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ARTICLE

Brief Inventory of Phytoseiidae (Parasitiformes) Found on Vegetable Crops in the Republic of Congo

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ABSTRACT

According to the last revised catalogue of the mite family Phytoseiidae and the online Phytoseiidae database, only six species of predatory mites have been identified to date in the Republic of Congo (RC). Two species were reported on cassava (*Manihot esculenta*), two on coffee (*Coffea* spp.), one on lemon (*Citrus* spp.), and one on unidentified plants. In this study, we catalogued predatory mites on five plants of economic interest in the RC. Two hundred and forty-seven mite specimens were collected on *Manihot esculenta*, *Solanum lycopersicum*, *Solanum melongena*, *Abelmoschus esculentus*, and *Capsicum* spp. Traditional taxonomy was used to identify the collected specimens. The morphological characteristics of the females were analyzed, including the lengths of the dorsal setae, presence or absence of dorsal and ventrianal setae, shape of insemination apparatus, leg chaetotaxy, and cheliceral dentition. Six species belonging to two sub-families (Phytoseiinae and Amblyseiinae) and five genera (*Amblyseius*, *Euseius*, *Paraphytoseius*, *Phytoseius*, and *Iphiseius*) were identified. Among these six species, only one had previously been observed in the RC; the remaining five species are reported.

1. Introduction

Phytoseiid mites are well known worldwide for their ability to control several pests in vegetable crops. Moreover, the success of biological control programmes greatly depends on the reliability of the specific taxonomic knowledge; indeed, each species has its own bio-ecological characteristics, including predator-prey relations, which determine their effectiveness in biological control programmes [1,2]. Therefore, their accurate identification is crucial. In the case of predatory mites, identification is based on the morphological characteristics of the female; specifically, the lengths of the dorsal setae, the presence or absence of the dorsal and ventrianal setae, the shape of

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the insemination apparatus, leg chaetotaxy, and cheliceral dentition [3]. However, this morphological identification system exhibits a major limitation; when only immature stages and/or males are encountered, accurate identification is impossible. In recent years, integrative taxonomy combining morphological and molecular data has enabled many taxonomic questions within the family Phytoseiidae to be solved [4-6]. This approach has made it possible to confirm the status of specimens belonging to the genus *Euseius* originating from the Republic of Congo (RC), which are morphologically close to the species *Euseius fustis* and *Euseius neodossei* [7].

Few studies have evaluated the biodiversity of Phytoseiidae in the RC [8,9] including a single study that reported six species of predatory mites: *Euseius fustis* Pritchard & Backer, *Typhlodromalus aripo* De Leon, and *Typhlodromalus saltus* (Denmark & Matthyse) on *Manihot esculenta* Crantz (Euphorbiacea), *Euseius neodossei* (Moraes, Ueckermann & Oliveira) and *Euseius baetae* (Meyer & Rodrigues) on *Coffea* spp. and, *Amblyseius sundi* (Pritchard & Backer) on an unidentified plant species [10]. *Typhlodromalus aripo* and *Typhlodromalus saltus* have previously been reported in Cameroon on *Manihot esculenta.* Both *Euseius fustis* and *Amblyseius sundi* have previously been reported in the Democratic Republic of Congo, on *Manihot esculenta* and *Ficus polita* respectively. *Euseius fustis* has also been recorded in Uganda on *Manihot esculenta*. *Euseius neodossei* has been recorded in Kenya on *Cassia* spp. and in Burundi on *Gmelina* spp. [10]. *Euseius baetae*, in Central Africa was only observed in RC. This inventory of the biodiversity of Phytoseiidae mites is incomplete as it was conducted randomly and with the primary objective of studying the pest mites present on *Manihot esculenta*. Other inventories have been conducted in Central Africa, predominantly in the neighboring DRC, where 47 species have been recorded to date [8,11-13], and in Cameroon, where 39 species have currently been recorded [11-13].

In this study, we record the predatory mites identified on five host plants widely consumed by the Congolese population from RC: cassava, tomato, aubergine, okra, and chilli.

2. Materials and Methods

2.1 Acari Survey

Three rounds of sampling were carried out from 2016 to 2019. Mite samples were collected from five host plants namely: *Manihot Esculenta* Crantz 1766 (Euphorbiacea), *Abelmoschus esculentus* Moench 1794 (Malvaceae), *Capsicum* spp Linnaeus 1753 (Solanaceae), *Solanum melongena* Linnaeus 1753 (Solanaceae) and *Solanum lycopersicum* Linnaeus 1753 (Solanaceae), in the south of Brazzaville at four site: Moungali (1), Kombé (2) (Agri-Congo), Groupement Jean Felicien Mahounda (GJFM) (3) (JFM) and Jardin (4) (experimental site) (Figure 1).

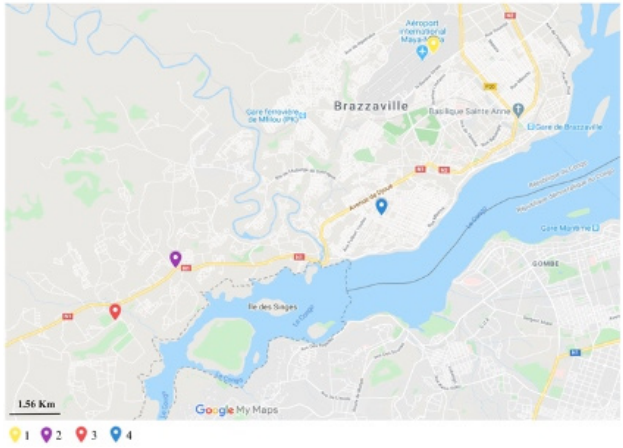

Figure 1. Three rounds of mite sampling from 2016 to 2019

Some females per population (Table 1) were collected directly from the leaves using a fine, clean hairbrush and immediately put in alcohol (70%) in plastic vials. The name of the host plant, sites of collection with GPS coordinates and number of individuals per population were noted on each vial. Males and immature stages were not collected because specific identification is impossible owing to the lack of discriminating characters.

2.2 Morphological Analysis

2.2.1 Traditional Taxonomy

Two hundred and forty-seven adult females were mounted glass sides in Hoyer's medium for later examination under a phase and differential interference contrast microscope (Sony Carl Zeiss Sonnar T* FE 55 mm f/1.8 ZA) at a magnification of 400×. One hundred and four adult females were considered in this study. Terminologies for chaetotaxy used in this paper follow those proposed by Lindquist and Evans (1965) [14] as adapted by H. J. Rowell, D. A. Chant, and R. I. C. Hansell [15] for dorsal idiosomal setae of Phytoseiidae for ventral idiosomal setae. All measurements are presented in micrometres [16]. To differentiate the specimens collected, individuals were measured; the setae considered were those of the sub-tribe of Paraphytoseiina [17], Amblyseiina [18], Euseiina [19], and finally the sub-family Phytoseiinae [3,9]. These specimens were compared with measurements derived from the original descriptions of the following species: *Phytoseius amba* Pritchard and Backer, *Paraphytoseius horrifer* Pritchard and Backer, and *Amblyseius swirskii* Athias-Henriot.

Table 1. Characteristic of different populations of *Amblyseius swirskii*, *Amblyseius* spp., *Iphiseius degenerans*, *Paraphytoseius horrifer, Phytoseius amba and Euseius fustis.*

Number of females considered	Species	Number of site	Site name	Latitude	Longitude	Host plant
22	*Amblyseius swirskii*	*1*	Moungali	4.248112	15.260441	Tomato
1	*Amblyseius swirskii*	*3*	Kombé	–4.248112	15.260441	Okra
2	*Amblyseius swirskii*	*1*	Moungali	–4.248112	15.260441	Cassava
4	*Amblyseius swirskii*	*1*	Moungali	–4.248112	15.260441	Chili
1	*Amblyseius swirskii*	*2*	GJFM	–4.310978	15.187236	Cassava
8	*Phytoseius amba*	*2*	Kombé	–4.326431	15.170045	Aubergine
1	*Phytoseius amba*	*1*	Moungali	–4.248112	15.260441	Okra
30	*Euseius fustis*	*1*	Moungali	–4.248112	15.260441	Cassava
10	*Euseius fustis*	*4*	Experimental site	4.248112	15.260441	Cassava
13	*Euseius fustis*	*2*	GJFM	–4.310978	15.187236	Cassava
15	*Euseius fustis*	*3*	Kombé	–4.248112	15.260441	Cassava
12	*Iphiseius degenerans*	*1*	Moungali	–4.248112	15.260441	Chili
5	*Iphiseius degenerans*	*1*	Moungali	–4.248112	15.260441	Okra
3	*Iphiseius degenerans*	*2*	GJFM	–4.310978	15.187236	Cassava
6	*Iphiseius degenerans*	*4*	Experimental site	4.248112	15.260441	Cassava
1	*Iphiseius degenerans*	*3*	Kombé	–4.248112	15.260441	Aubergine
17	*Paraphytoseius horrifer*	*3*	Kombé	4.248112	15.260441	Aubergine
2	Amblyseius spp.	*1*	Moungali	–4.248112	15.260441	Chili
1	Amblyseius spp.	2	Kombé	–4.248112	15.260441	Aubergine

The morphological characteristics considered were those currently used for the identification of phytoseiid mites [3]; specifically, continuous variables used to distinguish species belonging to the tribu Euseiini [20]. In our study we did not measure individuals belonging to the species *Iphiseius degenerans* due to 1) this species has very small setae and 2) can be identified quite easily. The specimens measured for morphometric analyses were deposited as voucher specimens in the mite collection of the laboratory of Biodiversity and Animal Ecology, Faculty of Science and Technology, Marien Ngouaby University. A donut chart with a hole inside, showing the percentage of each species observed within each host plant studied will be made using the R software and the ggplot2 package [21].

2.2.2 Statistical Approaches

We performed the statistical approach proposed by M-S Tixier [22] to identify continuous variables that can establish boundaries to distinguish between intra- and inter-specific variability based on the lengths of the Phytoseiidae setae on the dorsal shield.

3. Results and Discussion

3.1 Traditional Taxonomy and Statistical Approaches

Six species were identified in this study as belonging to two subfamilies and four genera: Phytoseiinae (*Phytoseius amba*); Amblyseiinae (*Amblyseius swirskii, Amblyseius* spp., *Euseius fustis*, *Iphiseius degenerans, and Paraphytoseius horrifer*). Cinq of these species have been recorded for the first time in this country.

3.1.1 *Amblyseius swirskii* Athias-Henriot 1962

Examined material/locality: Individuals of this species were found at site 1, 2, and 3.

Distribution: This species was found in proportions of 73.33, 13.33, 10.00 and, 3.33% on *Solanum lycopersicum, Capsicum* spp., *Manihot esculenta,* and *Abelmoschus esculentus,* respectively (Figure 2a).

Taxonomic remarks

D. A. Chant and J. A. McMurtry [18] proposed the following stable characteristics for identifying species of the genus *Amblyseius*. A lightly sclerotized idiosoma;

rarely brownish in color; very short z2, z4, Z1, S2, S4, S5, j4, j5, j6, J2, and J5 setae, approximately subequal in length; typically extremely elongate Z4, Z5, and s4 setae. Zannou et al. 2007 identified 23 species of the genus *Amblyseius* native to sub-Saharan Africa [13]. The continuous characteristics measured on the dorsal shield and the ventrianal shield have the same values as those reported in the original description. However, there is a slight difference in some discontinuous characteristics such as the morphology of Z4 and Z5 setae. The latter is smooth whereas those in the original description are serrated. Moreover, the number of teeth on the mobile digit part varies between six and nine whereas, in the original description, between nine and ten teeth are reported.

3.1.2 *Amblyseius* spp.

Examined material/locality: Individual of this species was found only on site 1.

Distribution: This species was found in proportions of 50 % on *Solanum melongena* and *Capsicum* spp. respectively (Figure 2b).

Taxonomic remarks

Individuals identified as *Amblyseius* spp. typically possess the following characteristics: setae Z1 is absent and setae Z5 and macrosetae on leg IV are shorter than *Amblyseius sundi.* Spermatheca has a tubular calyx and a slightly wider atrium (Figure A1, G × 400). Regarding the chelicera, the movable digit bears three teeth and the fixed digit bears nine teeth (Figure A2, G × 400).

3.1.3 *Paraphytoseius horrifer* Pritchard and Backer 1962

Examined material/locality: Individual of this species was found only on site 3.

Distribution: This species was found in proportions of 100 % on *Solanum melongena* (Figure 2c).

Taxonomic remarks

Three species of the genus *Paraphytoseius* have been reported from sub-Saharan Africa. They have an idiosomal setal pattern 10A: 5D/JV-3/ZV; seta J2, S2, S4, and S5 are absent; one pair of round pre-anal pores posterior is almost in line with JV2; and one pair of metapodal shields. The individuals observed belong to the species *Paraphytoseius horrifer* because they have the following morphological characteristics: dish-shaped calyx of spermatheca, subpentagonal ventrianal shield with an almost straight anterior margin and a lateral margin with light construction, the peritreme extending to the level of j1, and the last macrosetae absent on leg III. Regarding the chelicera, the movable digit with two-three teeth and the fixed digit with ten-eleven teeth (Figure A3, G×400).

3.1.4 *Euseius fustis* Pritchard and Backer 1962

Examined material/locality: Specimens of this species were found on site 1, 2, 3, and, site 4.

Distribution: This species was found in proportions of 100% on *Manihot esculenta* (Figure 2d).

Taxonomic remarks

Forty-four species belonging to this genus have been reported in sub-Saharan Africa [11]. Specimens identified as *Euseius fustis* possess the following characteristics: an imbricate, smooth dorsal shield; smooth and serrated Z5 seta; seta Z1 is present the peritreme reaches almost to seta z2; spermatheca with filamentous, trumpet-shaped calyx; and a small and barely distinguishable atrium.

3.1.5 *Iphiseius degenerans* Berlese 1889

Examined material/locality: Individuals of this species were found on the site 1, 2, 3 and 4 **Distribution**: This species was found with a proportion of 44.44, 33.33, 18.51 and 3.70% on *Capsicum* sp., *Abelmoschus esculentus*, *Manihot esculenta* and, *Solanum melongena* (Figure 2e).

Taxonomic remarks

Specimens identified as *Iphiseius degenerans* are characterized by the minute size of all dorsal setae (j3, j4, j5, j6, J2, J5, z2, z4, z5, Z4, Z5, s4, S2, S4, and S5), except for some short vertical setae (j1, Z5). All setae are smooth. The spermatheca has a narrow tubular calyx. Regarding the chelicera, the movable digit bears 1-2 teeth and the fixed digit bears 6-8 teeth.

One species in this genus has been reported in sub-Saharan Africa [23].

3.1.6 *Phytoseius amba* Pritchard and Backer 1962

Examined material/locality: Individuals were found on the site 1 and 2 .

Distribution: This species was found with a proportion of 88.88 and 11.11 % on *Solanum melongena and, Abelmoschus esculentus* (Figure 2f).

Taxonomic remarks

Individuals identified as *Phytoseius amba* typically possess the following characteristics: all setae of species in the genus *Phytoseius* are present (in this genus, setae

Z1, S2, S4, and S5 are typically absent and both setae z3 and s6 are present), except setae Z1, S2, and S5 (Chant and McMurtry 1994); setae j1, j3, z3, Z4, Z5, s4, s6, and r3 are thick, serrated, and long, whereas the other setae are small and smooth; the peritreme extends anterolaterally up to setae j1; and a spermatheca with a slender calyx flares towards the vesicle. Regarding the chelicera, the movable digit bears 1-2 teeth and the fixed digit bears 2-3 teeth. Macrosetae irregularly expanded distally on genu, tibia and basitarsus and knobbed on telotarsus (Figure A4, G × *400)*. Thirteen species of this genus have been reported in sub-Saharan Africa [24].

3.2 Discussion

Among the six phytoseiid species belonging to two subfamilies and three genera: Amblyseiinae *(Amblyseius sundi*, *Euseius neodossei*, *Euseius baetae*, and *Euseius fustis*) and Typhlodrominae (*Thyphlodromalus aripo, Thyphlodromalus saltus*) identified in previous study [10] only the *Euseius fustis* species was identified in our study. This has already been reported in the RC, in the Pool and Bouenza regions and twice at Kombé (1986 and 1987). The absence of species such as *Amblyseius sundi*, *Euseius neodossei*, *Euseius baetae*, and *Euseius saltus* may have been due to the difference in sampling locations and range between the two studies. In this study, we collected specimens only from the south of Brazzaville, whereas (Gutierrez and Bonato 1994) conducted sampling surveys in most regions of the country. All species reported in this paper have been previously described [24,25]. Three individuals of questionable status belonging to the genus *Amblyseius* were also identified. Individuals 12, 13 and, 14 were morphologically close to three species: *Amblyseius sundi* Pritchard & Baker and *Amblyseius parasundi* Blommers. According to the Phytoseiidae database [25], two species of the genus *Amblyseius* have been identified in the RC, *Amblyseius genya* Pritchard & Baker and *Amblyseius sundi*. However, the first species has only been observed in the DRC, Cameroon, and Kenya [12,13,26]. Only the *Amblyseius sundi* has previously been reported in the RC. Individuals 12, 13 and 14 sampled in our study differed from this species in the lengths of the Z4, Z5, and s4 setae and the macrosetae of leg IV. Indeed, we

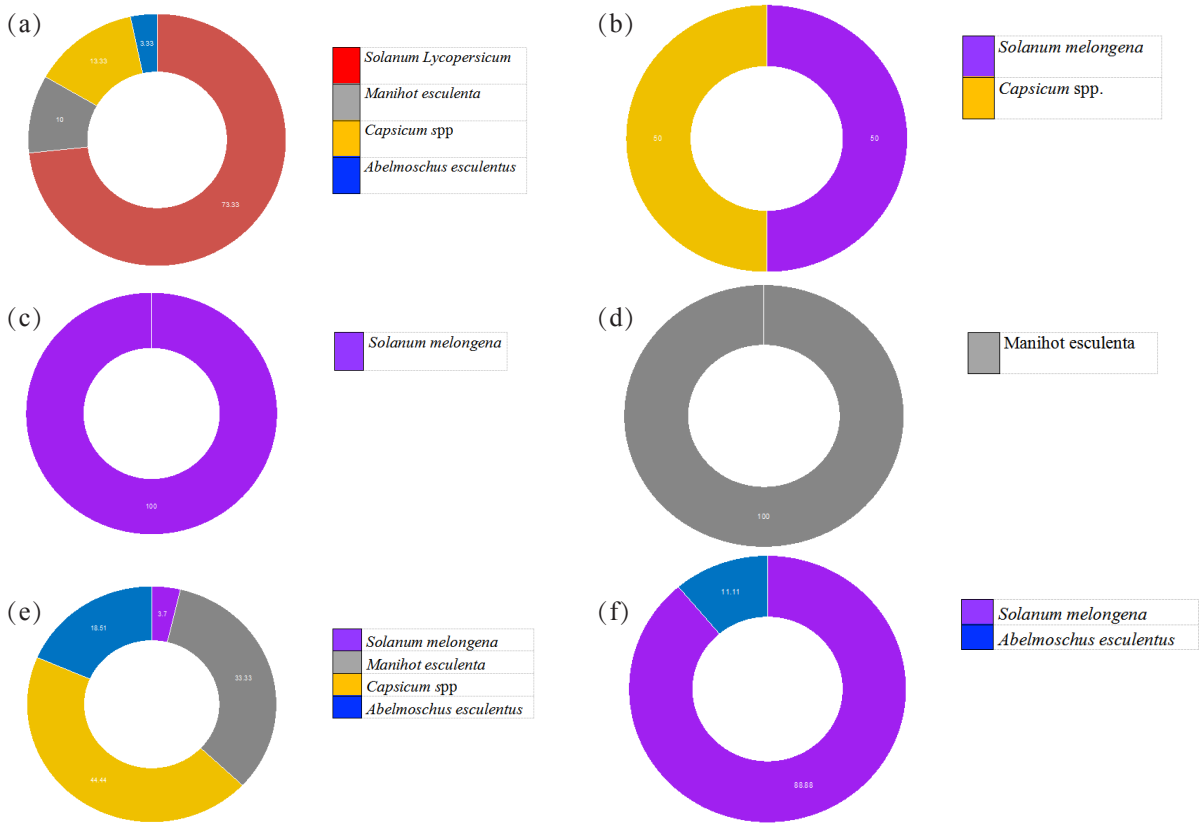

Figure 2. A donut chart with a hole inside, showing the percentage of Phytoseiidae (Parasitiformes) observed within each host plant studied
(*a*) *Amblyseius swirskii*; (b) *Amblyseius* spp.; (c) *Parahytoseius horrifer*; (d) *Euseius fustis*; (e) *Iphiseius degenerans*; (f) *Phytoseius amba*

observed significant differences with the following mean, minimum, and maximum values: 113 (112.5-115), 245 (230-270), 84 (80-87.5), 100 (100-100), 74 (72.5-75), and 62.5 (75-69), whereas the revised description of Zannou et al 2007 [13] gave the following values: 172 (144-208), 445 (336-547), 165 (133-206), 209 (157-270), and 156 (112-208) for Z4, Z5, s4, Sge IV, and Sti IV setae, respectively. Zannou et al. 2007 differentiated *Amblyseius parasundi* from *Amblyseius sundi* based on a Z5 setae and macrosetae on the much shorter leg IV [13]. However, these specimens were consistently larger than those observed in this study. Indeed, the specimens of *Amblyseius parasundi* had values of 170, 430, and 165 for setae Z4, Z5, and s4, respectively, and values of 190, 140, and 85 for the macrosetae of leg IV, Sge IV, Sti IV, and STIV. Zannou et al 2007 considered that the specimens identified by as *Amblyseius parasundi* were a synonymous species of *Amblyseius sundi*, and that these variations correspond to intraspecific variability. However, when we apply the procedure proposed by M-S Tixier [22], we observed that the Z4, Z5, and s4 setae and the macrosetae of leg IV can be used to distinguish *Amblyseius sundi* and *Amblyseius parasundi,* and that these variations correspond to inter-specific variability. Indeed, for setae greater than 65 µm, a difference greater than or equal to 31.74 µm is considered to be inter-specific variability.

