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Nr albumu: 247928

Long-term dynamic changes in the haemoparasites community, and description and characterization of a novel *Babesia* species and co-infecting blood parasites, of rodents from the Sinai massif (Egypt)

Rozprawa doktorska
w zakresie nauk biologicznych w
dyscyplinie: ekologia

Praca wykonana pod kierunkiem
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Warszawa, grudzień 2016

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Data Podpis autora pracy

Słowa kluczowe

Babesia, Bartonella, Haemobartonella, Hepatozoon, Trypanosoma, Acomys, Dipodillus, dynamic changes, Sinai, Egypt.

Wieloletnia dynamika zarażeń, opis i charakterystyka nowego gatunku Babesia i współwystępujących pasożytów krwi u gryzoni z Masywu Synaju (Egipt)

Szczególne podziękowania kieruję do mojej promotor,
profesor Anny Bajer, za niezłomną pomoc, motywację i wsparcie.
Dzięki jej zaangażowaniu i charyzmie udało się to, co wydawało się niemożliwe.
Prof. Jerzemu Behnke dziękuję za poświęcony czas i cierpliwość oraz służenie radą.
Dziękuję dr Renacie Welc-Falęciak za pomoc, życzliwość i cenne rady.
Dziękuję wszystkim tym, którzy byli przy mnie przez ostatnie 11 lat
i niezawodnie mnie wspierali.
Finally I thank my Mom, who always gives me unconditional support, my aunt Najeha and my brothers (Ali and Mustafa) who are my most faithful supporters.

Niniejszą pracę dedykuję mojemu Ojcu

Spis treści

1. Streszczenia

Wieloletnie parazytologiczne badania środowiskowe pozwalają na szeroki wgląd w ekologiczne i ewolucyjne kształtowanie się układu pasożyt-żywiciel (Alsarraf et al., 2016; Bajer et al., 2014b). Małe ssaki, a w szczególności gryzonie, są dobrymi podmiotami takich badań, ponieważ ich populacje są bardzo liczne, heterogenne i bardzo zależne od czynników środowiskowych, włączając w to dostęp do pożywienia oraz warunki klimatyczne (Bujalska and Hansson, 2000; Flowerdew JR, 1984; Turner et al., 2014). Zespół pasożytów krwi gryzoni jest dobrze zbadany na terenie Europy, Ameryki Północnej, Ameryki Południowej i Azji, natomiast niewiele informacji na ten temat pochodzi z terenu Afryki. Zgrupowania hemopasożytów występujących u gryzoni egipskich, takich jak kolcomysz arabska (*Acomys dimidiatus)*, kolcomysz złota (*Acomys russatus*) i myszoskocz arabski (*Dipodillus dasyurus*) podlegają zmianom w zależności od szeregu czynników i złożonych oddziaływań pomiędzy nimi (Alsarraf et al., 2016). Czynnikami biorącymi udział w kształtowaniu zgrupowań pasożytów u gryzoni z terenów pustynnych i półpustynnych Egiptu mogą być czynniki zewnątrzpopulacyjne, takie jak zespół unikalnych czynników środowiskowych (m.in. wilgotność i temperatura), charakterystycznych dla danego roku badań (dalej opisywany jako zmienna 'rok badań'), czy miejsce odłowu żywicieli (powierzchnia badawcza, dalej opisana jako zmienna „dolina badań" lub „Wadi'). Innymi czynnikami odpowiedzialnymi za strukturę zgrupowań pasożytów u gryzoni są czynniki wewnątrzpopulacyjne, do których należą, między innymi, płeć i wiek żywiciela. Na podstawie wymiarów i masy ciała oraz dojrzałości płciowej żywiciela, odłowione gryzonie zostały przypisane do trzech klas wiekowych (osobniki młodociane, młode dorosłe, dorosłe).

Badano wpływ wymienionych czynników wewnątrz- i zewnątrzpopulacyjnych na występowanie 5 rodzajów pasożytów krwi: *Babesia*, *Bartonella*, *Haemobartonella* (*Mycoplasma*), *Hepatozoon* i *Trypanosoma* u kolcomyszy arabskiej (*A. dimidiatus*) z Masywu Synaju. Wieloletnie badania (2000-2012) w czterech dolinach odizolowanych od siebie łańcuchami górskimi w okolicy miasta Świętej Katarzyny na Masywie Synaju (Egipt) rozpoczęto na przełomie sierpnia i września 2000 r. i powtarzano co 4 lata.

Moją rozprawę doktorską podzieliłem na dwie części; pierwszą stanowiły badania nad wieloletnią dynamiką występowania hemopasożytów u kolcomyszy arabskiej (*A. dimidiatus*)

reprezentatywnego gatunku gryzoni z gór Synaju. Drugą część badania stanowił opis nowego gatunku *Babesia*, wykrytego u myszoskocza *D. dasyurus.*

Postawiłem następujące hipotezy:

1. W warunkach pustynnych i półpustynnych można zaobserwować wieloletnią dynamikę występowania pasożytów krwi u gryzoni w Masywie Synaju.
2. W czterech odizolowanych dolinach, zasiedlonych przez podobne zgrupowania gryzoni, możemy zaobserwować różne zespoły pasożytów krwi, a izolacja populacji żywicieli może wpływać na różnorodność genetyczną i specyficzność żywicielską pasożytów.
3. Czynniki wewnątrzpopulacyjne (wiek i płeć żywiciela) mają wpływ na ekstensywność i intensywność zarażenia pasożytami krwi u gryzoni.
4. Niezbadane gatunki gryzoni mogą być zarażone nieopisanymi dotychczas pasożytami krwi.

