units composing the courtship pattern: starting unit, main unit and ending unit. The main units were further splitted in two phases that recurred in cycle (*P. proxima*: "stepping" and "jerking" phases; *P. vlijmi*: "springing" and "swinging" phases) (fig. 2).

The starting unit was performed only once and it differed between the two species. *Pardosa proxima* raised the first pair of legs (leg raising), which were kept high. The male waved the palps once or twice at the same time (palp waving) while vibrating the abdomen (abdomen twitching). Conversely, males of *P. vlijmi* performed horizontal and vertical movements of the whole body, progressively increasing in intensity (body bouncing). In both species the main unit was characterized by a "hopping" sequence (in accordance with Den Hollander and Dijkstra, 1974) towards the female, which was repeated several times (from 1 to 31 in *P. proxima*, mode= 4; inform one to thirty–two times in *P. vlijmi*, mode= 9) (fig. 3). However, the two behaviours were qualitatively different. In *P. proxima* each "hop" (body hopping) was characterized by two phases: i) the "stepping" phase characterized by the rising of the first pair of legs (leg raising), that were tapped immediately afterwards on the substrate (leg tapping) together with palps rubbing (cymbium rubbing) and the ii) "jerking" phase, in which the male jerkily moved the body while the abdomen vibrated (abdomen twitching) and the palps were rubbed again on the substrate (cymbium rubbing) for one to five times.

In contrast, during the hopping sequence, males of *P. vlijmi* performed up–and–down movements of the whole body. More specifically, the cephalothorax was raised due to the movements of the 2nd, 3rd and 4th pairs of legs (body hopping). The 1st pair was held near the body, parallel to the palps. The palps remained still. Also in this case we recognized two phases. In the "springing" phase (i) the abdomen was shaken with a quick up–and–down movement (abdomen twitching) when the maximum elevation was reached and then the male lowered the body touching the substrate with the palps (cymbium rubbing). After that, ii) one or two fast jerky horizontal movements of the body characterized the "swinging" phase, with the palps touching the substrate (cymbium rubbing).

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The ending unit was almost identical in the two species, namely the palps were rubbed on the substrate (cymbium rubbing). If no copulation occurred, the male ran quickly backwards and started over the courtship pattern with the starting unit.

A linear succession among the three different courtship units was observed for both species. Males performed the starting and the ending units only once at each courtship pattern, while the main unit was repeated several times in both species. The latter followed itself in 83.8% of cases for *P. vlijmi* and 84.2% of cases for *P. proxima*. In *P. vlijmi* the sequence "starting unit to main unit" occurred in 8.7% of cases while "main unit to ending unit" occurred in 7.1% of cases. Similarly, in *P. proxima* the sequence "starting unit to main unit" occurred in 10.8% of cases, while "main unit to ending unit" occurred in 4.8% of cases. In both species, the sequences "ending unit to main unit" and "main unit to starting unit" were unusual and occurred with frequencies lower then 0,1%. Results are illustrated in figure 2.

3.2. Motion analysis (fig. 4)

When looking at the speed profile plot of *P. proxima,* the main unit was characterized by a series of hops repeated in sequence, namely an alternation of "stepping" and "jerking" phase (fig. 5). The first was easily recognizable by a wide peak, while the second often consisted of one to three peaks within the speed profile plot. The "stepping" phase had a duration of 0.42 ◈ 0.06 seconds, (CV= 0.14) while the "jerking" phase lasted 0.56 ◈ 0.30 seconds (CV= 0.53) considering all specimens (N= 11, NC= 809). The "stepping" phase resulted highly variable in terms mean duration (CV was 3.78 times higher in respect to the "jerking" phase) (table 2).

The speed profile plot of the main unit of *P. vlijmi* was also characterized by the repetition of hopping in sequence, however it was qualitatively different from that of *P. proxima*. The observation of the speed profile plot revealed two main peaks related to the "springing" phase: the first was related to the rising and the second to the lowering of the body. The two behaviours were spaced by a short pause due to the absence of movement. The subsequent small peak was related to the "swinging" phase (fig. 6). The "springing" phase lasted in total 0.36 ◈ 0.04 seconds (CV= 0.11) while the "swinging" phase lasted 0.24 ◈ 0.17 seconds (CV= 0.71) considering all specimens (N= 7, NC= 1366). When

considering the whole group of specimens, a high variability was noticed with a CV of the "swinging" phase 6.45 times higher than in the "springing" phase (table 3). Results for each species are plotted respectively in figure 5 and figure 6.

For both species, the duration of "stepping" and "springing" phases did not show significant variations at individual (p= 0.0622) and pattern levels (p= 1). For the duration of the "jerking" and "swinging" phases, significant variations at individual (p= 0.006) and pattern levels (p< 0.0001) were found.