In view of the criteria listed in Phytoseiid mites of the subtribe Amblyseiina (Acari: Phytoseiidae: Amblyseiini) from sub-Saharan Africa [13], we assume that the status of *Amblyseius* spp. raises questions therefore, its status should be validated by further molecular and morphological analyses.

Using integrative taxonomy with the help of molecular markers should help improve the reliability of specific diagnosis in future studies. Indeed, very few DNA sequences from this region exist in the GenBank database [27]; the only current sequences being those presented in our recent study [7]. Acquiring knowledge of these parameters is important for the successful implementation of biological control as an alternative pest control method for farmers in the RC. Of the six phytoseiid species recorded in the RC, there is only one species whose life history traits have been thoroughly studied. A controlled laboratory study previously highlighted the potential of *Euseius fustis* in the regulation of cassava green mites (*Mononychellus progresivus* Doreste) when reared using *Mononychellus progresivus* Doreste, as well as pollen from cassava, maize, or castor varieties as food sources [28]. Future studies should aim to better understand the life history traits of the other 10 phytoseiid species found in the RC to improve the evaluation of their potential roles in biological control programmes in the inter-tropical zone.

4. Conclusions

Thus, the findings of this study fill some existing gaps in our knowledge in terms of the biodiversity of predatory mites belonging to the family Phytoseiidae in the RC. The number of known mites in the RC is now 10. However, this number is lower than that in the neighboring DRC, where 36 species have been reported. Therefore, it is necessary to conduct further studies to explore the biodiversity of predatory mites in the RC by expanding the study area and number of host plants.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

We would like to thank the market gardeners for allowing us to collect the various host plants on which predatory mites live. Without them, this work would not have been possible. We also thank the Pesticides Alternatives association for allowing us to use their phase contrast microscope and camera to identify and capture different individuals collected.

Appendix

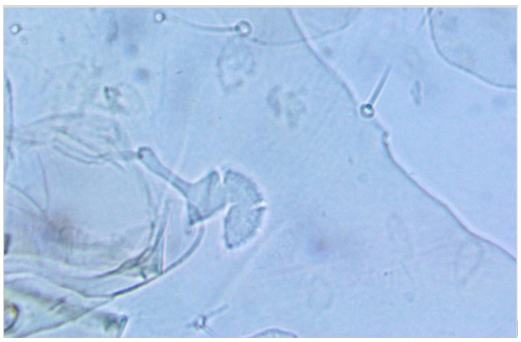

Figure A1. *Amblyseius* spp.(spermatheca)

Figure A2. *Amblyseius* spp.(chelicera)

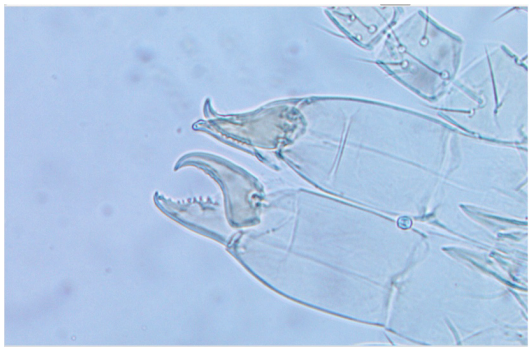

Figure A3. *Paraphytoseius horrifer*

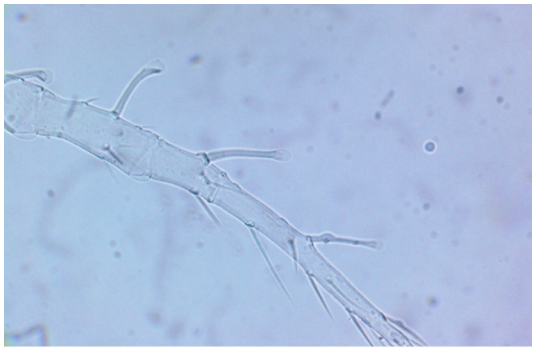

Figure A4. *Phytoseius amba*

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ARTICLE

Relationship between Dog Walking Behaviour and Owner-Dog Attachment Using the Lexington Attachment to Pets Scale

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ABSTRACT

Human-dog attachment is a special relationship and has been studied from various perspectives. Attachment or social bonding has a positive effect on the psychological and physiological wellbeing of a dog owner, increasing physical health and quality of life. Attachment is idiosyncratic, induced by neuroendocrinological functions like an oxytocin increase after an interaction, but also based on subjective perceptions of the quality of bonding and relationship. Dog-owner attachment was measured in this study using the Lexington Attachment to Pets Scale in its validated German version as a tool to compare owner perception with factual movement data of their dog. The question posed was whether the perceived dog behaviour impacted on the attachment score as assessed through the LAPS. The authors could show that perceived problematical or unwanted conduct, like hunting behaviour, had a negative effect on LAPS scores whereas perceived obedient behaviour had a positive effect upon attachment. The authors found that actual walking data of the dogs were not in congruence with owner assessments. Thus, owner reports alone possibly will not be a sufficient measure of dog-human relationships and animal behaviour.

1. Introduction

Establishing companionship and attachment parameters or merely interacting with dogs has been linked to several health benefits for humans such as enriched affective, physiological and psychological wellbeing [1,2]. Attachment to dogs has been shown to affect dog owners' social facilitation and quality of life [3]. Attachment bonds of the dog-human dyad are understood to be analogous to those that describe human caregiver-infant interactions [4,5] and these relationships are bidirectional [6].

Analysed was the exploration and orientation behaviour of off-leash pet dogs (n=30) while on a walk with their owner and without being signaled or called to. The dogs were off the leash at all times and their roaming behaviour was measured by means of GPS in the course of four consecutive walks. Additionally, the dog owners completed two

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questionnaires. First, an owner questionnaire from which data were used to bear comparison to individual owner estimations (e.g. how far/long does your dog explore) with factual dog exploration data and secondly the Lexington Attachment to Pets Scale (LAPS) by Johnson et al. (1992) [7], in its validated German version [8].

Dog companionship has undoubtedly the potential to sustenance health and personal development by way of the dog satisfying its owner's psychological needs for self-sufficiency as well as affiliation as represented by several attachment levels [9]. Ownership contentment with their dog implicates numerous characteristics of their relationship for instance dog-owner attachment strength [10]. Owner satisfaction is established through a number of behavioural traits exhibited by their dogs. Perceived problematical or undesirable activities, like hunting behaviour, have an adverse effect on the owner-dog affiliation and attachment [11] perceived obedient behaviour, however, leads to a positive consequence on owner affection [12].

The observed values of the owner-dog dyads herein showed high attachment and bonding scores and positive correlations could be observed between the time period of the walks and LAPS scores. Owners of male dogs exhibited greater attachment values compared to owners of female dogs. Moreover owners of intact dogs displayed greater scores in general attachment and lesser values in the Subscales "People Substitution" and "Animal rights/ welfare" compared to owners of neutered dogs. Between owners of purebred dogs and owners of mixed breed dogs significant differences could be shown at the animal rights subgroup and LAPS total.

Aim of this study was to gather data in order to measure duration and distance pet dogs spent away from their owner during a walk and on the other hand to evaluate whether dog owners accurately judged their dog's movement patterns as well as time and distance parameters. Furthermore, to show the value of reflecting subjective emotional responses and to display the influence our judgment and concurrent behavioural reaction has on dog-owner attachment. Pet attachment plays a significant part in how owners care for their dogs. Subjective perception impacts on owner (re-)actions in interspecific situations and a direct link between perception and behaviour exists. Thus the subjective evaluation of their dogs' behaviour even though not borne out in facts may alter dog owners' conduct. The classification of "problem behaviours" is plainly influenced by individual owner perception and as such might well cause compromised dog welfare and an increased risk of relinquishment.

2. Methods

GPS data were collected of freely exploring, off lead pet dogs (n = 30) on (n = 3145) trials while walking with their owners on four consecutive walks, two in known and two in unknown regions in North Rhine Westphalia, Germany (n = 120). Dogs were of different size, sex, breed, reproductive status and age. The average length (median) was 1:17:17h per walk; 5:24:53h over all walks. For each dog-owner dyad 51 parameters were recorded for every walk**.** The GPS used were a Garmin Astro® 320 and the dog collars DC™ 50, and T5 Mini, Garmin International Inc., Kansas, USA. Video camera used was a Garmin VIRB® Elite. Size: (H × B × T): 32 mm × 53 mm × 111 mm; weight 170 g. Datatype: MP4; 1080p-HDVideo: 1920 × 1080; 30 fps. Video data were also displayed on Garmin BaseCamp™ 4.5.2.1. The video camera could merely be attached onto the larger dogs' harnesses because it was too weighty and problematic to fasten to the small dogs harnesses. Main interest here was to establish whether dogs were hunting prey. Eighteen owners participated, fifteen female (83%) and three male (17%). Dog-owner teams were recruited via social media.

Owners had to answer a questionnaire with 57 dog-related items prior to the walks regarding the breed (in case of mixed breeds the owner description was used), date of birth, reproductive status, training and obedience status, duration of being with the family, attachment level, whether it was used for hunting purposes (in which case the dog was excluded), see Supplement Table S1 for details. The sociodemographic details are displayed in Supplement Table S2.

In addition, the owner was asked to appraise the dogs hunting tendencies on a scale from 1-6 (very weak to very strong) and an attachment scale (1-6). The owner moreover had to estimate the distance their dog would travel on any given exploration (maximal distance away from owner) as well as the duration their dog would be beyond his or her vision (minutes). Owners also had to estimate how often their dog would move beyond the range of 20 m (in%) Supplement Table S3. Measured were the time and distance from a starting point of a dogs exploration >20 m away from owner to a point of return (outbound) and the route back from the point of return to the owner (inbound).

Length of runs >20 m were at variance significantly, hence three different dog groups were determined to define roaming patterns more detailed and specify idiosyncratic movement patterns variance between the dogs: The majority of dogs moved within a maximum radius of 150 m encircling the owner (13 of 30 dogs =43%

Group 1) at all times on all walks; eight (27%) of the dogs exhibited a range between >150 m and 350 m (Group 2) away from the owner; nine of the 30 dogs (30%) had at least one run >350 m away from their owner (Group 3).

Correlation measures like distance and duration as well as speed and exploration patterns utilized by the dogs were applied to the LAPS scores.

LAPS - Lexington Attachment to Pets Scale

The LAPS questionnaire was employed to measure the owner-dog attachment. LAPS and socio-demographic data were recorded and online and paper-pencil questionnaires were used. The Lexington Attachment to Pets Scale (LAPS) by Johnson et al. (1992) [7] is a commonly employed instrument to measure attachment of owners to their pets [13,14]. Herein the validated German translation from Hielscher et al. (2019) [8] was applied for all dog owners. The LAPS questionnaire comprises 23 items in total on a scale of 0 to 69 and measures the overall value of owner attachment. The LAPS questionnaire contains three subscales: "General Attachment" (11 items, manifestation from 0 to 33), "People Substituting" (7 items, manifestation from 0 to 21) and "Animal Rights/ Animal Welfare" (5 items, manifestation from 0 to 15) and a total LAPS Score. The coding of the items lies between 0 (strongly disagree) and 3 (strongly agree). Each owner (n = 18) in this study completed a LAPS questionnaire for each individual dog. Therefore in total n = 30 questionnaires were obtained. 11 different dog breeds participated in this study.

3. Statistical Analysis

Discrete variables were summarized as absolute and relative frequencies. For graphical visualization Boxplots were used. To measure the relationship of two continuous or ordered variables Spearman correlation coefficients were applied. Mann-Whitney-U Test was used to compare LAPS between independent groups. For comparison between three or more groups Kruskall-Wallis Test was applied. For correction of multiple testing Standard Bonferroni correction was obtained. Level of significance was set to a = 0.05. All tests are performed two-tailed using SPSS version 25, IBM Inc.

Statistical tools to determine the reliability and validity of the components assessed by multivariate analysis of questionnaire data are in a similar way to their application in canine behavioural testing. Reliability, in the context of a questionnaire, refers to the internal consistency and the degree to which individual questions associated with a specific construct are correlated. Cronbach alpha coefficient is used as an estimate of internal consistency, where measures greater than 0.7 are generally considered acceptable [15].

4. Results

For all dog-owner dyads LAPS total revealed a mean of 57.1 (6.6 SD) with a median of 57 and lower and upper quartile of 55 and 61. The observed values showed high to very high attachment and bonding of all owner-dog dyads considering the range from 0 to 69. All subscales revealed high values: for LAPS "General Attachment" a median of 29 (quartiles: 27 and 31) - with the theoretically achievable maximum of this subscale of 33- was observed. For LAPS "People Substitution" the median was 15 (quartiles: 13 and 18) compared to the theoretical maximum of 21. The observed median of the subscale "Animal right/welfare" was 13 (quartiles: 13 and 15) compared with a theoretically achievable maximum of 15, see Table 1. In comparison, in his study Hielscher et al., (2019) [8] postulated a somewhat lower but similar dog-owner attachment (M = 55.5, SD = 8.2) with men (M = 52.3, SD = 8.9) scoring lower than women (M = 55.3, SD = 8.5). In this study it was not differentiated between men and women as only two owners were men.

Table 1. LAPS scores for Subscales General attachment; people substitution; Animal right/welfare and LAPS total for all questionnaires (n = 30) mean and median and range.

n = 30	mean	SD	Q25	median	Q75	range
LAPS: general attachment	29.00	2.84	27	29	31	23-33
LAPS: People substitution	14.70	3 .57	13	15	18	8-20
LAPS Animal right/ welfare	13.40	1.65	13	13	15	9-16
LAPS total	57.10	6.55	55	57	61	42-67

Distance Measures and Correlations

Looking at all values a positive correlation could be demonstrated between the total walking distance of the owner walk and the subscale "Animal Welfare". Between the total distance of the dog walk and "People substitution" "Animal rights" and LAPS total significant correlations were found. Between the total distance of the dog walk and "People Substitution" "Animal rights/ Welfare" and LAPS total, significant correlations could be shown, see Table 2. Thus, higher owner attachment scores and lengthier walks were correlated with a higher score of LAPS.

Table 2. LAPS and Subscales correlated with total walking distance; owner walking distance and dog walking parameters.

		LAPS: General Attachment	LAPS: People Substitution	LAPS Animal right/ welfare	LAPS total
total distance walk owner total in m	r	–0.053	0.110	**0.266**	0.134
	p	0.568	0.230	**0.003**	0.143
	p*			**0.072**	
total distance dog walk in m	r	0.036	**0.414**	**0.304**	**0.380**
	p	0.696	**<0.001**	**0.001**	**<0.001**
	p*		**<0.024**	**0.024**	**<0.024**
total runs>20 m in m	r	–0.081	0.097	–0.117	0.022
	p	0.381	0.294	0.202	0.812
	p*				
Max distance of run in m	r	–0.012	**0.182**	–0.088	0.118
	p	0.893	**0.047**	0.337	0.201
	p*		**>0.999**		

Spearman correlation coefficient (r) and corresponding p-value. P*: Bonferroni correction of p-value. Correction factor for Bonferroni: 4×6= 24; only significant uncorrected p-values are affected.

Owner Assessment of Hunting Behaviour

Table 3 displays the correlative values between the LAPS subscales, LAPS total and perceived hunting behaviour estimates and reliance on owner scores. The hunting value was negatively correlated with the Subscales "General attachment" "People Substitution" as well as LAPS total. The hunting score had a high negative correlation with a value of r = –0.538 ($p < 0.001$; p*= < 0.012) for the Subscales "General Attachment" and "People Substitution" as well as LAPS total. Owner reliance which also assessed through a six rating scale was negatively correlated with the Subscale "General Attachment".

LAPS subscales values and sex of the dog(s)

With respect to dog behaviour sex differences have been proposed [16]. The correlative values between the LAPS Subscales, LAPS total and assessment of owners of male versus owners of female dogs displayed significant variations for the Subscales "General Attachment", "Animal Rights/Welfare" as well as LAPS total. Owners of male dogs' displayed higher values in these subscales compared to owners of female dogs. The differences were about 1 unit/point for "General Attachment" as well as "Animal Right/Welfare" and 3 units/points for LAPS total signifying different attachment levels.

LAPS subscales values and reproductive status of the dog(s)

Behavioural differences based on the reproductive status of dogs have been proposed and animal welfare matters are also often associated with neutering issues in dogs. The attachment of owners and the correlation with the reproductive status of their dog was therefore measured. Significant differences of attachment scores between owners of intact and owners of neutered dogs were found at the Subscales "General Attachment", "People Substitution" ($p = 0.045$) and at the "Animal Rights/Welfare" level. Owners of neutered dogs had lower values in "General Attachment" (about 1.5 units/points)

Table 3. LAPS and Subscales correlated with Attachment, Hunting behaviour estimates and reliance on owner.