Tak postawione hipotezy dotyczące wieloletniej dynamiki zarażeń hemopasożytami u kolcomyszy arabskiej zostały zweryfikowane przez realizację następujących celów badawczych:

1. Określenie dynamiki zespołu pasożytów krwi u *A. dimidiatus* na przestrzeni 12 lat na podstawie danych uzyskanych na temat ekstensywności, intensywności i bogactwa gatunkowego zarażeń w badanych populacjach gryzoni w poszczególnych latach.
2. Określenie różnorodności genetycznej i specyficzności żywicielskiej pasożytów krwi gryzoni w odizolowanych od siebie dolinach górskich masywu Synaju.
3. Określenie różnorodności genetycznej i stopnia pokrewieństwa między gatunkami pasożytów krwi u *Acomys dimidiatus.*
4. Opisanie i charakterystyka nowych gatunków pasożytów występujących u innych gryzoni.

Gryzonie odławiane były przez 4-5 tygodni na przełomie sierpnia i września w latach 2000, 2004, 2008 i 2012 w pobliżu miasta Święta Katarzyna w Górach Synaj (Egipt). Odłowy prowadzono na terenie czterech dolin górskich (Wadi Arbaein, W. Gebal, W. Gharaba i W. Tlah), odizolowanych od siebie łańcuchami górskimi. Gryzonie (N=1041, 857 kolcomysz arabskich, 73 kolcomyszy złotych i 111 myszoskoczów arabskich) były odławiane za pomocą pułapek żywołownych Shermana i oznaczone co do gatunku, płci oraz przypisane do odpowiedniej klasy wiekowej. Pobierano próbki krwi bezpośrednio z serca do EDTA w celu izolacji DNA oraz wykonania dwóch rozmazów krwi do analizy mikroskopowej. Pasożyty krwi obserwowałem pod mikroskopem świetlnym i zliczałem zarażone komórki krwi na 200 polach widzenia przy powiększeniu 1000x w przypadku *Babesia, Bartonella, Haemobartonella*

i *Trypanosoma*. Natomiast w przypadku *Hepatozoon* zliczałem leukocyty zarażone w puli 50 kolejnych leukocytów w celu określenia ekstensywności, intensywności oraz bogactwa gatunkowego pasożytów krwi. DNA pasożytów wyizolowałem za pomocą zestawu DNAeasy Blood & Tissue kit (Qiagen, USA) albo MiniPrep Blood kit (AxyGen, USA). Do analiz molekularnych i filogenetycznych wykorzystałem marker 18S rRNA w celu określenia różnorodności genetycznej u *Hepatozoon* [fragment genu o wielkości 660 pz; (Inokuma et al., 2002)] i *Trypanosoma* [fragment genu 520 pz; (Noyes et al., 1999)], a także fragment genu *rpoB* w przypadku *Bartonella* [o wielkości 333 pz; (Paziewska et al., 2011)]. W celu opisania nowego gatunku *Babesia behnkei* wykorzystałem 18S rRNA [1700 pz; (Matjila et al., 2008; Oosthuizen et al., 2008)] oraz rejony ITS1 [615 pz; (Blaschitz et al., 2008; Nijhof et al., 2003)] i ITS2 [315 pz;(Blaschitz et al., 2008; Nijhof et al., 2003)].

Część I: wieloletnia dynamika zarażeń hemopasożytami u kolcomyszy arabskiej *A. dimidiatus* (Alsarraf *et al.* 2016)

Do realizacji celów 1-3 zbadałem 835 kolcomyszy arabskich. Ogólna ekstensywność zarażenia pasożytami krwi u *A. dimidiatus* wynosiła 76.2%, przy czym zaobserwowałem istotny spadek ekstensywności zarażenia hemopasożytami na przestrzeni lat – najwyższą ekstensywność zarażenia odnotowałem w roku 2004 (91.2%), natomiast najniższa była w roku 2012 (53%). Zaobserwowałem różnicę w ekstensywności zarażenia hemopasożytami pomiędzy populacjami kolcomyszy arabskich z różnych dolin badań – najwyższą ekstensywność odnotowałem w W. Tlah (88.5%), a najniższą w W. Arbaein – 68.8%.

Wieloletnia dynamika zarażenia *Babesia* sp. w populacjach *A. dimidiatus* z czterech dolin

Ogólna ekstensywność zarażenia *Babesia* sp. wynosiła 3.1%. Badania dynamiki występowania *Babesia* sp. u *A. dimidiatus* wykazały ogólny spadek w ekstensywności zarażenia na przestrzeni lat. Najwyższą ekstensywność zarażenia *Babesia* odnotowałem w 2004 roku – wynosiła 6.9% i spadła do 0% w 2012 r. Odnotowałem różnice w dynamice zarażeń *Babesia* u kolcomyszy arabskiej między dolinami. Ekstensywność zarażenia *Babesia* sp. u *A. dimidiatus* – najwyższy odsetek zarażenia (8.2%) odnotowałem w W. Gebal, a najniższy (0.5%) w W. Gharaba.

Wieloletnia dynamika zakażenia *Bartonella* sp. w populacjach *A. dimidiatus* z czterech dolin

Ogólna ekstensywność zakażenia *Bartonella* sp. wynosiła 3.6%. Badania dynamiki występowania *Bartonella* sp. u *A. dimidiatus* wykazały zmiany ekstensywności zakażenia na przestrzeni lat. Najwyższą ekstensywność zakażenia *Bartonella* (8.3%) odnotowałem w roku 2004, a najniższą (0.8%) w roku 2008. Najwyższą ekstensywność zakażenia *Bartonella* sp. w zależność od dolin badań odnotowałem na poziomie 6.9% w populacji z W. Gebal, a najniższą (1.6%) w W. Gharaba. Badania dynamiki występowania *Bartonella* sp. u *A. dimidiatus* wykazały różnice między dolinami gdzie odnotowałem wzrost ekstensywności zakażeń *Bartonella* sp. w W. Arbaein i spadek występowania we wszystkich innych dolinach na przestrzeni 12 lat badań.