4. Discussion

Modularity, namely the properties of discreteness and dissociability among parts (West–Eberhard, 2003), namely the degree to which developmental/evolutionary processes can be independent for each modules, is thought to enhance evolvability and promote complexity by facilitating the origin of novelty through the reuse and reorganization of existing parts. Modularity is a common propriety of organisms and an important aspect in their evolution (Wagner, 1996; Wagner and Altenberg, 1996; Prum and Dyck, 2003; West–Eberhard, 2003). The grasshopper *Syrbula admirabilis* (Orthoptera, Acrididae) provides some examples on modularity in behavioural context. In this species, the remarkable modular organization and the presence of several units permit a condition–sensitive flexibility in the production of the courtship by the male (Otte, 1972). In vertebrates, the bird of paradise (Aves, Paradisaeidae) was intensively studied on modular organization, highlighting an increasing complexity of courtship from the common ancestor due to duplication, reorganization and independent evolution of modules (Scholes 2008a, b).

Modular organization in courtships is also a common feature in wolf spiders (Araneae, Lycosidae). The *Pardosa lugubris–group* is composed of six species that show remarkable sets of courtship units (Töpfer–Hofmann, 2000) ranging from the simple visual display of *P. lugubris* to the highly complex courtship behaviour of *P. saltans*, the latter composed of several units (Töpfer–Hofmann et al. 2000). Another example is provided by the *P. wagleri*–group, in which the sibling species *P. wagleri* and *P. saturatior* perform two different courtships consisting of two units: a main unit and an ending unit (Chiarle et al., 2010).

Clear modular organization was also recognized when comparing *P. proxima* and *P. vlijmi*. In particular, we identified three different courtship units that occurred in linear succession (starting, main and ending units) and, in addition, two recurring phases nested within the highly repeated main unit. In contrast Den Hollander and Dijkstra (1974) described the display of *P. proxima* as "*uniform*" in comparison to *P. vlijmi,* for which they recognized an "initial" and a "terminal" phase. Although sharing a common general structure, qualitative differences were evident when comparing the two species,

As observed by several authors (Wagner, 1996; Raff and Sly, 2000; West–Eberhard, 2003; Schlosser and Wagner, 2004; Emlen et al., 2005) each module can evolve independently without correspondingly large effects on neighboring modules. In this respect, we can hypothesize that the actual differences in courtship behaviour of *P. proxima* and *P. vlijmi* were obtained by the independent evolution of each courtship unit. Nonetheless, when comparing the qualitative aspect of courtship units between the two species, a different degree of divergence/similarity is observed (i.e. the ending unit is almost identical while starting and main units show obvious differences).

As stated above, both *P. proxima* and *P. vlijmi* males performed the starting and ending units only once within a courtship pattern while they spent more than eighty percent of the time performing repetitions of the main unit. On closer inspection, the highly repeated main unit appears rather complex. The main unit can be clearly splitted in two phases recurring in cycle: "stepping" and "jerking" phase for *P. proxima* and "springing" and "swinging" phase for *P. vlijmi*. Both "stepping" and "springing" phases are strongly stereotyped, on the other hand "jerking" and "swinging" phases showed high variability, as attested by the high values of coefficient of variation in mean durations. Signal features that convey information on species identity show little variation among individuals within a species, while signal attributes that convey information on individual quality show high stereotypy within an individual but larger variation among individuals. Signal repetition rate or intensity may indicate motivation or condition and thus provide fundamental information for mate choice (Bee et al., 2001). Several studies were performed on *Anolis* lizard (Reptilia, Polychrotidae) showing highly stereotyped specie–specific courtship display. On the other hand, a high degree of variability in certain aspects of courtship was observed (Stamp and Barlow, 1973; Crews, 1975;

Jenssen 1979; Partan et al., 2011). In our model, we observed one highly variable and one remarkably stereotyped phase nested in the main unit. The latter could be interpreted as a signal related to species identity or an honest signal linked directly to the signaler's quality, however we cannot determine which one was due to the similar life history, rearing temperature and diet of the spiders. Future studies will thus focus on the response of females to courting males reared under different conditions in order to highlight how these display features reflect the male's honest signals about his quality. The high variable phase may not indicate mate viability or direct benefit, but facilitate signal detection and assessment by the female reducing choice costs or errors. In these categories are included cues arisen from Fisherian runaway selection (Pomiankowski and Iwasa, 1993; Pomiankowski and Iwasa, 1998), female sensory exploitation and remaining of past selection (Candolin, 2003). A tight correlation between signals, namely the ability of the male to generate one signal after another, could be informative for the female that estimates the overall complexity of the signal. More simply, one signal could also alert the receiver of the presence of another signal decreasing the latency in female response (Hebets and Papaj, 2005). Another possibility is that the association of different signals is useful to increase the probability of detection and discrimination of a signal making it more conspicuous (Hasson, 1990, 1991).