		LAPS: General Attachment	LAPS: people substitution	LAPS Animal right/welfare	LAPS total
Attachment (1-6)	r	**–0.246****	**–0.438****	**–0.228***	**–0.403****
	p	0.007	<0.001	0.012	<0.001
	p*	0.084	**<0.012**	0.144	**<0.012**
Hunting behaviour	r	**–0.538****	**–0.197***	–0.055	**–0.325****
	p	<0.001	0.031	0.551	<0.001
	p*	**<0.012**	0.372		**<0.012**
Reliance on owner	r	**–0.204***	–0.166	–0.022	–0.125
	p	0.026	0.070	0.814	0.172
	p*	0.312			

Spearman correlation coefficient (r) and corresponding p-value. P*: Bonferroni correction of p-value. Correction factor for Bonferroni: 4×3= 12; only significant uncorrected p-values are affected.

but higher values in "People Substitution" (about 1 unit/point) as well as "Animal Rights/Welfare" (about 1.3 units/points).

LAPS subscales values comparing owners of pure breed versus owners of mixed breed dogs

Significant differences were established at the Subscale "Animal Rights/Welfare" with a difference of about 1 unit/point as well as LAPS total with a difference about 1.5 units/points between the owners of purebred and the owners of mixed breed dogs. Owners of purebred dogs displayed lower values in the Subscales and in LAPS total compared to owners of mixed breed dogs.

LAPS and radius of exploration (3 Groups)

The three different radius groups showed very varied travelling behaviour and therefore it was examined whether owner attachment was influenced based on the distance their dog traversed. Between owners of dogs of the three radius groups significant differences (Mann Whitney U test) were found at the Subscale "People Substitution" and LAPS total (Figure 1).

Post hoc-tests revealed that LAPS Subscale "People Substitution" as well as the total LAPS score contrasted between owners of dogs belonging to different radius groups. Divergence was found in particular for the radius >=350 m, Group 3 dogs, compared to Group 1 and Group 2 dogs. Group 3 dog owners, thus owner of dogs having a large radius >= 350 m, displayed higher scores at the Subscale "People Substitution" as well as LAPS total in comparison to Group 1 and Group 2 owners. Between Group 1 and Group 2 dog owner no difference became apparent. Hence owners of far ranging Group 3 indicated higher attachment values with their dogs.

Comparison of owner assessment and GPS measurements

In the owner questionnaire every owner had to estimate the duration their dog(s) would explore on each run >20 m. Figure 2 demonstrates the difference in time measures. As may be perceived owners greatly overestimated the duration of runs >20 m. Displayed are the outbound route (from start of exploration >20 m away from owner to point of return) and the inbound route (from point of return back to owner).

Clearly, great discrepancies exist between owner estimate of duration and factual exploration data recorded with respect to time explored.

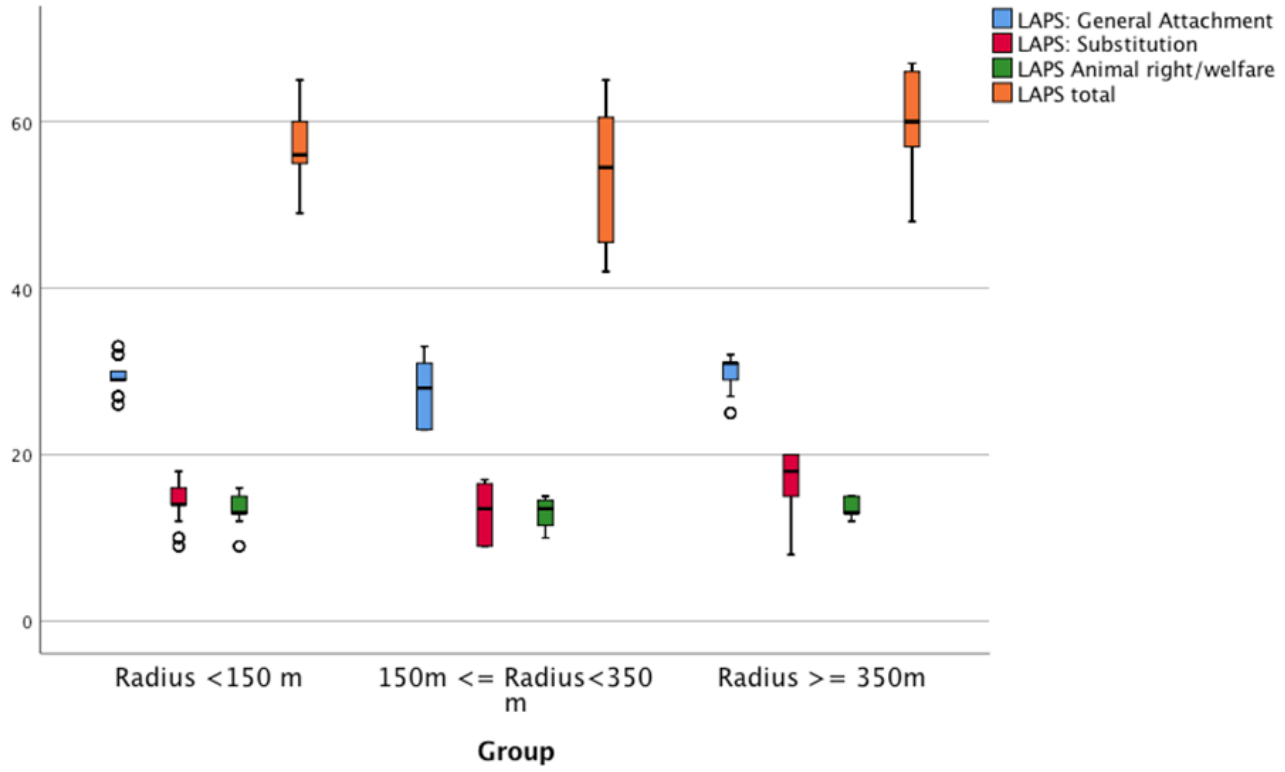

Figure 1. Comparison of dog owners with dogs belonging to Group 1, Group 2 and Group 3, correlated to Subscales General Attachment, People Substitution, Animal rights/Welfare and LAPS total.

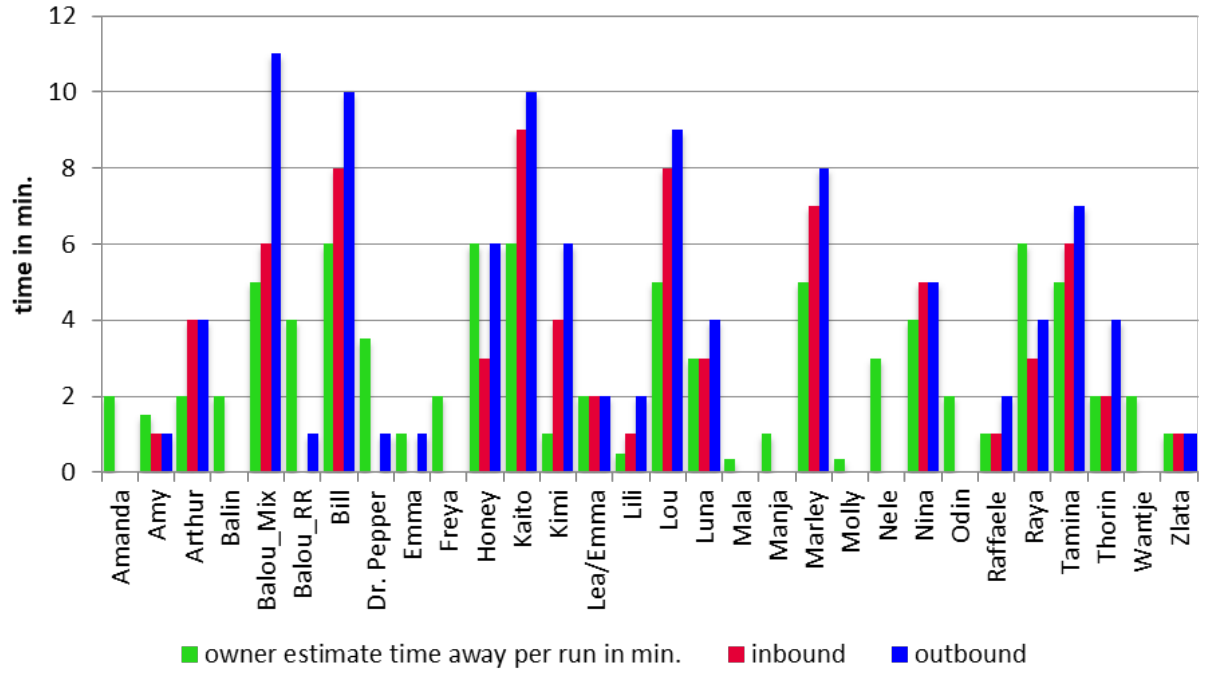

Figure 2. Variance between factual exploration time in minutes of all dogs (n=30) of all runs >20 m, back to the owner=inbound (red) and away from the owner to a point of return=outbound (blue) compared with owner assessment (green: estimate of duration per run in minutes).

5. Discussion

Attachment to and bonding with dogs are a widespread phenomenon. The quality of the attachment between owner and dog clearly affects the dog owners' (and the dogs) quality of life in a number of aspects. Frequently dog ownership is connected with mental, physical and psychosocial health benefits, for instance greater levels of physical activity like dog walking compared to non-owners [3,17]. Dogs may serve as a safe haven with attachment function [5] or act as a secure base [18,19] thereby providing comfort to their owner in distressing situations. Green et al., (2018) [20] proposed that attachment-related behaviours were associated with attachment dimensions. Owners may turn to their dog as a replacement for human companionship or feel less emotionally secure without their dog. Therefore, attachment theory may illuminate individual differences in relationship dynamics within the dyad [20].

Herein for all dog owners the perceived LAPS scores presented high attachment and bonding values of the owner to their dog(s). All Subscales indicated high to very high values. In their study Hielscher et al., (2019) [8] found a slightly lower German owner-dog attachment with men scoring lower than women. In this study no differentiation between men and women was made. This may of course have skewed results as female dog owners generally score higher than men dog owners [8,13]. Moreover, male owners have reduced odds of reporting problem behaviour(s) in their dog(s) compared to female owners [21]. Reevy & Delgado (2015) [13] using LAPS proposed that numerous variables were related to the degree of affection for one's dog. Greater scores of neuroticism and conscientiousness correlated significantly with greater LAPS values, as did gender (being female).

In addition attachment to dogs and other animals appears to be culturally predisposed. Average LAPS total and Subscale values in this study were higher compared to the results of the studies of Hayama et al., (2016) [22] in which exclusively male owners were studied, and Hielscher et al., (2019) [8], German male and female owners, as well as Johnson et al., (1992) [7], and Weiss & Gramann (2009) [23]. The studies of Reevy & Delgado (2015) [13] and Stephens et al., (2012) [24] published comparable values, whereas Kruger et al., (2014) [25] established higher scores in their study. Singer et al., (1995) [26] found varied effects with an overall score higher than the values in this study. All the above-mentioned studies that used the LAPS were executed in the USA. Hielscher et al., (2019) [8] conducted their LAPS study (cats and dogs) likewise in Germany, with results comparable to the ones presented herein. Comparable to this study Hielscher et al., (2019) [8] only had a small sample size (30 LAPS total). As one shortcoming the

LAPS values were less reliable due to a lack of statistical power. Owner subjectivity is also an important study limitation. While owner reports are arguably effective, interobserver reliability has been shown to vary depending on the particular trait being rated [27].

Studies have shown that attachment values correlate with the motivation to go on walks with their dog thereby increasing physical activity levels in dog owners [3,28]. We correspondingly found significant correlations between the total distance of the dog walk and the Subscales "People Substitution", "Animal rights/Welfare" as well as LAPS total. Cause and effect are difficult to distinguish since this was a correlation study. However, it seems plausible that owner attachment correlates with longer walking distances with their dogs and higher outcome of LAPS are correlated accordingly. To spend and enjoy increased quality time with their dog resulting in a closer bonding and higher attachment values is in accordance with findings of Kotrschral et al., (2009) [29] and Miklósi et al., (2014) [30]. Quality time spent with the dog is associated with experiencing the relationship with the dog as close [31] and may result in fewer behavioural problems [11] which in turn would also affect attachment positively.

An additional factor influencing attachment may be oxytocin as it plays a significant part in the dog-owner relationships. Oxytocin plays an important role in bond formation [32], and increased oxytocin levels are interconnected with an improved and augmented relationship from the owner perception [32]. Frequent affiliative interactions between owner and dog reinforce and fortify the attachment bond. The quantity of time that owner and dog spend together is reported to have a critical influence on both functional dog-human relationships [29] as well as dogmanship [33] and this may be one physiological explanation that may be reflected in the correlation between the walking duration and the LAPS measures.

Attachment strength is furthermore associated with owner satisfaction [10] which is based on different behavioural traits displayed by their dog. A number of dog-associated characteristics influence this owner satisfaction and consequently the dog-owner attachment strength such as preferred traits for example obedience, physical closeness and affection [34] with an owner preference of having a dog that is approachable, obedient, calm and friendly [35]. In the past dog-owner attachment has been associated with canine behaviour [36] and owner perception may influence how they view their dog's conduct and the severity of a "problem behaviour" which may objectively not be substantiated. Owners are more likely to judge behaviours as undesirable which negatively impact on their daily lives, such as poor recall, or behaviours which may be embarrassing or socially unacceptable to them [21]. We therefore expected a high hunting value to correlate negatively with owner attachment. In the main the opinion on high hunting activities or poor recall of pet dogs is acknowledged as undesirable and associated with owner stress and anxiety. The majority of owners in this study expected their dog to be interested in or to actually exhibit hunting behaviour. Significant negative correlations between a high hunting score assessment by the owner and attachment values could be perceived for all subscales and the total LAPS scores. Hence perceived problematic, negative, or unwanted dog behaviour clearly results in adverse effects on the dog-owner attachment values and relationship. Interestingly, data analysis of the dogs travelling movements as assessed through their GPS collars and video cameras (all medium to large dogs carried a camera) provided no evidence of any hunting or chasing activity by the dogs.

LAPS subscales values and sex of the dog(s)

Significant differences were also found between the predominately (83%) female owners of male and female dogs regarding the LAPS Subscales "Animal rights/ Welfare" and "General Attachment" and LAPS total. Owners of female dogs' showed lower values in these Subscales compared to owners of male dogs signifying distinctive levels of attachment depending on the sex of their dog. This result is thought-provoking as it has been claimed that male dogs tend to be more independent, showing more less-appreciated behaviour such as straying tendencies [37] compared to female dogs. Male dogs have been found to exhibit higher levels of separation-related behaviours and aggression compared to their female counterparts [38] and it has been postulated that male dogs interact less with their owners [39,40], which would have led to the expectation that female dogs would receive higher attachment scores. Inconsistencies in the literature regarding personality and canine sex, however, indicate that further research is needed.

LAPS subscales values and reproductive status of the dog(s)

Behavioural differences based on the reproductive status of the dog have also been proposed, including exploration behaviour [41]. Moreover animal welfare questions are frequently connected with neutering issues. Results reflected significant variances of attachment values at the Subscale "Animal rights/Welfare" between owners of intact and owners of neutered dogs. Owners

of intact dogs had lower values in the Subscale "Animal rights/Welfare".

Owners of intact dogs showed higher values in the Subscale "General attachment" and lower values in the Subscale "People substitution" compared to owners of neutered dogs. Arguably general attachment values were higher for owners of intact dogs because many intact dogs are purebred dogs and have been with their owner from an early age. On the other hand neutered dogs are in many cases adopted and come to their owner later in life. Studies show that one characteristic related to owner satisfaction is the dog's age with an owner preference to acquire a puppy [42].

The same holds true with respect to welfare issues: Purebred dogs are often not neutered whereas adopted dogs are frequently neutered for greater welfare purposes like decreasing the dog population. Awareness in owners of adopted dogs may thus be disparate in that they place a higher value on the non-reproductive status of their dog.

LAPS subscales values between owner of mixed versus owner of pure breed dogs

Data were analysed regarding attachment values of owners of purebred versus mixed bred dogs. Hielscher et al., (2019) [8] in their study established no significant differences between owners of purebred versus owners of mixed breed dogs. Herein significant differences between the two groups could be demonstrated at the Subscale "Animal rights/Welfare" and LAPS total. Owners of mixed breed dogs presented higher scores in these subscales compared to owners of purebred dogs.

It has been proposed that more extrinsically motivated dog owners display behaviour focused on earning external rewards and social acknowledgment (i.e., status) and that these owners tend to acquire a dog as part of a personal identity, thus frequently owning "designer" or purebred dogs [42]. Conversely, owners with an intrinsic motivation towards dog ownership are more likely to own a mixed breed dog. They may be more concerned with their dog's innate qualities rather than the breed or appearance [42]. Furthermore, to acquire a puppy increases owner satisfaction [34]. Mixed-breed dogs are likely to spend more time in shelters prior to adoption and the stressors of shelter life may lead to additional behavioural problems. Bir et al., (2017) [43] stated that women were more inclined to rescue a dog and favour adoption compared to men. Another factor pertaining to owner demographics is their educational level. Respondents with at least a college degree are more likely to adopt a dog from a shelter than those without a college degree [43]. Pogány et al., (2018) [44] similarly described a connection between how dog owners' define themselves and their dog breed choice. People who professed their race and personal behaviour as central in defining their sense of self rated the dog's breed as an important feature in their acquisition choice.

LAPS and radius of exploration

Owners of Group 3 dogs with a substantial walking radius (at least one run > 350 m away from owner) had the highest values at people substitution and total LAPS in comparison to Group 1 and Group 2. The far ranging Group 3 dogs had a type of owner with a higher score on the Subscales "People Substitution", demonstrating an increased value of their dog(s) company compared to people. Connections between dog behaviour and owner attachment style suggest that dogs established different strategies in their exploration and walking behaviour. This may be based on the type of support they got from their owner in the past during challenging or novel situations and by experience from their previous interaction history [45,46]. It has been publicized that owner-dog dyads with a secure attachment style similar to that of securely attached children and their caretakers have dogs that display a comparable behavioural response. They view their owner as safe haven and secure base and thus are able to engage in exploration behaviour [47]. Dogs, in contrast, which got less social support from their (insecure) owners, tend to become overly dependent on them, showing reduced exploration behaviour and staying closer to their owner most of the time. High attachment scores in Group 3 dogs therefore may reflect a secure attachment style within that dyad. These owners arguably support their dog's endeavors to independently solve problems, encouraging learning processes and thus resulting in more confident, experienced and self-reliant dogs that explored longer distances.