Wieloletnia dynamika zakażenia *Haemobartonella* sp. w populacjach *A. dimidiatus* z czterech dolin

Wśród zbadanych pasożytów najczęściej występował rodzaj *Haemobartonella* sp. Ogólna ekstensywność zakażenia wynosiła 57.8%. Zaobserwowałem ogólny spadek w zarażeniu *Haemobartonella* sp. na przestrzeni lat – najwyższy odsetek wyniósł 85.2% w roku 2004, a najniższy - 27.9% w roku 2012. Badania dynamiki występowania *Haemobartonella* sp. u *A. dimidiatus* wykazały różnice między dolinami gdyż odnotowałem najwyższą ekstensywność zakażenia *Haemobartonella* sp. w W. Gebal –71.7%, a najniższą w W. Gharaba (44.8%).

Wieloletnia dynamika zarażenia *T. acomys* u *A. dimidiatus* w zależności od roku, dolin badań i wieku żywiciela oraz analiza molekularna i filogenetyczna pasożytów

Ogólna ekstensywność zarażenia *T. acomys* wyniosła 15.8%. Najwyższą ekstensywność zarażenia (22.7%) odnotowano w roku 2004, a najniższą (11.9%) w roku 2012. Ekstensywność zarażenia *T. acomys* była najwyższa w W. Tlah (34.8%) i W. Gharaba (21.9%), zaś najniższa w W. Arbaein (0.4%). Najwięcej zarażonych *T. acomys* osobników stwierdziłem w drugiej klasie wiekowej (26.6%), a najniższy odsetek zarażeń- u dorosłych z trzeciej klasy wiekowej (10.4%).

Analiza molekularna i filogenetyczna 45 izolatów *T. acomys* z wykorzystaniem markera 18S rDNA pozwoliła wyróżnić dwa warianty – A i B. Wariant A *T. acomys* wykryto u 44 kolcomyszy, a wariant B tylko w jednym izolacie DNA. Różnica między wariantami wynosiła 7 nukleotydów. Analiza filogenetyczna wykazała, że oba warianty *T. acomys* grupują się

z innymi gatunkami *Trypanosoma* z różnych żywicieli i z różnych części świata, tworząc grupę monofiletyczną. Reprezentatywne sekwencje tych wariantów zostały wprowadzone do bazy GenBank NCBI jako pierwsze sekwencje tego gatunku.

Wieloletnia dynamika zarażenia *Hepatozoon* sp. u *A. dimidiatus* w zależności od roku i dolin badań oraz analiza molekularna i filogenetyczna reprezentatywnej grupy izolatów z *A. dimidiatus* i *A. russatus*

Ogólna ekstensywność zarażenia *Hepatozoon* sp. u *A. dimidiatus* wyniosła 29.7%. Najwyższą ekstensywność zarażenia (40%) odnotowałem w roku 2008 a najniższą (20.6%) w roku 2000. Był to jedyny pasożyt, dla którego zaobserwowano wzrost zarażenia na przestrzeni 12 lat badań. Większość zarażeń *Hepatozoon* sp. wykrywano w dwóch dolinach, W. Gharaba i W. Tlah, podobnie jak zarażenia *T. acomys*, co jest związane z występowaniem wektorów – pcheł *Parapulex chephrensis*, w tych dwóch dolinach (Bajer A., 2006). Najwyższą ekstensywność zarażenia odnotowałem w W. Tlah (58.9%) a najniższą w W. Gebal (6.3%).

Uzyskane sekwencje 18S rDNA *Hepatozoon* sp. z Egiptu porównywałem z innymi sekwencjami z bazy GenBank, w tym z sekwencjami *H. erhardovae,* występującego u nornicy rudej w Polsce (Alsarraf, 2012; Bajer et al., 2014b).

W celu określenia poziomu zmienności międzygatunkowej. Po przeprowadzeniu analiz molekularnych i filogenetycznych, wyróżniłem wśród swoich prób dwa warianty fragmentu genu 18S rDNA: A i B. Wariant A był reprezentowany przez 37 sekwencji i wykazał podobieństwo/ homologię rzędu 96.06% do *H. ayorgbor* z pytona z Ghany. Wariant A był szeroko rozpowszechniony i występował we wszystkich dolinach u *A. dimidiatus* i *A. russatus.* Drugi wyróżniony wariant B (N=2) wykazał podobieństwo na poziomie 96.75% do *H. ayorgbor*. Wariant B występował tylko u kolcomyszy złotej *A. russatus* w W. Tlah w 2012 r. i W. Gharaba w 2004 r. Warianty A i B *Hepatozoon* różniły się 11 nukleotydami w porównywanym fragmencie genu, a analiza filogenetyczna otrzymanych sekwencji wykazała, że tworzą jedną parafiletyczną grupę, która była odrębna od wszystkich innych gatunków *Hepatozoon.* Ze względu na niskie podobieństwo do opisanych gatunków *Hepatozoon* i brak sekwencji referencyjnych z *H. acomys* z rodzaju *Acomys,* opisanego na podstawie cech morfologicznych przez Mohammeda i Saounda (1972) za (Smith, 1996), nie jestem na razie w stanie określić, czy jest to nowy gatunek z rodzaju *Hepatozoon,* czy jest to właśnie *H. acomys*. Wymaga to dalszych badań.