Although the two displays show similar structure, they are clearly different both from the qualitative and quantitative points of view, especially if compared with the few morphological differences among males and in particular in females. Behavioural change can "buffer" or mediate morphological change of the organism in its environment. As observed by Randall and Di Fiore (2007), behavioural traits are exposed to selection pressure more readily or more directly than morphological traits. In this scenario behaviour precedes modification of morphology. Several examples exist in this sense, such as nest building behaviour in birds (Hansell, 2000), differences in prey choice (Evans, 1953) and nest architecture (O'Connor et al., 2011) for wasps, among social insects mostly regarding nest structure (Emerson, 1956; Sakagami and Yoshikawa, 1968) and regarding courtship behaviour of spiders (Stratton and Uetz, 1981, 1983, 1986).

Pardosa proxima and *P. vlijmi*, originally reported as "*morphologically indistinguishable*" (Den Hollander and Dijkstra, 1974), are often found in syntopy. One interesting question regards the role of

courtship behaviour in the isolation of the two species. Though heterospecific mating trials can be a good tool to verify the role of courtship behaviour in species delimitation, the syntopic occurrence, the impossibility to separate females in the field did not allowed us to perform laboratory trials.

In conclusion, we observed different layers of complexity: (i) a modular complexity, with the alternation of modules or behavioural units independent from each other in respect of the functional and evolutionary point of view and (ii) a hierarchical complexity, with different kinds of behaviours nested within highly repeated courtship units and an organization from the general to the specific (i.e. courtship session, courtship pattern, courtship units and courtship elements). Moreover, our model performs a multimodal communication during courtship behaviour, namely the males communicate via composite signals using more than one sensory channel simultaneously. Multimodal communication is commonly encountered in the animal kingdom. Works on multimodal communication were performed extensively on wolf spiders of the genus *Schizocosa* (Stratton and Uetz, 1983; Scheffer et al., 1996; Hebets and Uetz, 1999, 2000; Uetz, 2000; Pruden and Uetz, 2004; Hebets, 2008, 2005;), but also in other taxa such as for example frogs (Lewis et al., 2001; De Luna et al., 2010; Grafe et al., 2012), birds (Baptista, 1978; Beletsky, 1983; Lombardo et al., 2008), crickets (Stoffer and Walker, 2012). In respect to this, the genus *Pardosa* can also be a good model for investigating the function of the different communicative channels and their role in mate choice and species recognition.

Even though further data are necessary, all of these aspects contribute to the first attempt in understanding the evolution of these particular and complex courtships that almost certainly play a role in species delimitation and speciation.

Acknowledgement

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Figures legend

Fig. 1. Schematic representation of a courtship pattern composed of two courtship units (A and B). Unit A is characterized by two phases: phase 1 is composed of five courtship elements and phase 2 is composed of four. The two phases recur circularly within the courtship unit. Unit B is composed of five courtship elements grouped in a single phase.

Fig. 2. Courtship pattern analysis for *Pardosa vlijmi* and *P. proxima*. The arrows with percentage values indicate the number of cases in which a certain unit follows another within a courtship pattern

Fig. 3. Occurrence of consecutive repetitions of the main unit (x axis) within each courtship pattern (y axis) in *Pardosa vlijmi* and *P. proxima.*

Fig. 4. *Pardosa proxima* (A, B and C) and *P. vlijmi* (D, E and F) motion analysis. Example of speed profile plot (i) and single video frame (ii) corresponding to the motion. The numbers (1–3) correspond to the body movement illustrated in (ii).

Fig. 5. *Pardosa proxima* motion analysis. Mean duration and standard deviation of "stepping" and "jerking" phases in all specimens (n= 11). Error bars represent 95% confidence interval.

Fig. 6. *Pardosa vlijmi* motion analysis. Mean duration and standard deviation of "springing" and "swinging" behaviours (n= 7). Error bars represent 95% confidence interval.

Table 1

Definition of courtship elements of Pardosa proxima and P. vlijmi.

Table 2

Pardosa proxima univariate ANOVA analysis results. For each specimen mean duration (M), standard deviation (SD) and coefficient of variation (CV) of both "stepping" and "jerking" phases are reported. N= number of cases.

Table 3

Pardosa vlijmi univariate ANOVA results. For each specimen mean duration (M), standard deviation (SD) and coefficient of variation (CV) of both "springing" and "swinging" is reported. N=number of cases.