Comparison owner assessment and GPS measurements

The time the dogs explored was greatly over-estimated by their owners. Dogs explored significantly shorter periods and returned faster than predicted by their owner. A number of possible reasons for the overestimation are plausible. Moods for example affect perceptions [48] in that a downcast mood leads to systematically overestimating times and distances [49] and perception differs with the perceiver's biases and capability. It has been shown that emotional responses such as fear influence perception of distance [50]. Emotional reactions engage motivational systems produced by stimuli which are highly significant

to the individual and that developed throughout evolution. Consequently, if the stimuli elicited the owners' expectation of potential danger and uncontrollability it resulted in the owner's estimation being increased and perceiving the dog as travelling longer and moving further away. Thus, emotions influence how and what we see and possibly expect to see and suggest an acute rousing influence on how the environment is perceived [51]. Supposing the adaptive function of fear is to protect an individual or organism from danger it could be expected that stimuli relevant to that risk may be enhanced and thus imprecise. Emotions may stimulate owners to focus on particular sources of information or cues or to perceive these information in a distinctive manner in the environment. That in turn could then produce changes in their time and/or distance estimates. Stefanucci & Storbeck (2009) [52] stated that an emotional state of arousal (whether positive or negative) influences perception, signifying that arousal may be a sufficient cue for changing duration and distance cognizance, which is in agreement with the findings herein. Time perception is also prone to distortions and illusions based on emotional dynamics and features [53]. Studies have shown that ostensible periods of emotionally arousing events are usually inaccurate based on the valence of the occurrence when compared to neutral stimuli [54]. In general, time estimations increase as arousal intensifies with the presentation of affective stimuli. Unlike positive valence, negative valence like discomfort or fear is generally correlated with time overestimations [53].

6. General Conclusions

Physiological, psychological and affective benefits result from an enriching and positive dog-human relationship and these encompass both parties of the dyad. The dyads have comparable qualities as interpersonal affiliations in that both members may be significant attachment figures for one another [55]. Owners' perception of their dog as affectionate, caring and accepting, are qualities which can satisfy a person's basic need for self-worth [56]. Green et al., (2018) [20] proposed that attachment dimensions and affection-related behaviours were associated such as turning to one's dog as an auxiliary for human companionship or feeling less emotionally secure without one's dog. Attachment theory therefore does illuminate individual differences in relationship dynamics within a dog - owner dyad [20].

It is likely that dogs, analogous to children, have varying attachment styles towards their owners and vice versa. Improved awareness and understanding of these attachment manners could shed light on the aspects and elements having an effect on the establishment of a particularly positive and fulfilling dog-human relationship. Of course, such approaches may be overly simplistic, as attachment dimensions alone may fail to capture the influence of specific human behaviours, such as affiliation and perceptions. Nevertheless, the dog-human bond may be described as a symbiotic relationship, potentially benefitting owner and dog.

Implications for Practice

The dog-human bond is multifaceted and numerous components influence the attachment level. Practical implications may be that these factors should be taken into consideration prior to owning a dog. Dog professionals such as trainers or veterinarians may be of help to find effective strategies to improve the dog-owner connection to ensure a lasting and rewarding relationship, thereby increasing dog welfare.

Conclusions

In summary, positive, affirmative emotions, assenting dispositions and constructive behaviour towards their dog clearly enhance a strong owner-dog relationship improving and stabilizing their attachment level. This attachment and bonding is bidirectional, exemplifying the benefits that may arise from a positive and sustaining owner-dog relationship for both parties. The beneficial welfare implications should encourage the cultivation of such an affiliation. In contrast, adverse attitudes, misinterpretation of dog behaviour, insecure attachment, and feelings of arousal which influence perception and may lead to erroneous interpretations have the potential to disrupt the relationship. The impact on the dyadic functionality may be negative results for both partners. More information on the determinants of dog ownership satisfaction facilitates the identification of factors in relatively fulfilled dog owners' relationships and the lack thereof may on the other hand also be used as early warning signals for a compromised owner-dog association.

Compliance with Ethical Standards

Non-invasive observational studies on dogs are allowed to be done without any special permission in Germany according to the "German Animal Welfare Act 2013".

Conflict of Interest

The authors declare there are no conflicting interests.

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ARTICLE

Invertebrates Diversity in Arabuko-Sokoke Forest and Nearby Farmland at Gede, Kilifi County, Kenya

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ABSTRACT

Insectivorous bats mainly feed on various types of invertebrates. The authors studied the abundance and diversity of invertebrates in the farmland in the eastern part of Arabuko-Sokoke Forest, mainly to assess their availability to insectivorous bats occurring in the two study sites. Solar powered light traps were used to attract aerial invertebrates to a white suspended cloth sheet used as a landing surface. The sampling was conducted for four hours in one trapping station each night, and in twelve different stations both in the ASF and farmland. A total of 6,557 invertebrates individuals were trapped, which included 48% in ASF and 52% in the farmland. The two most common invertebrate orders were *Hymenoptera* (ants, bees, wasps and sawflies) represented by 38.1%, and *Coleoptera* (beetles, 28.1%). The interior of ASF had higher invertebrate species diversity (Shannon-Weiner index 1.72 ± 0.1), than the farmland (1.41 ± 0.1). Although the farmland (260.5 ± 52.9, N=12) had higher mean number of invertebrates trapped per night, than the interior of ASF (200.3 ± 36.4, N=12), there was no significant difference between the medians of invertebrates captured in the two study areas (Mann-Whitney U-Test, U=61: P>0.544). Thus, the farmland and the interior of ASF had the same invertebrate abundance. This study indicates the value of human-modified areas (agricultural and human settlements) landscapes, always ignored in biodiversity surveys, in sustaining diverse invertebrates that are preyed by different species of insectivorous bats that occur in the two study areas.

1. Introduction

Vegetation structure is one of the important factors that explain the distribution pattern of species, insectivorous bats included [1,2]. Nevertheless, vegetation features exclusively may not explain the distribution patterns of animals as well as their relationships with other features of a habitat [3]. Thus, it's needed to assess resource availability which are linked to the habitat where animals occur [3]. Insectivorous bats mainly feed on different types

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of invertebrates [4]. In a forested habitat, understanding the relationships between invertebrate prey availability and feeding insectivorous bats is crucial [5]. Moreover, insectivorous bats activity is influenced by vegetation characteristics and invertebrate prey availability [6]. Even though dense vegetation structure is associated with high density of invertebrates, and can subsequently increase the feeding activity of by insectivorous bats [7], the effects of habitat structure might mainly be independent of the invertebrate abundance [8]. This study investigated the diversity and abundance of invertebrates, which are preyed by insectivorous bats in ASF and the adjacent farmland. Insectivorous bats studies, previously conducted both in the interior of ASF and farmland (Table 1), had shown that the farmland had higher activity of insectivorous bats and individual bat captures than in the interior of ASF [9-11]. Therefore, we predicted that invertebrate would be more abundant in the farmland than in the interior of ASF.

2. Materials and Methods

Study area

The study was undertaken in the interior of Arabuko-Sokoke Forest (ASF) and nearby agricultural area dominated by crop cultivation and human settlements occurring east of ASF; in this study broadly denoted to as "farmland" (Figure 1). Arabuko-Sokoke Forest occurs in Kenya, north of Mombasa City, in Gede-Kilifi County (–3.5167S, 39.8167E, less than 80 m above sea level) [12,13]. The forest is a protected area managed by Kenya Forest Service (KFS), jointly with National Museums of Kenya (NMK), Kenya Forestry Research Institute (KEFRI) and Kenya Wildlife Service (KWS) [14]. The ASF has three main distinct broad vegetation types namely: 1) Mixed Forest (MIXFo), a variety of fairly impenetrable, tall and multiple tree species covering an estimated area of about 7000 ha; 2) *Brachystegia* woodland (BRA) covers about 7636 ha running in as a middle band through the ASF; and 3) *Cynometra* forest (CYNO), which covering an estimated area of 23,500 ha, occurring to the west on red Magarini soils, and is dominated mainly by *Cynometra webberi* [15]. The ASF is a globally renown biodiversity repository [16], particularly for the conservation of endemic, and rare globally threatened bird species in Kenya and Africa [17]. Invertebrate surveys were conducted in the interior of MIXFo, BRA and CYNO, as well as in farmland in the eastern part of ASF (Figure 1). In the forest, invertebrate sampling stations were established on the roads used to access different parts of the study area. These roads (maximum 4 m wide) are potential insectivorous bats pathways/flight paths. The farmland around ASF is the major habitat type in the study area (Figure 1), and is characterised mainly by areas of crops farming, human settlements, infrastructure development (village markets and small towns), and social amenities (access roads, villages paths, hospitals and schools). Household farms were cultivated with cashew nut (*Anacardium occidentale*), mangos (*Mangifera indica*) and coconut (*Cocos nucifera*). Some household farms were solely planted with either mangos or coconut trees, while others had a mixture of both trees in varying proportions. Other trees occasionally found in these farms were Neem (*Azadirachta indica*), Casuarinas (*Casaurina equesitifolia*), and Sugar-apple (*Annona squamos*). We selected farms dominated by mango, coconut or mixture of both trees and other trees described above. The mango farms (MAN) had 70.7% dominance by mango trees, coconut farms (COC) 89.3% coconut trees, while the mixed (MIXFa) had 52.9% coconut trees, Cashew nuts 23.2% and Mango 18.6%. The trees in the farms (especially, coconuts, mangos, casuarinas, neem trees) were more than 20 m in height. Invertebrate sampling stations were established in the open areas of selected farms. The 12 invertebrate sampling stations each, in the farmland and in the interior of ASF, were established in the general areas, that had been previously been used to sample insectivorous bat species [9-11]. In addition, a number of bat roosts actively used by bats to roost, during invertebrate survey occurred in the sampling areas in the farmland [18].

Invertebrate inventorying methods

Before the inventory of invertebrate was undertaken, six expeditions to sample insectivorous bats with mist-nets and their activity with detectors had previously been undertaken in the two study sites in between November 2014 to 2016 (Table 1). Results of these studies indicated that, insectivorous bats abundance and activity were higher in farmland than in the ASF (Table 1). Therefore, we predicted that invertebrate would be more abundant in the farmland than in the interior of ASF. Many nocturnal invertebrates, especially the different species of beetles and moths are easily attracted by artificial light at night [19]. Light traps are widely used to sample different invertebrate species which are active at night [20]. In the current study, light bulbs powered by a small inbuilt battery charged with solar panels were used to sample invertebrates [21] in the two study sites. In each sampling station in the ASF or farmland, four lights were used to attract invertebrates. In one sampling station the lights were deployed at least at a distance of 70m from each other.

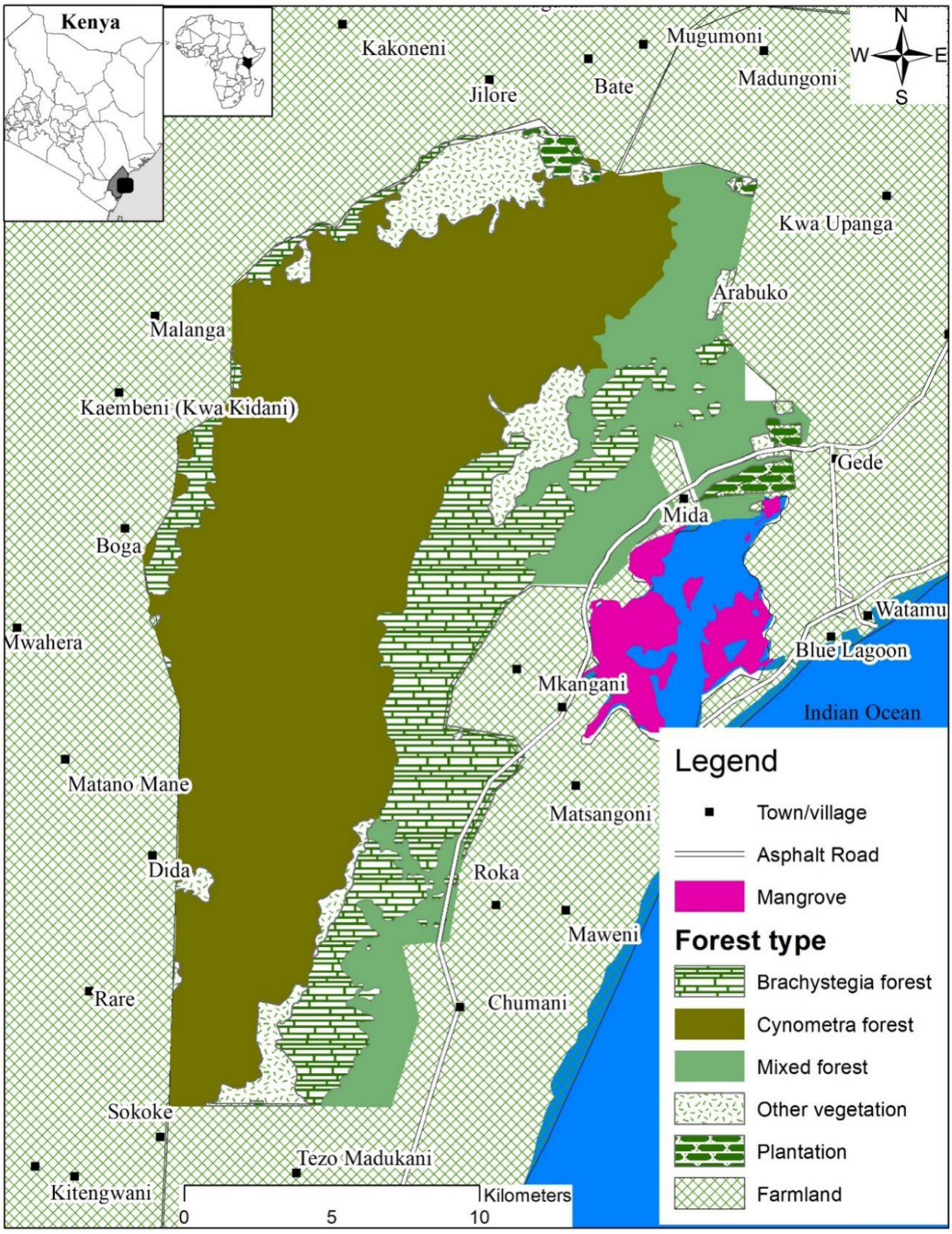

Figure 1. A map of the study areas showing the various vegetations types in the interior of Arabuko-Sokoke Forest and farmland where invertebrate sampling was conducted.

The solar powered lights (DP Light DP-6005A) http://en.dpled.com, consisted of a small battery (Figure 2), which attained a voltage of 110 V ~240 V (50/60 Hz). The battery was charged with a small solar panel (9 V/3.5 W). When the battery was charged from morning (0800 hr) to afternoon (1600 hr) during days of full sunlight, the bulb would be kept bright without dying for the night continuous sampling of four to five hours.

Figure 2. A mounted solar light and battery (DP Light DP-6005A) which was used to attract air-borne invertebrates active at night.

Invertebrate sampling stations were established in the interior of ASF at the middle of different roads used to access various vegetation types. The set up included a light trap suspended with strings, a white cloth sheet and a polythene sheeting spread on the ground (Figures 3-5). In ASF strings to suspend light traps, were tied 1.5 m above the ground on selected two trees across the road. In the farmland a sampling station was established where there were two nearby trees. A light trap was suspended at the centre of the tied string. After this a white cotton piece of cloth measuring (2 m long by 1.5 m wide), was tied at the edges with other strings and suspended facing the light trap light source with these strings at an approximate distance of a meter from the light trap (Figure 3). The white cloth which was hung about 30 cm from the ground, was used as a landing surface for nocturnal invertebrates attracted by the light trap [22]. In addition, a plastic sheet was spread under the suspended white sheet, to make it easier to collect invertebrates which dropped to the ground after colliding with the cloth barrier (Figure 3). In each sampling station the four light traps were monitored for four hours from 1900hr to 2300hr each night (Figure 4). A total of 12 light traps were used to sample invertebrates in the interior of ASF. These included four sampling stations each in *Cynometra* Forest (CYNO), Mixed Forests (MIXFo) and *Brachystegia* woodland (BRA). In the farmland 12 different stations were used to sample invertebrates, including four each in the farms dominated by mango (MAN) trees, coconut (COC) trees and other farms with multiple trees (MIXFa) species. The light traps were monitored twice each hour. Any individuals of moths captured were collected in a clear glass jar and killed with chloroform vapour soaked in cotton wool [21]. The dead moths were later removed from the glass jar, and preserved using toilet nappies and stored in plastic containers, in order to ensure that their delicate wings were not damaged. All other invertebrates excluding moths were collected in plastic jars and preserved in a fluid solution of 70% ethanol. The sample of invertebrates collected each hour was stored separately [23] in each vegetation type in the farmland and interior of ASF, so as later to assess their abundance at the end of the trapping operation.

Figure 3. The operational set up of solar light trap and battery (DP Light DP-6005A) with a transparent plastic sheeting spread on the ground (A) and suspended white cloth screen (B) for invertebrates landing

Table 1. The number of insectivorous bats echolocation calls (passes) counted with detector, and individuals captured in mist-nets in six different sampling trips in the farmland and interior of ASF in between November 2014 to June 2016.

	Survey	Sampling	Farmland	ASF Forest	Farmland	ASF Forest
	Dates/Trip	**Seasons**	**bat passes/trip**	**bat passes/trip**	**bat captures/trip**	**bat captures/trip**
1	Nov-2014	Short rain season	1775	231	161	17
2	Feb-2015	Dry season	2420	862	197	52
3	Jun-2015	Long rain season	1808	461	140	31
4	Nov-2015	Short rain season	2103	603	190	21
5	Feb-2016	Dry season	1437	871	120	15
6	Jun-2016	Long rain season	1009	1147	92	7
	TOTAL		**10,552**	**4,175**	**900**	**143**

Figure 4. The solar light and battery (DP Light DP-6005A) in operation at night attracting air-borne invertebrates to the white cloth sheet (black dots)

The individual moths from each trapping station and vegetation type were collected and stored together and not separated into hours. Any captured individuals of large moths trapped, were killed and stored in envelopes. The size of invertebrates collected measured 5 mm~40 mm in their body length. Invertebrates of small size (<5 mm) were not sampled because they are unlikely to be detected by feeding insectivorous bats [24], and those bigger (>40 mm in length) are unlikely to be consumed by them [25]. No individuals of invertebrate that were more than 30 mm by width were collected, because they were perceived to be too large a prey for Striped Leaf-nosed Bat (*Macronycteris vittata*), the largest insectivorous bat, found in the study area. Invertebrate sampling was carried interchangeably, with one night in the forest interior, followed by the next in the farmland to spread any sampling bias associated with variations in weather conditions (humidity, temperature etc.) between the ASF and the farmland. A combination of trap types and different survey methods are required for a detailed inventorying of invertebrates in an area, even for a single taxon [26]. Nevertheless, this method is problematic to implement in most field surveys, because it is expensive and time consuming [27], the reason it was not used in the current study.

Data analysis

Individuals of invertebrates were totalled and identified to taxonomic order by use of specimens collected in the past from ASF and preserved with Invertebrate Section of National Museums of Kenya. Species diversity of invertebrates was calculated using Shannon-Wiener index of diversity [28]. The total number of individuals of invertebrates in each of the 12 sampling stations in ASF and farmland was counted. To test for the differences in sample medians of invertebrates captured in each station per night in the farmland and in the ASF, a Mann-Whitney U-test non-parametric statistical test was used. To estimate the size of invertebrate (to the nearest 0.25 mm), individuals were measured using a ruler from the head (exclusive of antennae) to the tip of the abdomen (short of inclusion of cerci) [29]. Thereafter, the individuals of invertebrates were clustered into four different size groups: from the smallest of size 5 mm~10 mm, 11 mm~21 mm, 22 mm~32 mm, to the largest >33 mm. To assess the pattern of invertebrate activity during the four (1900 hr~2300 hr) sampling hours, the total number of individuals of invertebrates not including moths captured in each hour was counted. Moths were omitted from this analysis, because the individuals from each trapping station and vegetation type were collected and stored together and not separated into hours. PAST statistical program [30] was used to analyse collected data.