Część II: Opis nowego gatunku *Babesia* (*Babesia behnkei*) u *D. dasyurus* (Bajer et al. 2014)

Obserwacje mikroskopowe rozmazów krwi myszoskocza pozwoliły wyróżnić trofozoity *Babesia* sp., które były mniejsze od trofozoitów *B. microti* King's College, referencyjnego szczepu utrzymywanego w myszach szczepu BALB/c w naszym zakładzie. Ze względu na rozmiar trofozoitów początkowo gatunek zaklasyfikowałem do rodzaju *Theileria*, jednakże przeprowadzone przeze mnie analizy molekularne i filogenetyczne pozwoliły ustalić, że jest to nowy gatunek *Babesia,* odrębny od rodzaju *Theileria* i znanych gatunków *Babesia*. Pasożyty te występowały tylko u myszoskocza *Dipodillus dasyurus* w dwóch dolinach W. Arbaein i W. Gebal, a ogólna ekstensywność zarażenia tego gatunku gryzonia była wysoka i wynosiła 39% (Bajer et al., 2014a).

W celu opisania gatunku *B. behnkei,* zmierzyłem średnicę trofozoitów *B. behnkei* (N=212) i porównałem z wymiarami trofozoitów *B. microti* King's College. Średnica trofozoitu *B. behnkei* wynosiła 1.26 μm, a *B. microti* 1.46 μm. Analiza molekularna i filogenetyczna z wykorzystaniem 3 markerów genetycznych, prawie całego genu 18S rRNA (1700 pz), rejonu ITS1 (615 pz) i rejonu ITS2 (315 pz), wykazała odrębność genetyczną tego pasożyta od wszystkich innych opisanych gatunków *Babesia*. Na podstawie analiz 18S rDNA, *B. behnkei* wykazała pewne podobieństwo (96% homologii) do *B. lengau* z geparda z Południowej Afryki. Analiza filogenetyczna wykazała, że *B. behnkei* zgrupowała się z gatunkami *Babesia* o afrykańskim i amerykańskim pochodzeniu, w tzw. grupie Duncani, zawierającej także gatunki patogenne dla ludzi (Bajer et al., 2014a; Lack et al., 2012). Analizy filogenetyczne rejonów ITS1 i ITS2 dały bardzo podobny wynik do analizy 18S rDNA. *Babesia behnkei* jest trzecim po *B. microti* i *B. rodhaini* gatunkiem *Babesia* występującym u gryzoni.

Dyskusja

Główną rolę w kształtowaniu zgrupowań pasożytów krwi u gryzoni pełniły czynniki zewnątrzpopulacyjne. Zaobserwowałem wyraźną dynamikę wieloletnią zarażeń oraz różnice w ekstensywności i intensywności zarażeń oraz w średnim bogactwie gatunkowym zespołu pasożytów pomiędzy izolowanymi dolinami/ populacjami na przestrzeni 12 lat. Natomiast powodem ogólnego spadku badanych parametrów (ekstensywności, intensywności i bogactwa gatunkowego) w ciągu 12 lat mogła być przewlekła susza, objawiająca się wyraźnym zmniejszeniem opadów. Odnotowana średnia opadów deszczowych to 42.5 mm/rok w latach 1970-1994, natomiast w latach 2001-2009 średnia opadów wynosiła tylko 15.5 mm/rok (Alsarraf et al., 2016). Podczas ostatnich wypraw badawczych 2008 i 2012 zaobserwowaliśmy

degradację pokrycia roślinnością i wysychanie/ pustynnienie ogrodów należących do Beduinów w tych dolinach. Te zmiany klimatyczne, szczególnie brak wody, mogą wpływać negatywnie nie tylko na populację gryzoni, ale również na przeżycie wektorów hemopasożytów, a więc i na spadek transmisji.

Zaobserwowałem występowanie *T. acomys* i *Hepatozoon* sp. najczęściej w dwóch dolinach, W. Gharaba i W. Tlah. Powodem tego może być obecność potencjalnego wektora – pchły z gatunku *Parapulex chephrensis,* która występowała najczęściej u kolcomyszy arabskich z W. Gharaba i Tlah, i była stwierdzona tylko jednorazowo w W. Arbaein w roku 2000 u dwóch osobników kolcomyszy (Alsarraf et al., 2016; Bajer A., 2006).

Z czynników wewnątrzpopulacyjnych wiek żywiciela miał istotny wpływ na ekstensywność i intensywność zarażenia *Hepatozoon* sp. i *T. acomys,* z maksimum w drugiej i trzeciej klasie wiekowej *A. dimidiatus.* Wynika to z tego, że dłużej żyjące gryzonie są bardziej (częściej) narażone na infestacje przez wektory.

W celu opisania nowego gatunku *Babesia* występującego u myszoskocza arabskiego *D. dasyurus* z Masywu Synaju (Egipt) wykonałem analizy mikroskopowe, molekularne i filogenetyczne. W oparciu o trzy markery genetyczne (18S rRNA, ITS1 i ITS2) wykazałem jego odrębność od *B. microti* i *B. rodhaini,* gatunków typowych dla gryzoni, jak również od innych znanych gatunków *Babesia* i *Theileria*. Nowy gatunek *Babesia* wykazywał ograniczony zasięg (dwie doliny w masywie Synaju) i specyficzność żywicielską w stosunku do *D. dasyurus*, jego patogenność dla ludzi jest nierozpoznana.