3. Results

Invertebrate richness and diversity

A total of 6,557 individuals of invertebrates were trapped, which included 52% in the farmland and 48% in the interior of ASF. The most abundant order was *Hymenoptera* (ants, bees, wasps and sawflies), which was represented by 38.1% of all sampled invertebrates, followed by the order *Coleoptera* (beetles (28.1%) and *Lepidoptera* (moths (15.7%) Tables 2-3). Majority of individuals of orders *Hymenopterans* and *Coleopterans* were found in the farmland than in the interior of ASF (Table 3). Many individuals of the order *Lepidopterans* and of larger sizes were found in the interior of ASF than in the farmland (Table 2). The interior of ASF had higher species diversity (Shannon-Weiner index 1.72 ± 0.1), than the farmland (1.41 ± 0.1). Although the mean number of invertebrates trapped per night in the farmland was larger (260.5 ± 52.9, N=12), than in the interior of ASF (200.3 ± 36.4, N=12), there was no significant difference between the medians of invertebrates captured in the two study areas (Mann-Whitney U-Test, U=61: P>0.544).

Invertebrate sizes and their activity pattern

Of the 6,557 invertebrate individuals captured, 68% of these were of small sizes (5 mm~10 mm), followed by those of size 11 mm~21 mm (29%) (Table 4). Most of the individuals of invertebrates in the two study sites were captured at 1900 hr. In the interior of ASF invertebrate activity (captures) underwent a steep declined from 1900 hr to 2000 hr. However, at the same in the farmland invertebrate activity maintained relatively stable decline. Finally, in both habitats invertebrate activity was lowest at 2300 hr (Figure 5).

Table 2. Abundance and diversity of orders of invertebrates sampled in three different habitat types in the interior of ASF.

	COUNT OF INVERTEBRATES IN EACH VEGETATION TYPE				
	ORDERS OF INVERTEBRATES	**MIXFo**	**BRA**	**CYNO**	**ASF**
1	Hymenoptera (Ants, bees, wasps and sawflies)	131	537	376	**1044**
2	Coleoptera (Beetles)	120	357	158	**635**
3	Hemiptera (Bugs, aphids and cicadas)	79	73	41	**193**
4	Blattodea (Cockroaches and termites)	68	108	62	**238**
5	Diptera (Flies and mosquitoes)	27	91	25	**143**
6	Orthoptera (Grasshoppers, crickets, katydids)	32	38	29	**99**
7	Mantodea (Praying mantids)	5	21	7	**33**
7	Neuroptera (Net winged invertebrate)	3	11	4	**18**
8	Odonata (Dragonflies and damselflies)	0	1	0	**1**
10	Lepidoptera (Moths)	159	280	325	**764**
Abundance		**624**	**1517**	**1027**	**3168**
Shannon_H		**1.85 ± 0.09**	**1.72 ± 0.08**	**1.57 ± 0.11**	**1.72 ±0.05**

Legend: Mixed Forest (MIXFo), *Brachystegia* Woodland (BRA), and *Cynometra* Forest (CYNO)

Table 3. Abundance and diversity of orders of invertebrates sampled in three different habitat types in the farmland.

		COUNT OF INVERTEBRATES IN EACH VEGETATION TYPE			
ORDERS OF INVERTEBRATES		**MAN**	**COC**	**MIXFa**	**FARMLAND**
1	Hymenoptera (Ants, bees, wasps and sawflies)	1157	202	93	**1452**
2	Coleoptera (Beetles)	414	247	547	**1208**
3	Hemiptera (Bugs, aphids and cicadas)	39	42	23	**104**
4	Blattodea (Cockroaches and termites)	122	10	18	**150**
5	Diptera (Flies and mosquitoes)	39	12	14	**65**
6	Orthoptera (Grasshoppers, crickets, katydids)	47	42	33	**122**
7	Mantodea (Praying mantids)	1	7	1	**9**
7	Neuroptera (Net winged invertebrate)	8	4	3	**15**
8	Odonata (Dragonflies and damselflies)	1	0	0	**1**
10	Lepidoptera (Moths)	135	46	82	**263**
Abundance		**1963**	**612**	**814**	**3389**
Shannon_H		**1.27 ±0.94**	**1.52 ± 0.15**	**1.16 ± 0.16**	**1.41 ± 0.06**

Legend: Mango farms (MAN), Coconut farms (COC), Mixed farms (MIXFa)

Table 4. The counts and percentages of invertebrates of different sizes sampled in the farmland and in the interior ASF

INVERTEBRATE BODY SIZE CLASSES					
	5-10 mm	**11-21 mm**	**22-32 mm**	**sizes >33**	**Total**
Total (Farmland and ASF) All other invertebrates minus Lepidoptera (Moths)	3,440	1,869	167	54	5530
Total (Farmland and ASF) Lepidoptera (Moths)	994	30	3	0	1027
Total (Farmland and ASF)	**4,434**	**1,899**	**170**	**54**	**6,557**
Percentage (%)	**67.6**	**29.0**	**2.6**	**0.8**	**100.0**

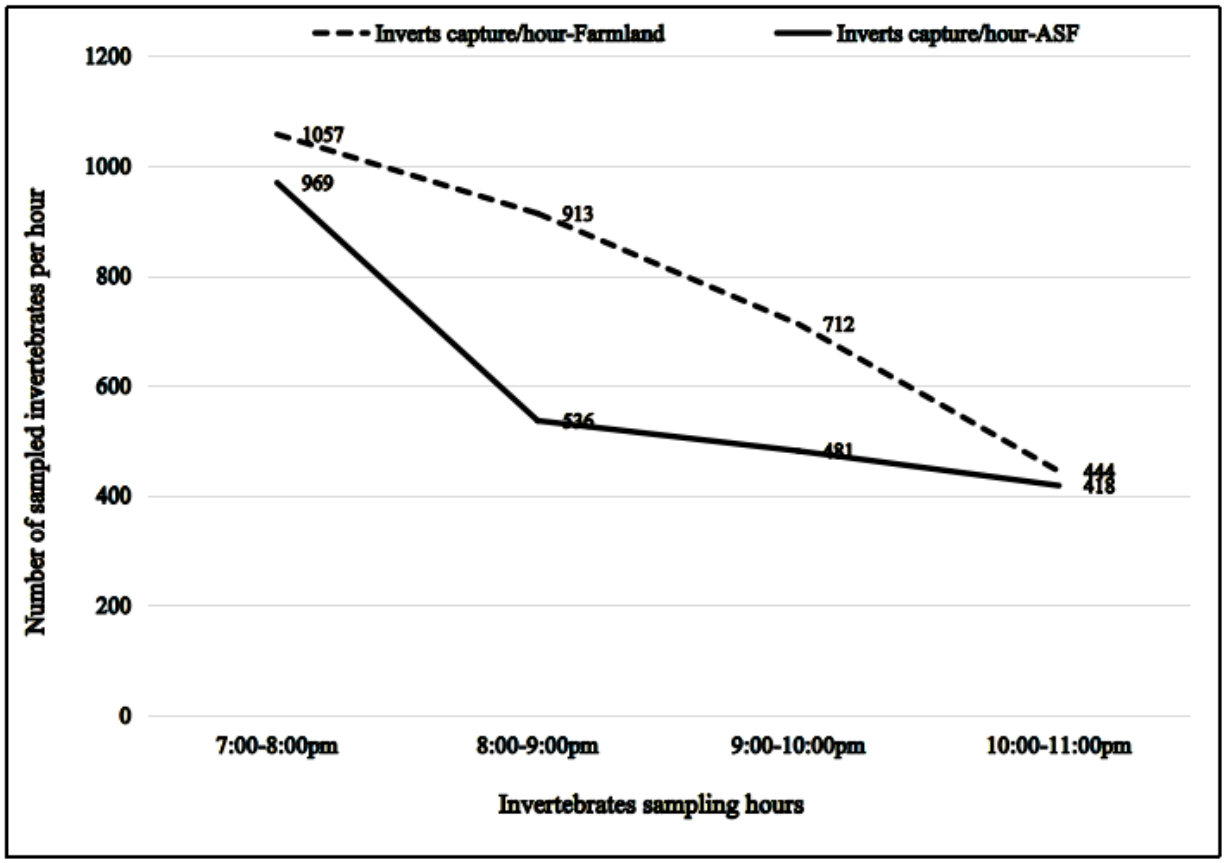

Figure 5. The hourly pattern in the activity (captures) of invertebrates (excluding moths) sampled in the farmland and the interior of ASF.

4. Discussion

The current study investigated diversity and abundance of invertebrate in the interior of ASF and surrounding farmland. The ASF is a legally protected area, characterised by indigenous vegetation typical of the east African coastal forests [10,17], while the farmland habitat is completely disturbed and modified into an agricultural landscape. Because the two contrasting habitats had been shown to host various types of insectivorous bat species [9,10], there was need to provide data on diversity and abundance of invertebrates, which are the primary food items eaten by these bats. The results of our study indicated that the farmland in the eastern part of ASF as well as the interior of ASF and had similar invertebrate abundance. However, the species diversity of invertebrate was higher in the interior of ASF than in the farmland. This was possibly because of the large number of individuals of two invertebrate orders (Coleopterans (1452), and Hymenopterans (1208) in the farmland which dominated most of the captures (Table 3), while in the forest interior the captures were fairly distributed among several orders (Table 2). The order Lepidopterans (moths) were more common and of larger sizes in the interior of ASF than in the farmland. Studies have shown that the abundance of moths decline with habitat disturbance especially fuelled by agriculatural intensification [31]. This is largely because less disturbed habitat as was the case of ASF, provides sheltered environments for moth species survival [32], as compared to the highly disturbed farmland habitat. The individuals of order *Coleoptera, Hymenoptera* and *Orthoptera* were more common in the farmland than in ASF. Comparable results were recorded in Malaysia at Kota Damansara Community Forest Reserve [33].

Among the four largest orders of invertebrates *Hymenoptera* is largest, followed by *Coleoptera*, *Lepidoptera* and *Diptera* [34]. This possibly may explain the large numbers of individuals of these orders recorded in the current study. Individuals of the order *Coleoptera, Lepidoptera, Diptera, Hymenoptera* and *Isoptera* are the most common groups of invertebrates preyed upon by insectivorous bat species [35]. In this study, representatives of these orders were captured in large numbers in both study sites. Nevertheless, many individuals of the order *Dipterans, Lepidopteran,* and *Blattodea* were more common in ASF than in the farmland. Therefore,

though the interior of ASF has been shown to have low insectivorous bats abundance and activity than the farmland [9-11], most of the invertebrate orders preyed by these bats were also common in the forest interior. This probably indicates that the farmland and the interior of ASF were suitable foraging habitats for the insectivorous bat species found in the two study sites.

There are a number of vegetation characteristics related factors which may explain the abundance and composition of invertebrate diversity recorded in a specific area. For example, the understorey vegetation structure may influence the abundance of invertebrates trapped with light traps. Specifically, the undergrowth (vegetation measured <3 m by height) understorey) openness possibly increases the efficiency of light-trapping area, particularly for nightly invertebrates [36]. For instance, in the interior of ASF, the light source from the light traps, was observable in a small area, as a result of the barriers occasioned by the impenetrable understorey vegetation cover. Consequently, a small area of ASF interior may have been sampled, the area immediately around the light source. Nonetheless, in an uncluttered habitat, such as in the farmland in the current study, the light trap was noticeable from far, and perhaps attracted invertebrates from a wide trapping range. Furthermore, canopy openness has been shown to have a strong influence on beetle compositions [37], but not moths [38]. The understorey and canopy of the interior of ASF is more cluttered than that of farmland [10]. Hence, even though closed habitats which are undisturbed have high abundance of invertebrate [39], the impediment of the light trap by thick canopy vegetation, possibly reduced light detection by air-borne invertebrates, and eventually reduced overall abundance of individuals of invertebrates trapped inside ASF. This perhaps may suggest that, though results of the current study showed that, both study sites had the same invertebrate abundance, the interior of ASF may be richer in invertebrate abundance. The activity of invertebrate peaked after nightfall (1900 hrs), and gradually or sharply deteriorated to the lowest level at 2300 hr in both study sites. The activity of insectivorous bats in and around ASF has been shown to be highest immediately after dusk (1900 hrs~2000 hrs) and is lowest after midnight [9,11]). This possibly, suggests that insectiverous bats in the study area, sychronize their foraging activity with the availbility and abundance of invertebrate prey.

5. Conclusions

The farmland was highly disturbed, and in continous habitat modification, and was expected to have a low abundance of invertebartes [40]. Nevertheless, the farmland had the similar invertebarte abundance with the comparatively less disturbed ASF. This may suggest that the two study sites provided suitable feeding areas for the insectivorous bat species found in the area. This study, highlight the value of human-modified areas, always ignored in biodiversity surveys, in sustaining diverse invertebrates that are preyed upon by different species of insectivorous bats that occur in the two study areas. In order to have a detailed documentation of invertebrate composition in the two study sites in future studies, it is recommended to employ a combination of different invertebrate sampling methods [26]. This is because the efficacy of light traps in invertebrates sampling varies between taxa [41]. Furthermore, light traps sample exclusively individuals of invertebrate taxa attracted to light [42].

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Conflict of Interest

There is no conflict of interest.

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ARTICLE

Anti-trypanosomal Activity of Bufonidae (Toad) Venom Crude Extract on *Trypanosoma brucei brucei* in Swiss Mice

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Haematological parameters

ABSTRACT

Trypanosomiasis afflicts about 6 ~ 7 million people globally and to a large extent impedes livestock production in Africa. Naturally, trypanosomal parasites undergo genetic mutation and have developed resistance over a wide range of therapies. The utilization of animals and plants products has presented therapeutic potential for identifying novel anti-trypanosomal drugs. This study evaluated toad venom for anti-trypanosomal potency in-vivo in Swiss mice. Toads were collected from July to August 2019. The acute oral toxicity and biochemical characterization of the toad venom were determined. The experimental mice were administered various doses (130 mg/kg, 173 mg/kg and 217 mg/kg) of the toad venom crude extract and 0.75 mg/mL of Diamizan Plus standard drug for the treatment of trypanosomiasis, once daily for 3 days. The in-vivo anti-trypanosomal activity was evaluated by a curative test, after infecting the mice with *Trypanosoma brucei brucei*. The pre-patent period was 72 hours before treatment commenced. The overall results showed that trypanosomal load was highest in the control group while the group treated with Diamizan drug had the least trypanosomal load. As such, the mean trypanosomal load in relation to treatments showed a very high significant difference ($P<0.05$). Also, the mean trypanosomal load in Swiss mice in relation to the highest dosage of toad venom versus Diamizan drug showed a very high significant difference ($P<0.05$). The mean change in relation to the haematological parameters across treatments groups varied significantly ($P<0.05$) with the exception of Hb which showed no significant difference ($P>0.05$) across treatment groups. The over 50% reduction in the trypanosomal load in the 130 mg/kg group in comparison with the control group brings to bare the anti-trypanosomal potency of the toad venom. The anti-trypanosomal activity demonstrated by the toad venom has provided basis for development of new therapeutic agents from different toad species. The study recommends further studies (both in-vivo and in-vitro) followed by the characterization of the active compounds present in the toad venom responsible for the anti-tyrpanosomal activity observed alongside the management and conservation of these species.

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1. Introduction

Trypanosomiasis is a disease caused by microscopic parasites belonging to the species *Trypanosoma brucei* which invades the blood plasma and various body tissues, lymph and cerebrospinal fluid. In sub-Saharan Africa, tsetse fly serves as the insect vector responsible for the transmission of this parasite. They appear to be harmful to only mammals including man though they also infect many animals [1]. Human African trypanosomiasis (HAT), or sleeping sickness, remains a life-threatening disease that mostly affects poor rural populations. Two subspecies of *Trypanosoma brucei* cause disease: *T. b. gambiense* in West and Central Africa, and *T. b. rhodesiense* in East Africa. HAT transmission requires the interaction of humans, tsetse flies and parasite reservoirs (humans, and domestic and wild animals) [2].

African animal trypanosomiasis (AAT) is a parasitic disease that causes very significant economic losses in livestock, from anemia, loss of condition and effects on reproduction whereas cattle production, accounts for a greater percentage of these losses [3]. Trypanosomes infect a large number of wild fauna (antelope species, warthogs, elephants, hippopotamus, lions, hyenas, jackals, caracals, and wild ruminants etc.) [1,4,5] and domestic ungulate species (cattle, sheep, goats, horses, pigs, camels and dogs) [6]. Infections in wildlife are influenced by species and habitat [7]. Trypanosome species commonly found in wildlife species include *T. vivax, T. brucei brucei, T. congolense* and *T. evansi* [5].

The human and animal trypanosomiasis continue to present significant global health burden in human and animal (domesticated and wildlife communities) thus far, chemotherapy and chemoprophylaxis represent the mainstay for its control [8,9]. Worryingly, the inherent potential of trypanosomal parasite to undergo genetic mutation has led to its ability to successfully develop resistance over a wide range of therapies.

Uptake of natural products from animals and plants presents explorable therapeutic potentials and earlier studies of toad venoms from different species and their chemical basis have demonstrated new perspectives for their pharmaceutical use including the development of new therapeutic agents [10]. Over the years yet, a novel licensed compound is unlikely to be available [1]; hence the rationale for this study.

2. Materials and Methods

2.1 Toad Collection

The toads used for this study were collected from the month of July to August 2019 in a well-ventilated container between 07:00 a.m. and 10:00 a.m. hours daily in the rice fields at Gandu, Lafia LGA, Nasarawa State and conveyed to the Laboratory unit of the Department of Zoology, Faculty of Science, Federal University of Lafia, Nasarawa State for extraction.

2.2 Ethical Permit

Ethical permit with the Project Identification Code (PIC) – FUL/FS/ZLY/2019/002 was obtained for the research from the Ethical Committee of the Department of Zoology, Faculty of Science, Federal University of Lafia, Nasarawa State.

2.3 Extraction of Crude Venom Extract from Toad

The extraction process of bufonidae was achieved by massaging and pressing the parotoids macro-glandules and the secretion was collected using a petri dish [11]. The collected secretion was lyophilized and stored in a freezer (–20 °C) at the Federal University of Lafia, Lafia, Nasarawa State.

2.4 Characterization of Bioactive Compounds

Determination of the quantitative and qualitative chemical components of the venom was achieved by Mass Spectrometry using GC-MS techniques at the Spectral Laboratory and Services, Tudun, Wada Kaduna South, Kaduna, Nigeria. The Shimadzu Fourier transform Infrared Spectrophotometer- FTIR 8400 S was used for the determination of the functional units present in the venom. Gas Chromatography Analysis of toad venom was done using gas chromatography (Perkin-Elmer 8500). The Scanning Electron Microscope energy dispersive X-ray spectroscopy (SEM-EDS) Phenom Prox, manufactured by phenom World Eindhoven (Netherlands) was used to carry out the morphology analysis (that is; analysis of the chemical elements present, in the toad venom).

2.5 Experimental Animals

Thirty Laboratory Swiss mice of the same age, weighing between 12 g to 45 g were purchased from National Veterinary Research Institute (NVRI) Vom, Jos, Plateau State. All animals were fed with formulated feeds and water was administered *ad libitum*. The caring and experimental use of the mice was in accordance with the National Institutes of Health Guidelines for Care of Laboratory Animals. The animals were acclimatized for 7 days prior to their randomization into the various experimental groups.

2.6 Coding and Weighing of Animals

Different codes were given to every animal that was

employed in this study using a permanent marker to create marks of identification on a particular part of the body that is head (HD), Bark (BK), Tail (TL), Right side (RS), Left Side (LS), Right ear (RE), Left Ear (LE) etcetera. The weight of each animal was taken using top animal precision balance.

2.7 Toxicity Study

The median oral lethal dose of the toad venom was determined in mice using Lorke's method 1983 [12]. This method has two phases.

Phase 1 requires 9 animals. The nine animals were divided into three groups of three animals each. Each animal was administered with different doses (10, 100 and 1000 mg/kg) of the toad venom and animals, placed under observation for 24 hours to monitor their behavior as well as mortality.

Phase 2 involves the use of 3 animals, distributed into three groups of one animal each. The mice were intoxicated with different doses (250 mg/kg, 500 mg/kg and 750 mg/kg) of the toad venom and observed for 24 hours for behavior and mortality as well.

Then the LD_{50} is calculated by the formula:

$$LD_{50} = \sqrt{(D_0 \times D_{100})}$$

D_0 = Highest dose that gave no mortality,
D_{100} = Lowest dose that produced mortality.

2.8 Parasite Species and Standard Inoculation

Parasite was obtained from National Veterinary Research Institute (NVRI) Vom, Plateau State, Nigeria. Parasites were maintained through serial blood passage in mice wherein the mice previously infected with Nigerian strain of *Trypanosoma brucei brucei* and with high parasitemia level served as the donor. Donor mouse was anaesthetized with chloroform and blood (1 mL) was extracted through cardiac puncture using 1ml needle and syringe and made up to 20 mL with normal saline. Blood samples were taken such that 0.2 mL injected subcutaneously into the experimental animals [13].

2.9 Determination of Parasites

Blood samples were collected by bleeding the tail vein of the infected mice. Thin blood smears were made on clean glass microscope slides. The films were dried in air and then fixed in methanol and stained with 10% Giemsa solution [14]. The stained film was then observed under the binocular compound microscope and viewed for parasitemia. The percentage parasitemia were determined by counting the number of parasites on four or five fields.

2.10 Preparation of Treatment Solution

The treatment doses of the venom were calculated based on the lethal dose (LD50), which is 30% of the LD_{50} [15]. Therefore, the study calculated 15%, 20% and 25% of the lethal dose as the curative doses, which gave 130 mg/kg, 173 mg/kg and 217 mg/kg respectively.

2.11 Curative Study

The animals were acclimatized 7 days prior to their randomization into various groups infected with *T. brucei-brucei* and then divided into five groups of five mice per group. The presence of parasites was confirmed in the mice 72 hours after inoculation and was taken as day 0, thereafter, treatment commenced once daily for three days. Group 1, 2, and 3 were orally treated with 130 mg/kg, 173 mg/kg, and 217 mg/kg dose of crude toad venom extract respectively while group 4 received 0.75 mg/mL of Diamizan Plus (treatment drug for trypanosomiasis) intradermally, whereas group 5 (infected and untreated) was regarded as the control. The parasitemia of experimental mice were established before treatment was administered [16].

2.12 Hematological Parameters

Using the methods described by Cheesbrough [17], the Packed Cell Volume (PCV), haemoglobin (Hb) and erythrocyte (RBC) counts were determined. These parameters were determined for each mouse before infection and after treatment. Blood samples were collected from the tail of each mouse with a heparinized capillary tube with one end sealed with plasticine.

2.13 Statistical Analysis

Data obtained were analyzed using R Console software (Version 3.2.2). Shapiro-Wilk normality test was carried out to determine normality in the distribution of the data. Thereafter, Kruskal-Wallis rank sum test was used to compare the mean of pooled trypanosomal load in Swiss mice in relation to toad venom treatments and standard drug. One-way analysis of variance was used to compare daily changes in the mean load of trypanosomes in Swiss mice in relation to toad venom treatments and diamizan plus standard drug. Mean change in body weight as well as in haematological parameters was compared using Kruskal-Wallis rank sum test. Level of significance was set at $P < 0.05$. Wilcoxon rank sum test with Bonferroni correction was used as post-hoc test for multiple pairwise comparisons of means where there was a significant difference between the treatments in pooled trypanosomal

load, change in weight and as well as haematological parameters. While Turkey's Honest Significant Difference (Turkey HSD) post-hoc test was used for multiple comparisons of means in daily trypanosomal load changes between the treatments.

3. Results

3.1 Determination of the Bioactive Compounds in the Toad Venom Crude Extract

The biocharacterization analysis of the toad venom crude extract involving GC-MS, revealed that the most dominant chemical compound present was 9,12-Octadecadienoic acid (Z,Z) at peak 3 as depicted in Figure 1 which had a rate of 38.615, at a proportion of 49.76% followed by n-Hexadecanoic acid (29.04%) then Octadecanoic acid (7.03%), Squalene (5.48%), 1-Hexadecyne (3.87%), Butyl 9,12-octadecadienoate (2.95%) while Hexadecanedioic acid was the least compound (1.87%). These findings are properly presented in Table 1.

Furthermore, as depicted in Figure 2, the characterization procedures using FTIR showed that the toad venom crude extract contains 18 active functional groups, in which Nitrogen-Hydrogen Oxygen stretch of 93.18% was the most active functional group. However, the toad

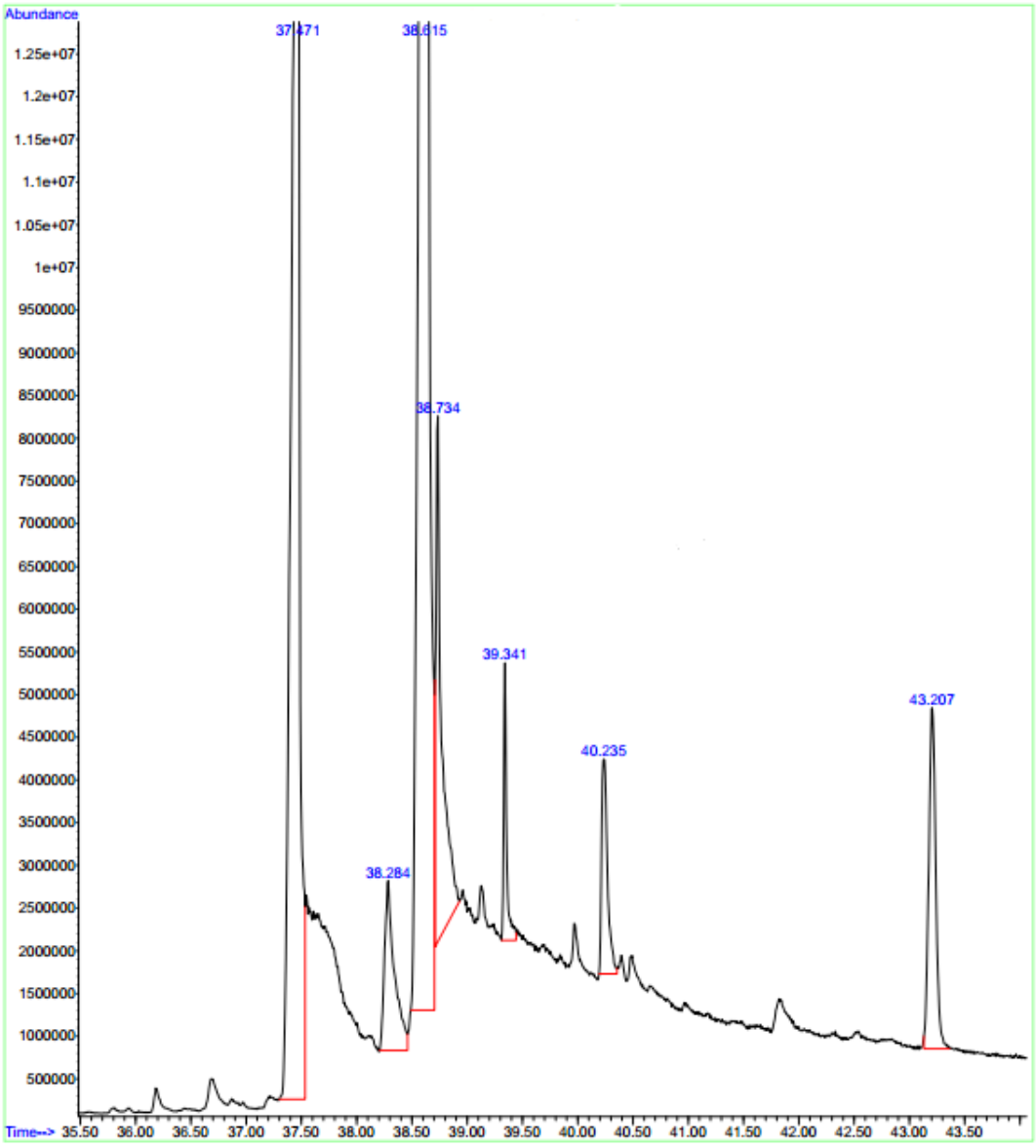

Figure 1. Mass spectroscopy of toad venom crude extract showing the abundance of the chemical compounds at different peak

venom crude extract was predominantly characterized by the presence of the amino group made up of 8 functional groups, followed by the nitro-group having 6 functional groups while the non- protein group was made up of only 4 functional group as shown in Table 2.

The SEM-EDS method uncovered the dynamics of the 11 chemical elements contained in the toad venom crude extract. Carbon was top of the list with atomic concentration and weight of 61.76 mol/dm^3 and 41.04 g/mol respectively, followed by oxygen (25.04 mol/dm^3 and 22.29 g/mol) while iodine and aluminium had the least atomic concentration and weight of 0.24 mol/dm^3 and 0.90 g/mol respectively. The details of these results were properly captured in Table 3.

Table 1. The chemical components in the crude extract of the toad venom using the GC-MS

Peak	Rate	Library/ID of Compounds	Percentage of Total Compounds (%)
1	37.471	n-Hexadecanoic acid n-Hexadecanoic acid n-Hexadecanoic acid	29.04
2	38.284	1-Hexadecyne 5-Hexadecyne 5-Eicosyne	3.87
3	38.615	9,12-Octadecadienoic acid (Z,Z)- 9,12-Octadecadienoic acid (Z,Z)-9,17-Octadecadienal, (Z)	49.76
4	38.734	Octadecanoic acid Octadecanoic acid Pentadecanoic acid	7.03
5	39.341	Hexadecanedioic acid Dodecanoyl chloride 4-Cyclopropylme thylbenzonitrile	1.87
6	40.235	Butyl 9,12-octadecadienoate 6-Dodecane 7,10-Hexadecadienoic acid, methyl ester	2.95
7	43.207	Squalene Squalene Supraene	5.8

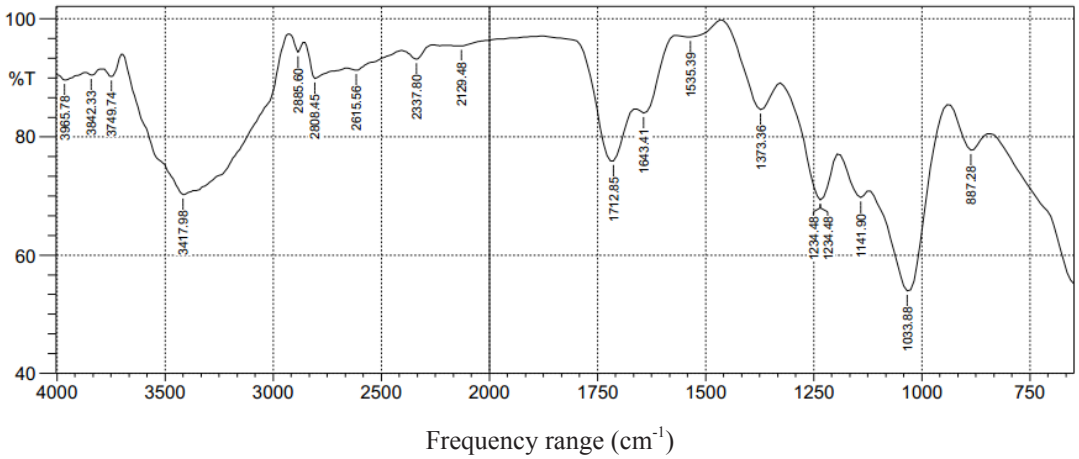

Figure 2. The functional groups present in the toad venom crude extract at different peaks

Table 2. The different protein functional groups present in the toad venom crude extract at different peaks using FTIR

Amino-group	Nitro-group	Non-protein group
Triethylamine	Nitro group with broad stretching of NO_2	Aromatic group (Peak 4)
Diethylamide	M-nitrotoluene	Aromatic group (Peak 14)
Aniline with a concentration of 84.653%	Nitrile group	Carbon Hydrogen stretch with strong intensity of 89.60%
Secondary Amines	Nitrogen Hydrogen Oxygen stretch of 93.18%	Olefins with weak band of 53.975%
n-butylamine & Benzamide with intensity (concentration) of 90.201%	Nitro Methane with concentration of 91.31%	
Nitrogen Hydrogen strong bond (amide) with concentration of 90.462	Nitrile group with Concentration of 89.86 %	
N-H bending		

Table 3. Chemical elements present in the toad venom crude extract using the SEM-EDS

Element Number	Element Symbol	Element Name	Atomic Conc.	Weight Conc.
6	C	Carbon	61.76	41.26
8	O	Oxygen	25.04	22.29
25	Mn	Manganese	4.12	12.59
82	Pb	Lead	0.94	10.84
24	Cr	Chromium	1.43	4.13
7	N	Nitrogen	2.67	2.08
53	I	Iodine	0.24	1.68
11	Na	Sodium	1.12	1.44
16	S	Sulfur	0.80	1.44
9	F	Fluorine	1.28	1.36
13	Al	Aluminium	0.60	0.90

3.2 Oral Toxicity (LD_{50}) of the Toad Venom Crude Extract on the Laboratory Swiss Mice

The oral toxicity study resulted in mortality of lab animals after one hour of administration of 1000 mg/kg dose of toad venom crude extract. Prior to their mortality, symptoms like hyperactivity, convulsion and constant stooling were observed before finally resulting in death. However, on the administration of 750 mg/kg, hyperactivity, diarrhea and sedation were observed within one hour, but there was no mortality recorded. Following the Lorke's method computation, the oral toxicity of the toad crude venom extract was established as 866 mg/kg.

3.3 Trypanosomal Load at Day Zero

Pre-patency was observed after 72 hours that was noted as day zero prior to the commencement of first treatment. Prior to treatment, the mean of trypanosomal load in Swiss mice at day zero in relation to treatments with toad venom and Diamizan plus drug showed no significant difference (F_{20} = 0.9545, Adjusted R^2 = –0.007644, P = 0.4537, Figure 3). Though trypanosomal load was found to be highest in mice group treated with 173 mg/kg of the toad venom (group 2) followed by those designated in group 1 (130 mg/kg), then individuals for group 4 treatment (0.75 mg/mL of Diamizan plus), whereas parasitemia was very low in those set aside for group 3 (217 mg/kg) trial.

3.4 Trypanosomal Load in Swiss Mice in Relation to Treatments with Toad Venom and Diamizan Plus Drug

A very high significant variation (Kruskal-Wallis χ^2 = 42.189, df = 4, P < 0.0001, Figure 4) was observed in the mean trypanosomal load in Swiss mice in relation to treatments with toad venom alongside Diamizan plus drug. Thus, the overall trypanosomal load was highest in group 2 treated with 173 mg/kg of toad venom crude extract followed by group 3 treated with 217 mg/kg then group 1 treated with 130 mg/kg while it was least in group 4 treated with Diamizan plus. Table 4 shows multiple comparisons between means of trypanosomal load in Swiss mice in which the highest dosage of toad venom versus

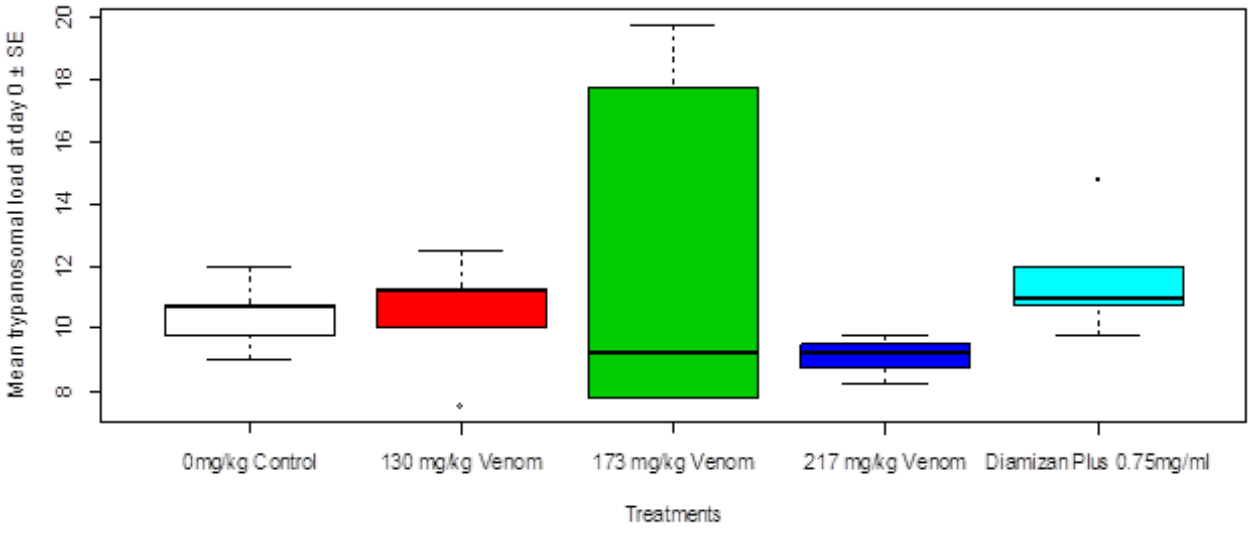

Figure 3. Mean trypanosomal load in Swiss mice in relation to treatments with toad venom and Diamizan plus drug at day zero

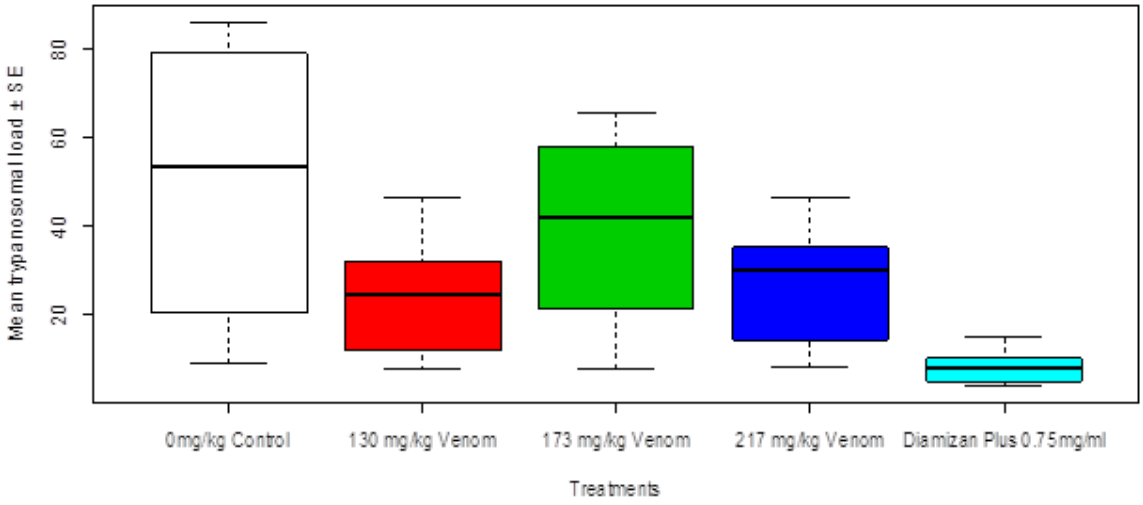

Figure 4. Mean Trypanosomal Load in Swiss mice in Relation to Treatment with Toad Venom and Diamizan Plus Drug

Table 4. Pairwise Comparisons between Means of Pooled Trypanosomal Load in Swiss mice in Relation to Treatments with Toad Venom and Diamizan Plus Drug using Wilcoxon Rank Sum Test

	Trypanosomal Load and Treatments			
	0 mg/kg	**130 mg/kg**	**173 mg/kg**	**217 mg/kg**
130 mg/kg	0.038	0	0	0
173 mg/kg	0.156	0.038	0	0
217 mg/kg	0.038	0.626	0.093	0
Diamizan Plus 0.75 mg/mL	1.0e-05	1.0e-05	1.0e-05	2.7e-05

P Value Adjustment Method: BH

Diamizan plus drug showed a very high significant difference ($P < 0.0001$). Also, trypanosomal load in Swiss mice between control group and the highest dosage of toad venom treatment was significant ($P = 0.038$).