Podsumowanie

Podsumowując dokonania w ramach mojej pracy doktorskiej:

- Zaobserwowałem spadek średniego bogactwa gatunkowego, ekstensywności i intensywności zarażenia hemopasożytami na przestrzeni 12 lat.
- Zaobserwowałem występowanie *Hepatozoon* i *Trypanosoma* tylko w dwóch izolowanych dolinach, w których stwierdzono odpowiednie dla nich wektory.
- Zaobserwowałem specyficzność żywicielską u *T. acomys,* która występowała tylko u kolcomyszy arabskiej (*A. dimidiatus*), natomiast *Hepatozoon* sp. wykazał pewną specyficzność żywicielską, gdyż wariant A stwierdzany był u obu gatunków z rodzaju *Acomys*, a wariant B tylko u kolcomyszy złotej (*A. russatus*).
- Wieloletnie zmiany warunków środowiskowych (susza) na przestrzeni 12 lat prawdopodobnie miały wpływ na występowanie wszystkich żywych organizmów, w tym na spadek transmisji pasożytów wektorowanych.

Otrzymane wyniki pozwoliły na sformułowanie następujących wniosków:

- W badaniach wieloletnich można zaobserwować wyraźną dynamikę zarażeń oraz potwierdzić występowanie powtarzalnych różnic w zespołach pasożytów krwi izolowanych populacji żywicieli.
- Zespół pasożytów krwi kolcomyszy arabskiej kształtuje się w zależności od właściwości badanych dolin oraz czynników wewnątrzpopulacyjnych.
- Dla niektórych gatunków pasożtów wykazano specyficzność żywicielską, skutek koewolucji układu pasożyt-żywiciel (*B. behnkei, T. acomys*)
- Mało przebadane gatunki gryzoni z trudno dostępnych rejonów świata mogą być zarażone nieznanymi nauce pasożytami.

2. Summary

Long-term field studies of parasite communities provide a powerful insight into ecological and evolutionary processes shaping host-parasite communities (Alsarraf et al., 2016; Bajer et al., 2014b). Small mammals, especially rodents, are good model hosts for such studies because their populations are abundant, heterogenous and highly dependent on environmental factors, including food availability and climatic conditions (Bujalska and Hansson, 2000; Flowerdew JR, 1984; Turner et al., 2014). Haemoparasites of rodents are well studied in Europe, North America, South America and Asia but there are comparatively few studies on the haemoparasites of rodents from Africa. The parasites communities infecting Egyptian rodents, such as spiny mice (*Acomys dimidiatus*), golden spiny mice (*Acomys russatus*) and Wagner's gerbils (*Dipodillus dasyurus*) are subject to change depending on a number of factors and complex interactions between them (Alsarraf et al., 2016). With regard to rodents from desert and semi-desert areas of Egypt, such factors were hypothesized to include unique environmental factors (such as humidity and temperature), specific for the year of the study (hereafter described as YEAR), or the place (dry valley, in arabic Wadi) where the rodents were trapped (hereafter described as SITE). Other factors affecting the structure of the haemoparasite communities of rodents are intrinsic factors that we took into account in our analyses (host age or sex). The rodent's age was classified in three classes (juvenile, adult and mature) based on the body size, weight and sexual maturity of the host.

We studied the effect of these external and intrinsic factors on the prevalence, abundance and species richness of 5 blood parasites species: *Babesia*, *Bartonella*, *Haemobartonella (Mycoplasma)*, *Hepatozoon* and *Trypanosoma* in spiny mice (*A. dimidiatus*) from the Sinai

Massif (Egypt). Fieldwork was conducted over 4–5 -week periods in August-September beginning in 2000 and repeated every 4 years.

My PhD thesis is structured in two sections: the first focuses on a long-term dynamic study of the haemoparasites of the spiny mouse (*A. dimidiatus*), the representative rodent species from the Sinai Mountains. The second section comprises a description of a novel species of *Babesia* from Wagner's gerbil (*D. dasyurus*).

I postulated the following hypotheses:

1. Under desert and semi-desert condition we should observed long-term dynamic variation in the presence of haemoparasites in rodents from the Sinai Massif.
2. In four isolated valleys (Wadis), inhabited by similar groups of rodents, we should observed a different combination of blood parasites, while the degree of isolation of the host population should have consequences for the genetic diversity and the host specificity of the parasites.
3. The intrinsic factors (age class and sex) should influence the prevalence and abundance of the haemoparasite infections in rodents.
4. The hitherto unaudited species of rodents could be infected with as yet unclassified novel species of blood parasites.

The hypotheses concerning long-term dynamics of haemoparasite infections in spiny mouse were verified by realization of the following research aims.

1. Determination of the dynamic presence of combinations of blood parasites in *A. dimidiatus* over a period of 12 years, based prevalence, abundance and species richness data in the populations of rodents in different years.
2. Determination of the genetic diversity and the host specificity of the rodents' haemparasite communities in the isolated mountain valleys in Sinai Massif.
3. Determination of genetic diversity and the relationship between the species of haemparasites in *A. dimidiatus*.
4. Description and characterization of novel species of parasites present in other rodents.