3.5 Trypanosomal Load at Day One

At the 24th hour, the mean trypanosomal load in Swiss mice showed a very high significant difference ($F_{20} = 19.43$, Adjusted $R^2 = 0.7544$, $P = 0.000001157$, Figure 5) in relation to treatments with toad venom and Diamizan plus. Therefore, the trypanosomal load at day one (after 24 hours of treatment) was highest in group 2, followed by group 1, then group 3, whereas it was least in group 4. Furthermore, the comparison of trypanosomal load in Swiss mice in relation to the highest dosage of toad venom versus Diamizan plus treatments in day 1 showed a very high significant difference ($P < 0.0001$) as shown Table 5.

3.6 Trypanosomal Load at Day Two

At 48 hours of treatment, the trypanosomal load was highest in group 2 followed by group 3, then group 1, whereas it was least in group 4. Thus, there was a very significant difference ($F_{20} = 107$, Adjusted $R^2 = 0.9464$, $P < 0.00001$, Figure 6) in the mean of trypanosomal load in Swiss mice at 48 hours of treatment with varying concentrations of toad venom and Diamizan plus drug. Trypanosomal load in Swiss mice in relation to multiple comparison of means between treatments at day 2 showed a significant difference ($P < 0.0001$) as shown in the Table 6.

3.7 Trypanosomal Load at Day Three

Group 2 at 72 hours of treatment had the highest trypanosomal load followed by group 1, then group 3 whereas it was least in group 4. Hence, mean trypanosomal load in Swiss mice in relation to treatments with toad venom and Diamizan plus drug at day three of treatment had a very significant difference ($F_{20} = 145.2$, Adjusted $R^2 = 0.9601$, $P < 0.00001$, Figure 7). Trypanosomal load in Swiss mice in relation to multiple comparison of means between treatments at day 3 showed a high significant difference ($P < 0.0001$) with the exception of between 130 mg/kg and 217 mg/kg as shown in the Table 7.

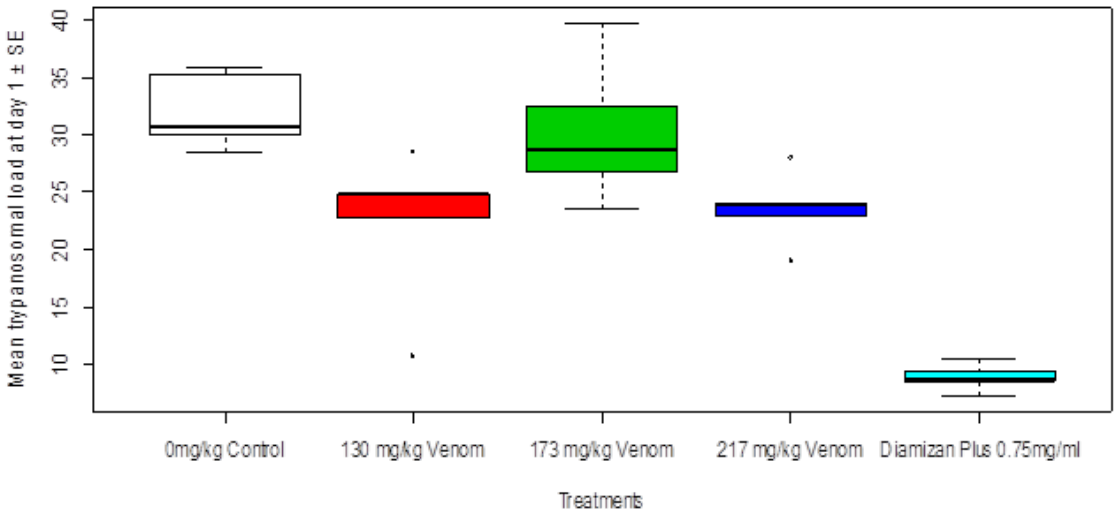

Figure 5. Mean trypanosomal load in Swiss mice in relation to treatments with toad venom and Diamizan plus treatments at day one

Table 5. Turkey Multiple Comparisons of Means of Trypanosomal Load at 95% Family-Wise Confidence Level –for Day One

Treatment	Diff	Lower	Upper	P – Adjusted
130 mg/kg – 0 mg/kg	–9.75	–18.5138779	–0.9861221	0.0246790
173 mg/kg – 0 mg/kg	–1.80	–10.5638779	6.9638779	0.9710613
217 mg/kg – 0 mg/kg	–8.45	–17.2138779	0.3138779	0.0621310
Diamizan Plus 0.75 mg/mL – 0 mg/kg	–23.15	–31.9138779	–14.3861221	0.0000013
173 mg/kg – 130 mg/kg	7.95	–0.8138779	16.7138779	0.870134
217 mg/kg – 130 mg/kg	1.30	–7.4638779	10.0638779	0.9913229
Diamizane Plus 0.75 mg/mL – 130 mg/kg	–13.40	–22.1638779	–4.6361221	0.0015377
217 mg/kg – 173 mg/kg	–6.65	–15.4138779	2.1138779	0.1954923
Diamizan Plus 0.75 mg/mL – 173 mg/kg	–21.35	–30.1138779	–12.5861221	0.0000043
Diamizan Plus 0.75 mg – 217 mg/kg	–14.70	–23.4638779	–5.9361221	0.0005646

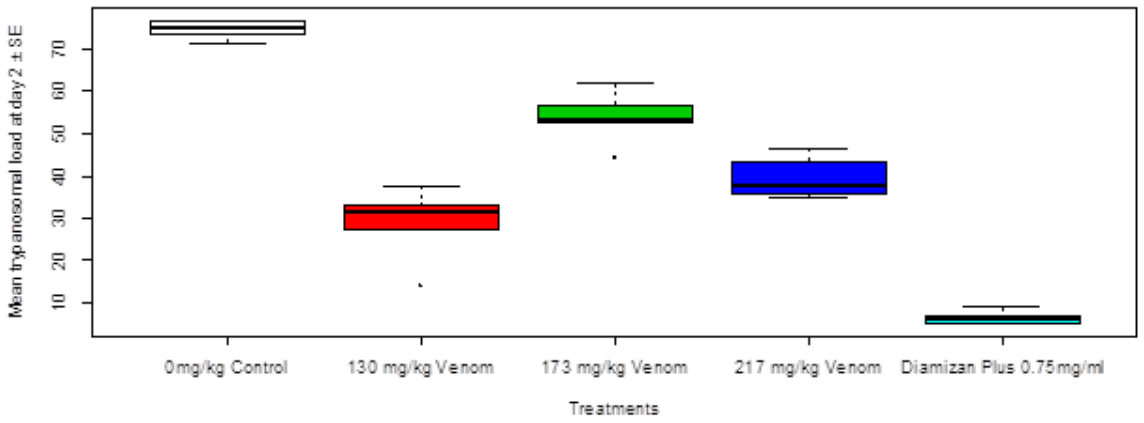

Figure 6. Mean trypanosomal load in Swiss mice in relation to treatments with toad venom and Diamizan plus treatments at day 2

Table 6. Turkey Multiple Comparisons of Means of Trypanosomal Load at 95% Family-Wise Confidence Level for Day Two

Treatment	Diff	Lower	Upper	P – Adjusted
130 mg/kg – 0 mg/kg	–46.02	–56.5505483	–35.489452	0.0000000
173 mg/kg – 0 mg/kg	–20.97	–31.5005483	–10.439452	0.0000705
217 mg/kg – 0 mg/kg	–35.07	–45.6005483	–24.539452	0.0000000
Diamizan Plus 0.75 mg/mL – 0 mg/kg	–68.32	–78.8505483	–57.789452	0.0000000
173 mg/kg – 130 mg/kg	25.05	14.5194517	35.580548	0.0000061
217 mg/kg – 130 mg/kg	10.95	0.4194517	21.480548	0.290893
Diamizane Plus 0.75 mg/mL – 130 mg/kg	–22.30	–32.8305483	–11.769452	0.0000313
217 mg/kg – 173 mg/kg	–14.10	–24.6305483	–3.569452	0.0055509
Diamizan Plus 0.75 mg/mL – 173 mg/kg	–47.35	–57.8805483	–36.819452	0.0000000
Diamizan Plus 0.7 5mg – 217 mg/kg	–33.25	–43.7805483	–22.719452	0.0000001

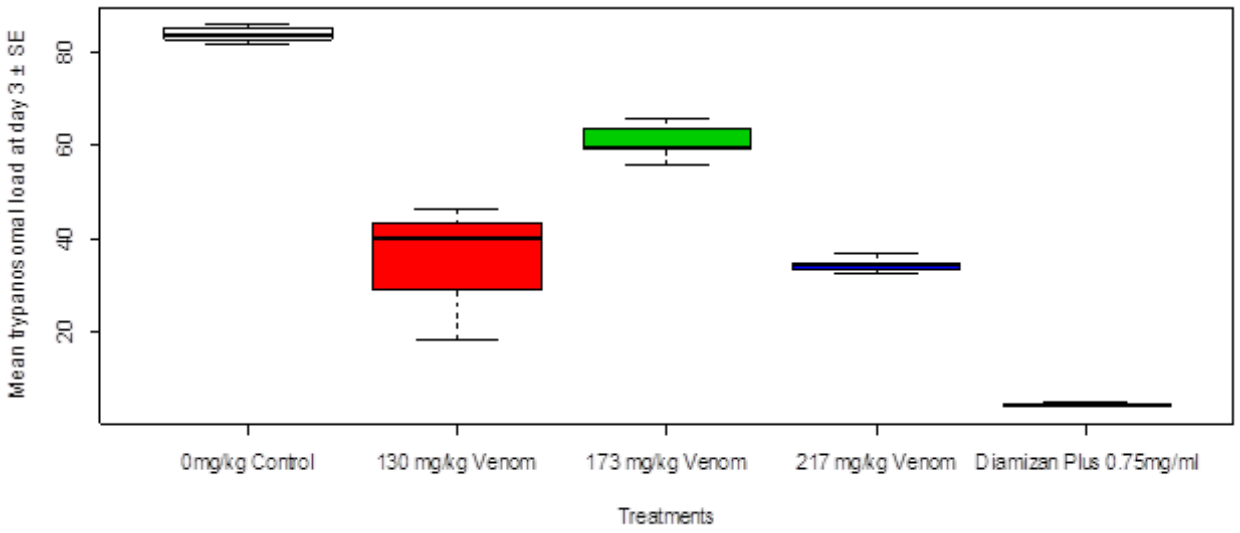

Figure 7. Mean trypanosomal load in Swiss mice in relation to treatments with toad venom and Diamizan plus treatments at day 3

Table 7. Turkey Multiple Comparisons of Means of Trypanosomal Load at Day 3

Treatment	Diff	Lower	Upper	P – Adjusted
130 mg/kg – 0 mg/kg	–48.45	–59.01312	–37.886875	0.0000000
173 mg/kg – 0 mg/kg	–23.00	–33.56312	–12.436875	0.0000214
217 mg/kg – 0 mg/kg	–49.50	–60.06312	–38,936875	0.0000000
Diamizan Plus 0.75 mg/mL – 0 mg/kg	–79.60	–90.16312	–69.036875	0.0000000
173 mg/kg – 130 mg/kg	25.45	14.88688	36.013125	0.0000051
217 mg/kg – 130 mg/kg	–1.05	–11.61312	9.513125	0.9981429
Diamizan Plus 0.75 mg/mL – 130 mg/kg	–31.15	–41.71312	–20.586875	0.0000002
217 mg/kg – 173 mg/kg	26.50	–37.06312	–15.936875	0.0000028
Diamizan Plus 0.75 mg/mL – 173 mg/kg	–56.60	–67.16312	–46.036875	0.0000000
Diamizan Plus 0.75 mg – 217 mg/kg	–30.10	–40.66312	–19.536875	0.0000004

3.8 Change in Body Weight of Swiss Mice after Treatment with Toad Venom and Diamizan Plus

After treatment with the toad venom crude extract of different dosages and Diamizan plus drug, change in body weight was highest in Group 4 treated with 0.75 mg/mL of Diamizan plus, followed by Group 3 treated with 217 mg/kg of toad venom, then Group 1 treated with 130 mg/kg of toad venom whereas it was least in Group 2 treated with 173 mg/kg of the toad venom. Thus, the mean change in body weight of Swiss mice after treatment in relation to toad venom as well as Diamizan plus drug respectively showed a high significant difference (Kruskal-Wallis χ^2= 15.779, df = 4, P = 0.00333, Figure 8). The multiple comparisons of means of change in body weight between 173 mg/kg treatment and Diamizan plus drug showed a significant difference (P< 0.05, Table 8).

3.9 Change in Haematological Parameters of Swiss Mice after Three Days of Parasitemia Treatment

Hemoglobin (Hb)

After treatment with the toad venom crude extract of different dosages and Diamizan plus drug, the result showed that there was no change in the blood concentration of hemoglobin of the Swiss mice in the different groups. However, the mean change in Hb level in Swiss mice after three days of trypanosomal treatment between dosages of toad venom and Diamizan plus drug showed no significant difference (Kruskal-Wallis χ^2 = 8.141, df = 4, P = 0.08655, Figure 9).

RBC

After treatment with the toad venom crude extract of different dosages and Diamizan plus drug, the result showed a change in the RBC level of the Swiss mice in the different treatment groups where group 4 had the highest level followed by group 3 then group 2 while group 1 had the least. Thus, the mean change in RBC level in Swiss mice after three days of trypanosomal treatment between dosages of toad venom and Diamizan plus drug showed a high significant difference (Kruskal-wallis χ^2 = 14.326, df = 4, P = 0.006324, Figure 10). Table 9 shows that multiple comparisons of means of change in RBC level between treatments between dosages of toad venom and Diamizan plus drug showed a high significant difference (P= 0.006324).

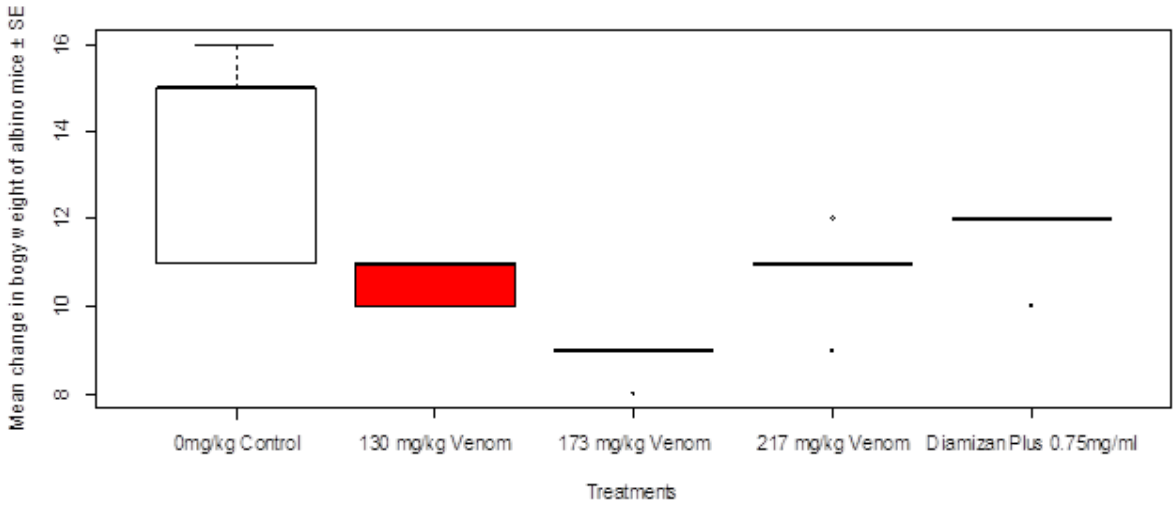

Figure 8. Mean change in body weight of Swiss mice after treatment with toad venom and Diamizan plus drug

Table 8. Turkey Multiple Comparisons of Means of Change in Body Weight Between Treatments

	Treatments			
	0 mg/kg	**130 mg/kg**	**173 mg/kg**	**217 mg/kg**
130 mg/kg	0.087	0	0	0
173 mg/kg	0.031	0.031	0	0
217 mg/kg	0.168	0.555	0.060	-
Diamizan Plus 0.75 mg/mL	0.428	0.128	0.031	0.219

P Value Adjustment Method: BH

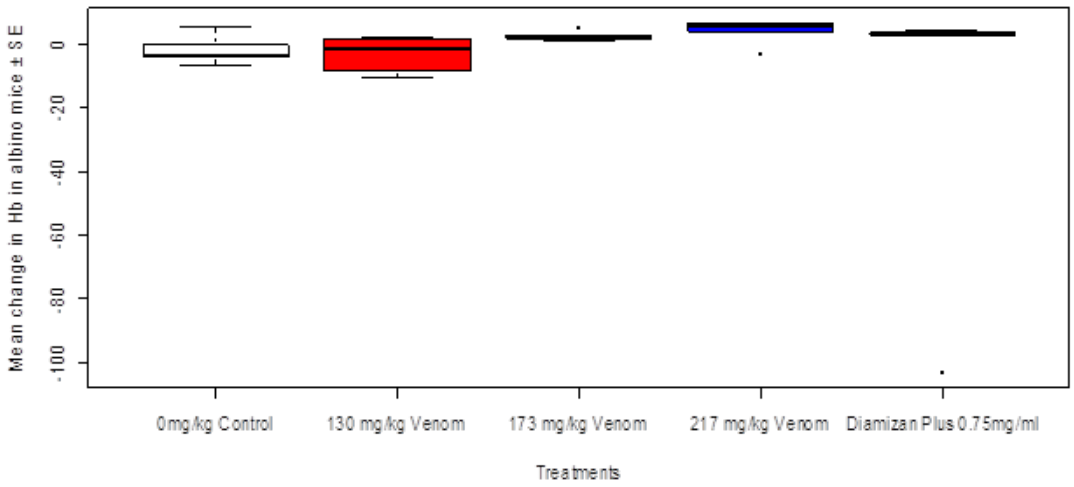

Figure 9. Mean change in hemoglobin level in Swiss mice in relation to treatments with toad venom and Diamizan plus drug after three days of trypanosomal inoculation