Rodent trapping was conducted over 4–5 -week periods in August September in 2000, 2004, 2008 and 2012, close to the town of St Katherine in the Sinai mountains, Egypt. Trapping was carried out in four montane dry valleys "wadis" (W. Arbaein, W. Gebal, W. Gharaba and W. Tlah), isolated from each other by mountain ranges. Rodents (N=1041, 857 spiny mice, 73 golden spiny mice and 111 Wagner's gerbil) were caught live in Sherman traps, identified to

species level, and sex and age class were recorded. A blood sample was taken directly from the heart into EDTA for DNA isolation and two blood smears were made for microscopic analyses. I examined the blood smears by light microscopy and I detected blood cells (erythrocytes), infected with *Babesia, Bartonella,*and *Haemobartonella*, The presence of *Trypanosoma* in the plasma was also recorded in 200 fields of vision for each blood smear. In the case of *Hepatozoon* I count the number of infected leucocytes in a total of 50 leucocytes to determination the prevalence, and abundance of this species. I isolated the parasites' DNA, using DNAeasy Blood & Tissue kit (Qiagen, USA) or MiniPrep Blood kit (AxyGen, USA). For the molecular and phylogenetic analyses I used the marker 18S rRNA to determine the genetic diversity of *Hepatozoon* [gene part 660 bp; (Inokuma et al., 2002)] and *Trypanosoma* [gene part 520 bp; (Noyes et al., 1999)], and gene part of *rpoB* for *Bartonella* [product size 333 bp; (Paziewska et al., 2011)]. For the description of the novel species of *Babesia behnkei* I used the markers, 18S rDNA [1700 bp (Matjila et al., 2008; Oosthuizen et al., 2008)], the ITS1 region [615 bp (Blaschitz et al., 2008; Nijhof et al., 2003)] and the ITS2 region [315 bp; (Blaschitz et al., 2008; Nijhof et al., 2003)].

I section: Long-term dynamics of haemoparasite infections in spiny mice *A. dimidiatus* (Alsarraf et al. 2016)

To realize aims 1-3, I examined 835 spiny mice, and found the overall prevalence of haemoparasites in spiny mice to be 76.2%. I observed a decrease in the prevalence of haemoparasites over successive years of the study. The highest haemoparasites prevalence was recorded in 2004 (91.2%), while the lowest was in 2012 (53%). I observed also marked differences in the prevalence of haemoparasites in spiny mice from different valleys (Wadis) – I recorded the highest in W. Tlah (88.5%) and the lowest in W. Arbaein (68.8%).

Long-term dynamic change in *Babesia* sp. in *A. dimidiatus* population from four valleys

The overall prevalence of *Babesia* sp. was 3.1%. In *A. dimidiatus* there was a gradual decrease in the prevalence of *Babesia* sp. over the years of the study. The highest prevalence of *Babesia* sp. was recorded in 2004 (6.9%) and this fell to 0% by 2012. I recorded also differences in prevalence of *Babesia* sp. in *A. dimidiatus* from different SITES, the highest prevalence being (8.2%) in W. Gebal and the lowest in W. Gharaba (0.5%).

Long-term dynamics of *Bartonella* sp. infection in *A. dimidiatus* population from four valleys

The overall prevalence of *Bartonella* sp. infection was 3.6%, however, the study found dynamic changes in the presence of *Bartoenlla* sp. in *A. dimidiatus* over the years of the study. The highest prevalence of *Bartonella* sp. infection was 8.3%, recorded in 2004 and the lowest in 2008 (0.8%). In W. Gebal I recorded the highest prevalence of *Bartonella* sp. infection (6.9%) and the lowest was in the population of *A. dimidiatus* from W. Gharaba (1.6%). There were also marked temporal differences in prevalence of *Bartonella* sp. infection between SITES with prevalence of *Bartonella* increasing in W. Arbaein and decreasing in all other valleys over the years of study.

Long-term dynamics of *Haemobartonella* sp. infection in *A. dimidiatus* from four valleys

From among all the recorded species of parasites *Haemobartonella* sp. was the most prevalent in the population of *A. dimidiatus*, overall prevalence being 57.8%. I observed a fall in the prevalence of *Haemobartonella* over the years of the study, with the highest prevalence recorded (85.2%) in 2004 and the lowest (27.9%) in 2012. There were also differences in the prevalence of *Haemobartonella* sp. infection between the valleys. I recorded the highest prevalence of *Haemobartonella* sp. infection in *A. dimidiatus* from W. Gebal (71.7%) and the lowest (44.8%) in W. Gharaba.

Long-term dynamics of *T. acomys* infection in *A. dimidiatus* depending on year, valley of study and the host age, and additional molecular and phylogenetic analyses of the parasite

The overall prevalence of the *T. acomys* infection was 15.8%. The highest prevalence of infection was 22.7% in 2004, and the lowest was 11.9% in 2012. The highest prevalence of *T. acomys* infection was in W. Tlah (34.8%) and in W. Gharaba (21.9%), but the lowest was in W. Arbaein (0.4%). I record the highest prevalence of infection in adult (second age class) *A. dimidiatus* (26.6%) and the lowest in the mature (third age class) of *A. dimidiatus* (10.4%).

Molecular and phylogenetic analyses of 45 isolates of *T. acomys* using a fragment of 18S rRNA gene revealed two genetic variants A and B. Variant A was identified in 44 spiny mice, while variant B was present in just one isolate. Alignment of our two *Trypanosoma* variants revealed a difference of 7 nucleotides between them. Phylogenetic analysis revealed that our *Trypanosoma* sequences grouped together with species of *Trypanosoma* derived from

other species of rodents from different parts of the world, and together they formed a monophyletic group. A representative sequence of each of these two variants is provided in the GenBank database, as the first sequence for *T. acomys*.

Long-term dynamics of *Hepatozoon* infection in *A. dimidiatus* depending on year and valley of study, and additional molecular and phylogenetic analyses of a representative group of isolates from *A. dimidiatus* and *A. russatus*

The overall prevalence of the *Hepatozoon* sp. infection in *A. dimidiatus* was 29.7%. The highest prevalence of infection (40%) was recorded in 2008 and the lowest (20.6%) in 2000. *Hepatozoon* sp. was the only parasite which showed an increase in prevalence over the12 years of study. Almost all cases of the presence of this parasite were recorded in W. Gharaba and W. Tlah, similarly to *T. acomys* , the prevalence of which was associated with the presence of the vector *Parapulex chephrensis* in these two valleys (Bajer A., 2006). The highest prevalence was recorded in W. Tlah (58.9%) and the lowest in W. Gebal (6.3%).

The 18S rDNA sequence of *Hepatozoon* sp. from Egypt was aligned with other representative sequences from the GenBank database to determine the diversity between the species. Among these sequences was one of *H. erhardovae,* which infects bank voles in Poland (Alsarraf, 2012; Bajer et al., 2014b). Molecular and phylogenetic analyses led to the identification of two variants of the 18S rDNA gene fragment: A and B. Variant A was represented by 37 sequences, these sequences showing 96.06% identity to *H. ayorgbor* from a python from Ghana. Variant A was widespread, identified in 32 *A. dimidiatus* and 5 *A. russatus* at all sites. Variant B was identified only in 2 *A. russatus* (1 from W. Gharaba, 2004 and 1 from W. Tlah, 2012) and showed 96.75% identity to *H. ayorgbor*. The differences between these two variants were 11 nucleotides and the phylogenetic analyses showed that the two variants clustered together forming a paraphyletic group, separated from all other species of *Hepatozoon.* Because of the low similarity of our isolate to the classified species of *Hepatozoon* and there being no reference sequence for *H. acomys* in GenBank which has been described only on morphological features by Mohammed and Saound (1972) from (Smith, 1996), I have not yet determined whether our isolate is a new species of *Hepatozoon* or whether it is in fact just *H. acomys*. This requires further study.

II section: description of a new species of *Babesia* (*Babesia behnkei*) from *D. dasyurus* (Bajer *et al.* 2014)

Microscopic observations of blood smears led to the identification of trophozoites of *Babesia* sp. which were smaller than trophozoites of *B. microti* King's College, the reference strain maintained in BALB/c mice in our department. Due to the size of the trophozoites, at first I classified them as *Theileria* sp. but when I completed the molecular and phylogenetic analyses I established that it was a new species of *Babesia,* distinct from *Theileria* and other classified species of *Babesia*. These parasites were recorded just in Wagner's gerbil *D. dasyurus* in two valleys W. Arbaein and W. Gebal. The overall prevalence was 39%. In order to provide a detailed description of the species *B. behnkei,* I measured the diameter of trophozoites of *B. behnkei* (N=212) and compared these measurements with the diameter of trophozoites of *B. microti* King's College. The mean diameter of the *B. behnkei* was significantly smaller 1.26 μm than *B. microti* 1.46 μm. For the molecular and phylogenetic analyses, 3 genetic markers were used, near-full-length of 18S rDNA (1700 bp), region ITS1 (615 bp) and region ITS2 (315 bp) and these analyses revealed that *Babesia behnkei* was genetically distinct from all other species of *Babesia.* A BLAST search of 18S rDNA of *B. behnkei* showed 96% identity to *B. lengau* from a Cheetah from South Africa. The phylogenetic analyses revealed that *B. behnkei* clustered in group Duncani with American and African species of *Babesia* (Bajer et al., 2014a; Lack et al., 2012). Phylogenetic analyses for ITS1 and ITS2 gave very similar results to 18S rDNA. *B. behnkei* is the third species of *Babesia* infecting rodents to be described, after *B. microti* and *B. rodhaini.*

Discussion

As predicted, external factors (site and year of study) had a much greater influence on the haemoparasites community compared with intrinsic factors. Long-term dynamic changes of haemoparasites were observed in prevalence, abundance and in mean species richness during the 12 years of the study and these could be associated with the chronic drought, reflected in a substantial reduction in rainfall over this period in this part of Egypt. The recorded average rainfall was 42.5 mm / year in the period 1970-1994, while in 2001-2009 the average rainfall was only 15.5 mm / year (Alsarraf et al., 2016). During the last expeditions to sample the rodent populations in 2008 and 2012, we observed degradation of plant cover and the drying / desertification of gardens belonging to the Bedouin in these valleys. Climate change, particularly lack of water, may adversely affect not only the population of rodents but also the survival of the vectors of haemoparasites and therefore result in a decrease in transmission.

I observed the occurrence of *T. Acomys* and *Hepatozoon* sp., mostly in just the two valleys, W. Gharaba and W. Tlah. The reason may be the presence of a potential vector - flea of the species *Parapulex chephrensis*, which appeared most often in the spiny mouse in W. Gharaba and W. Tlah, and was found only once in W. Arbaein in 2000 on two individual spiny mice. From among the intrinsic factors, age had a significant effect on the prevalence and abundance of *Hepatozoon* sp. and *T. acomys* infection, with maximum values for both parameters recorded in the second and third age classes of *A. dimidiatus*. This is due to the fact that the longer lived rodents are more (often) exposed to infestation by the vector. In order to describe the new species of *Babesia* from *D. dasyurus* from the Sinai Massif (Egypt) I used microscopic, molecular and phylogenetic analyses. Based on the three genetic markers (18S rRNA, ITS1 and ITS2) this showed the distinct identity of *B. behnkei* and its separation from the *B. microti* and *B. rodhaini*, typical species of rodent *Babesia*, as well as other known species and *Theileria*. The new species of *Babesia* had limited coverage (two valleys in the Sinai massif) and host specificity to *D. dasyurus*. Its pathogenicity for humans is as yet unstudied and unknown.