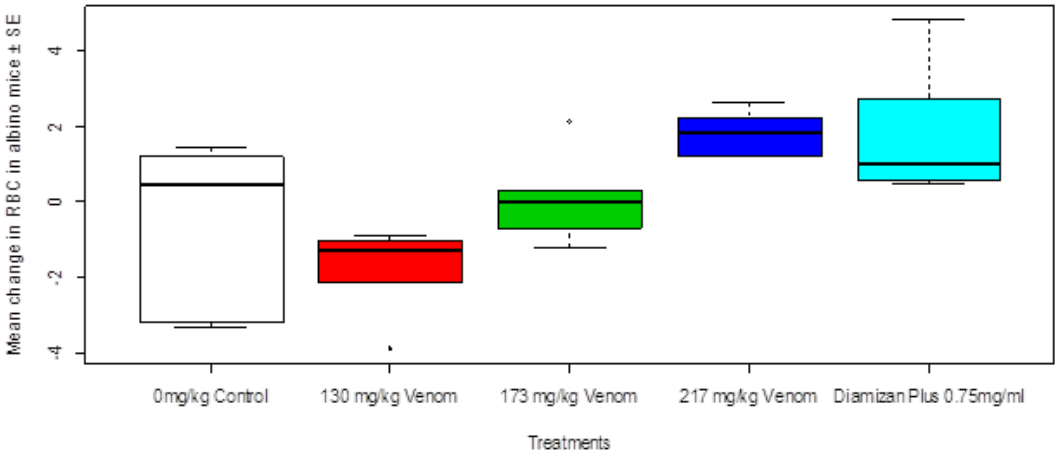

Figure 10. Mean change in RBC level in Swiss mice in relation to treatments with toad venom and Diamizan plus drug after three days of trypanosomal inoculation

Table 9. Turkey Multiple Comparisons of Means of Change in RBC Level between Treatments

	Treatments			
	0 mg/kg	**130 mg/kg**	**173 mg/kg**	**217 mg/kg**
130 mg/kg	0.526	0	0	0
173 mg/kg	1.000	0.079	0	0
217 mg/kg	0.099	0.060	0.099	0
Diamizan Plus 0.75 mg/mL	0.136	0.060	0.079	0.750

P Value Adjustment Method: BH

PCV

After treatment with the toad venom crude extract of different dosages and Diamizan plus drug, the result showed a change in the PCV level of Swiss mice in the various treatment groups in which group 3 had the highest PCV level, followed by group 4 then group 2 whereas group 1 had the lowest PCV level. Thus, the mean change in PCV level in Swiss mice after three days of trypanosomal treatment between dosages of toad venom and Diamizan plus drug showed a very high significant difference (Kruskal-Wallis $\chi^2 = 18.513$, df = 4, P = 0.0009793, Figure 11). Table 10 shows multiple comparisons of means of change in PCV level between treatments which the highest dosage of toad venom versus Diamizan plus drug showed no significant difference (P = 0.317).

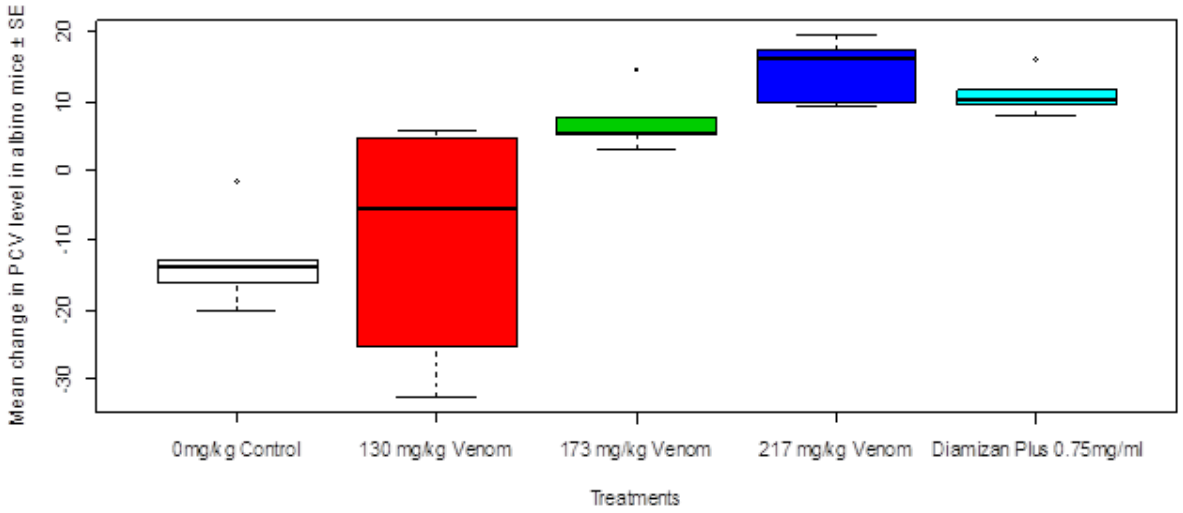

Figure 11. Mean change in PCV level in Swiss mice in relation to treatments with toad venom and Diamizan plus drug after three days of trypanosomal inoculation

Table 10. Turkey Multiple Comparisons of Means of Change in PCV Level Between Treatments

	Trypanosoma Load and Treatments			
	0 mg/kg	**130 mg/kg**	**173 mg/kg**	**217 mg/kg**
130 mg/kg	0.841	0	0	0
173 mg/kg	0.026	0.136	0	0
217 mg/kg	0.026	0.026	0.053	0
Diamizan Plus 0.75 mg/mL	0.032	0.032	0.139	0.317

P Value Adjustment Method: BH

4. Discussion

4.1 Bioactive Compounds Present in Bufonidae Toad Venom Crude Extract

The toad's parotoid secretions have been reported to be of a high chemical complexity. They have diverse types of biomolecules as recorded in this study, such as proteins, chemical components and elements, peptides, biogenic amines, and alkaloids and other unidentified classes. Previous studies had established that, bufadienolides have several activities against bacteria, fungi, protozoa, virus, HIV, cancer cells and some of its components identified as modulators of blood coagulation, neurotransmission, analgesic, and anti-inflammatory [18-21].

4.2 Biochemical Compounds Present in the Toad Venom Crude Extract

Using GC-MS techniques, compounds identified in this study like n-Hexadecanoic acid; 9,12-Octadecadieonic acid (Z,Z); 9,17-Octadecadienal (Z); Hexadecanoic acid 2-hydroxy-1-(hydroxymethyl) ethyl ester, are in harmony with the findings of Ajanaku *et al*. [22] who reported that the methanolic extract of the leaf of *C. adansonii* having the same compounds, has cancer preventive, anti-microbial, anti-fungal and other therapeutic properties which could suppose that toad venom crude extract equally possesses anti-trypanosomal therapeutic potentials as observed by Aparna *et al*. [23] and Sanni and Omotoyinbo [24].

4.3 The Protein and Non-Protein Functional Groups Present in the Toad Venom Crude Extract

According to Sakate and Oliveira [25], the various components that make up the toad venom can be divided into basic compounds (biogenic amines) and steroid derivatives. Consequently, the biochemical analysis using FTIR procedures employed in this study revealed the presence of amines like aniline and triethylamine which is in agreement with the earlier discoveries of Andrade *et al.* [26] who reported that amino acids and polyamines are established membrane transporters in *T. cruzi*. These findings apparently suggest that the amino-group (aniline

and triethylamine) were easily absorbed by *T. brucei brucei* and could be potential therapeutic targets due to the anti-trypanosomal activity of the toad venom crude extract [27]. Tempone *et al*. [28] reported antiparasitic activity of the steroids isolated from toad venom crude extract and only hellebrigenin from the biogenic amines was active against trypomastigote of *T. cruzi*, which could be attributed to the activity against *T. brucei brucei* as seen in this study.

4.4 The Chemical Elements Present in the Toad Venom Crude Extract

Except for lead (Pb) and aluminium (Al), every other element identified in this study using SEM-EDS are essential for the human body, and this agrees with the findings of Lingamaneni *et al*. [29] who stated that these elements function in the stabilization of cellular structures (immune systems) at normal levels but in deficiency, may stimulate alternate pathways and cause diseases. Thus, these elements could be considered to be chemotherapeutic and chemo-preventive agents which stimulate the immune systems in the treatment of other diseases and may be, trypanosomal infection as demonstrated in this study. However, variation in the toad's diet can influence the molecules uptake by feeding, thereby modifying the secretion composition as well as activity. Thus, this requires further and sustained research in diverse locations, looking for constituents of toad venoms from various species [30].

4.5 Oral Toxicity (LD_{50}) of the Toad Venom Crude Extract on the Laboratory Swiss Mice

Prior to the mortality of the laboratory animals, symptoms such as hyperactivity, convulsion and constant stooling were observed which suggests that the toad venom extract was lethal and even up to 1000 mg/kg after an hour, however on the administration of 750 mg/kg, hyperactivity, diarrhea and sedation were observed within one hour, but there was no mortality recorded. Following the Lorke's method computation, the oral toxicity of the toad crude venom extract was established as 866 mg/kg. This finding agrees with Tubaro *et al.* [31] who investigated the acute oral toxicity of a new palytoxin congener in mice which induced scratching, jumping, respiratory distress, cyanosis, paralysis and death in mice within 24 hours but not in accordance with Al-Afifi *et al*. [32] who reported that *Dracaena cinnabari* resin methanol extracts in rats, showed no treatment related mortality.

4.6 Pre-patency Period of *Trypanosoma brucei brucei*

The lack of variation observed at day zero prior to treatment in the mean of trypanosomal load in Swiss mice in relation to treatments with toad venom and diamizan plus drug respectively could be because the Swiss mice were yet to receive any form of treatment at this stage. The results possibly suggests that the pre-patent period of *Trypanosoma brucei brucei* was 3 days, which is in line with the findings by Turay *et al*. [33] and Udensi and Fagbenro-Beyioku [34].

4.7 Trypanosomal Load in Swiss Mice in Relation to Treatments with Toad Venom and Diamizan Plus Drug

The observed variation in the three (3) days pooled trypanosomal load in Swiss mice in relation to treatments possibly suggests that the standard drug (diamizan plus) is effective. This is in consonance with the finding of Ezeh *et al*. [35] who reported that treatment with Diminazene aceturate (Berenil) resulted in the reduction of parasitemia load in infected mice after two days of treatment. Although the toad venom did not actively reduce the *Trypanosoma* parasitaemia as much as diamizan plus drug did when both of their mean load was compared, however, the highest dosage of toad venom treatment still yielded over 50% reduction in trypanosomal load in Swiss mice in comparison with the control group. This suggests that the toad venom has the potential to relatively reduce trypanosomal load in the Swiss mice which corroborates with Tempone *et al*. [28] who reported antiparasitic activity of steroids (telocinobufagin and hellebrigenin) from toad venom crude extract and also leishmanicidal activity against *L. infantum* promastigotes with IC50 of 126.2 μg/mL and 61.2 μg/mL. This report possibly suggests that the activity of the steroids may involve mitochondrial degradation and perturbation of the parasite membrane, resulting in cell death. These results indicate the range of biochemical molecules and possible applications.

The observed variation after 24 hours (day one) of treatment in the mean trypanosomal load in Swiss mice in relation to treatments with toad venom and diamizan plus perhaps suggests that the diamizan plus drug was more effective in reducing trypanosomal load in the infected Swiss mice. The toad venom did not successfully reduce the trypanosomal load in the mice as evident in Figure 5. With respect to the highest dosage of toad venom and diamizan plus treatments at day one, the high significant difference recorded suggests that although the toad venom was able to reduce the trypanosomal load in the mice, diamizan plus drug actively and considerably reduced the trypanosomal parasitaemia in the Swiss mice examined. Freiburghaus *et al.* [36] have shown that the mean minimum inhibitory concentration value of common trypanocidal drugs is 10.7 mg/mL and that agent with minimum

inhibitory concentration value between 5 mg/mL ~ 20 mg/mL could be regarded as very active.

The high difference detected after 48 hours (day two) of treatment in the mean of trypanosomal load in Swiss mice in relation to treatments with toad venom and diamizan plus drug possibly connotes that the toad venom did not successfully reduce the trypanosomal parasitaemia in the mice models as much as diamizan plus drug did. This is possibly because of poor inactivation of the active components contained in toad venom. Thoughvariation was observed between the mean trypanosomal load of toad venom treatment and control group, which possibly suggests that the toad venom treatment could be used to reduce *Trypanosoma* parasitaemia. The present finding is not in agreement with Habila *et al*. [37] who examined the anti-trypanosomal potentials of *A. indica* seeds methanolic extract against *T. evansi* at 25 mg/mL, 50 mg/mL and 100 mg/mL and immobilized the parasites within 14 mins, 8 mins and 3 mins, respectively.

The 72 hours (day 3) decline in the trypanosomal load in Swiss mice in relation to treatments suggests that *Trypanosoma* become relatively susceptible to bufonid product over a longer period.

4.8 Impact of Trypanosomes on Body Weight of Swiss Mice after Three Days of Treatment

Body weight of Swiss mice after a three (3) day treatment in relation to toad venom as well as diamizan plus drug respectively was much more a positive increase in body weight for group treated with diamizan plus drugs than the toad venom groups. But, the change in the mean body weight in control (untreated) group went the negative direction. Kifleyohannes *et al*. [38] reported that the weight in the untreated infected mice group started to decrease after 12 days post infection till all the mice died by day 18 where as those standard drug and extract of *A. absinthium* and *M. stenopetala* treated mice generally showed a gradual increase in mean weight until the end of the experimental period. The finding is also in agreement with finding of Tadesse *et al*. [39] who found out that the treatment with the crude extracts of *A. absinthium* and *D. abyssinica* prevented loss in body weights, particularly at higher doses. The aqueous and methanolic extracts of *V. sinaiticum* were capable of improving body weight of treated animals on day 8–14 as compared to the untreated control group [40]. On the other hand, reports by Ngure *et al*. [41] showed that extract of *A. indica* and suramin-treated animal groups had a significant decline in body weight.

4.9 Impact of Trypanosomes on Haematological Parameters of Swiss Mice after Three Days of Parasitemia Treatment

Haemoglobin (Hb)

The lack of variation observed in the mean change in Hb level in Swiss mice after three days of trypanosomal treatment between dosages of toad venom and diamizan plus drug respectively suggests that the treatments has some haematopoetic property, and also trypanosomal infection in the Swiss mice has no effect on haemoglobin. This result is not in discordance with Alli *et al*. [42] who recorded high Hb concentration when he compared group treated with diminazene and *M. lucida* leaf extract increased haemoglobin.

Red Blood Cells (RBC)

The observed high variation in the mean change in RBC level in Swiss mice after three days of trypanosomal treatment between dosages of toad venom and diamizan plus drug possibly suggests that the presence of trypanosomes in the Swiss mice had a major effect on the RBC of the mice. It equally suggests that if left untreated, the impact of the infection on the mice would possibly be deleterious. This result is in consonance with the finding of Alli *et al.* [42] who recorded lowest RBC count in the group infected but not treated and this is in keeping with trypanosomal infection.

Packed Cell Volume (PCV)

The negative in the mean change in PCV level of the control (untreated) wiss mice group after three days of possibly suggests that the trypanosomes had a negative impact on the immunity of the mice with resultant effect on PCV. The trypanosomes affect the immune-response of animals as observed in this study leading to anaemia which is the most outstanding clinical and laboratory feature of African trypanosomiasis and the primary cause of death [43].

The PCV of the mice treated with the toad venom crude extracts and diamizan plus drug stayed within constant range. This result illustrates that toad venom crude extract has the capacity to improve the PCV even if it declines after relapse of parasites. The finding is in agreement with finding of Abubakar *et al*. [44] who reported *M. balsamina* and *S. longipenduculata* possess the highest anti-trypanosomal potential since they are able to control anemia by resisting sudden drop in PCV level.

The mean PCV between diamizan plus drug and different dosages of toad venom crude extract were relatively comparable which is in agreement with Kifleyohannes *et al*. [38] and Tadesse *et al*. [39] who studied on *A. absinthium* and *D. abyssinica* respectively in which PCV level was significantly improved with comparable potential to diminazene aceturate since they are able to control anemia as well as minimize the decline in PCV level.

5. Conclusions

Results of the in-vivo anti-trypanosomal activities of the toad venom crude extract on laboratory mice showed that the overall parasitemia was highest in the control group and the least parasitemia was observed in the group treated with Diamizan Plus drug. Toad venom has the potential to relatively reduce the *Trypanosoma* infection in Swiss mice as observed in one of the toad venom treatment group (130 mg/kg) where trypanosome parasitemia was about thrice less than the control group. The 3-day treatment shows that trypanosomal load in Swiss mice have a negative impact on the RBC and PCV levels. There is a need for further studies (both in-vivo and in-vitro) based on the output on trypanosomal potential of bufonidae product recorded in this study. Also, research should be conducted on the bio-active compounds of toad venom crude extract and their pharmacological activity.

Conflict of Interest

There is no conflict of interest.

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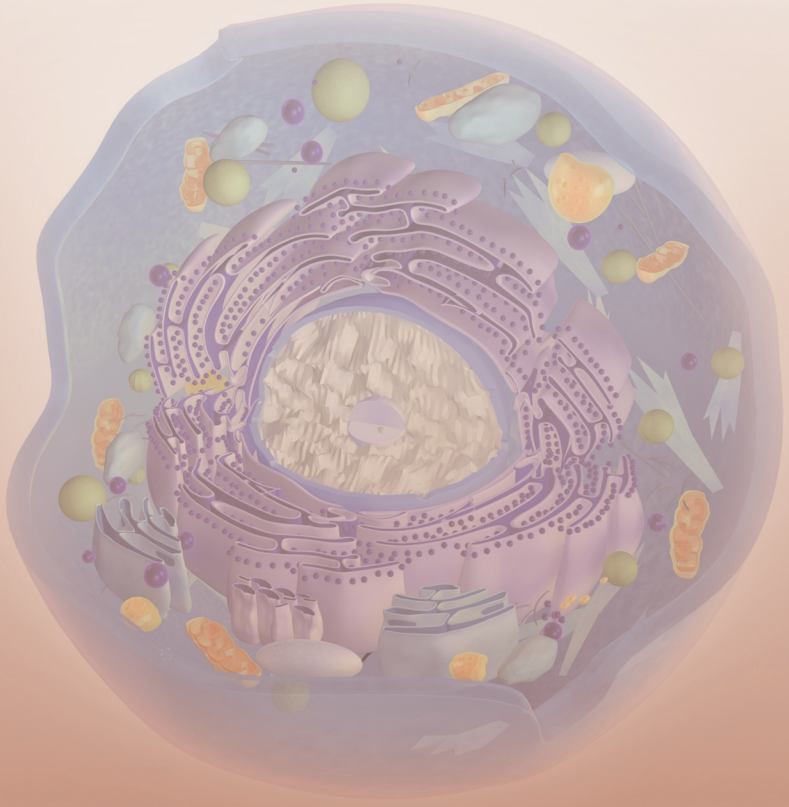

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