Summary

To summarize the achievements in my PhD thesis

- I have observed a decrease in the mean species richness, prevalence and abundance of haemoparasites infection over 12 years in our study sites.
- I have observed the presence of *T. acomys* and *Hepatozoon* sp. only in two isolated valleys, wherein the proposed vectors of these species are suitable for transmission.
- I observed host specificity of *T. acomys*, which appeared only in spiny mouse (*A. dimidiatus*), while *Hepatozoon* sp. showed some specificity for its hosts, since variant A was found in both species of the genus *Acomys*, but variant B only in golden spiny mice (*A . russatus*).
- Long-term changes in environmental conditions (drought) over 12 years probably have had an impact on the incidence of all living organisms, including a decrease in the transmission of rodent haemoparasites in the region.

The results led to the following conclusions:

- Long-term research, such as this recorded in the current thesis, is necessary to detect clear dynamic temporal patterns of change in the prevalence of infections and to

identify stable as well as dynamic features in the prevalence and abundance of combinations of blood parasites from isolated populations of hosts.

- The prevalence of blood parasites of spiny mice is dependent on ecological features of the valleys in which they live and host intrinsic factors.
- Some parasite species have been demonstrated to be host specific, the consequence of co-evolution of host and parasite (*B. behnkei*, *T. Acomys*).
- Wild rodents from remote places in the world which have not been studied well could harbor as yet unclassified novel species of parasites.

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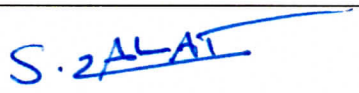
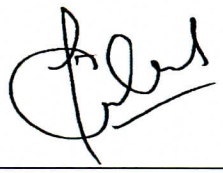
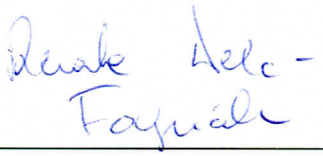
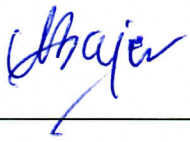
Long-term spatiotemporal stability and dynamic changes in the haemoparasite community of spiny mice (Acomys dimidiatus) in four montane wadis in the St. Katherine Protectorate, Sinai, Egypt

STATEMENT OF CO-AUTHORS OF JOINT PUBLICATION

Mohammed Alsarraf, Małgorzata Bednarska, Eman M. E. Mohallal, Ewa J. Mierzejewska, Jolanta Behnke-Borowczyk, Samy Zalat, Francis Gilbert, Renata Welc-Falęciak, Agnieszka Kloch, Jerzy M. Behnke, Anna Bajer (2016). Long-term spatiotemporal stability and dynamic changes in the haemoparasite community of spiny mice (Acomys dimidiatus) in four montane wadis in the St. Katherine Protectorate, Sinai, Egypt. *Parasites & Vectors* (2016) 9:195 DOI 10.1186/s13071-016-1471-z.

We, the undersigned, co-authors of the above publication, confirm that we are aware the above publication is included to the PhD thesis of Mohmmed Alsarraf.

Mohammed Alsarraf (70%)	performed microscopical, molecular and phylogenetic studies	
Małgorzata Bednarska	participated in detection of haemoparasites	
Eman M. E. Mohallal	performed the laboratory and field studies and drafted the manuscript	
Ewa J. Mierzejewska	performed the laboratory and field studies and drafted the manuscript	
Jolanta Behnke-Borowczyk	performed the laboratory and field studies and drafted the manuscript	

Samy Zalat	organized and supervised field work in Sinai	
Francis Gilbert	organized and supervised field work in Sinai	
Renata Welc-Falęciak	performed microscopical, molecular and phylogenetic studies	
Agnieszka Kloch	participated in detection of haemoparasites	
Jerzy M. Behnke	organized and supervised field work in Sinai	
Anna Bajer	designed the study and supervised laboratory and field analyses	

Alsarraf et al. Parasites & Vectors (2016) 9:195
DOI 10.1186/s13071-016-1471-z

Parasites & Vectors

RESEARCH Open Access

Long-term spatiotemporal stability and dynamic changes in the haemoparasite community of spiny mice (*Acomys dimidiatus*) in four montane wadis in the St. Katherine Protectorate, Sinai, Egypt

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Abstract

Background: Long-term field studies of parasite communities are rare but provide a powerful insight into the ecological processes shaping host-parasite interactions. The aim of our study was to monitor long-term trends in the haemoparasite communities of spiny mice (*Acomys dimidiatus*) and to identify the principal factors responsible for changes over a 12 year period.

Methods: To this end we sampled four semi-isolated populations of mice ($n = 835$) in 2000, 2004, 2008 and 2012 in four dry montane valleys (wadis) located in the Sinai Massif, Egypt.

Results: Overall 76.2 % of spiny mice carried at least one of the five haemoparasite genera (*Babesia, Bartonella, Haemobartonella, Hepatozoon, Trypanosoma*) recorded in the study. Prevalence of haemoparasites varied significantly between the sites with the highest overall prevalence in Wadi Tlah and the lowest in W. El Arbaein, and this changed significantly with time. In the first two surveys there was little change in prevalence, but by 2008, when the first signs of a deepening drought in the region had become apparent, prevalence began to drift downwards, and by 2012 prevalence had fallen to the lowest values recorded from all four sites over the entire 12-year period. The overall mean species richness was 1.2 ± 0.03, which peaked in 2004 and then dropped by more than 50 % by 2012. Species richness was highest among mice from Wadi Tlah and peaked in age class 2 mice (young adults). Site was the most significant factor affecting the prevalence of individual parasite species, with *Trypanosoma acomys* and *Hepatozoon* sp. occurring mainly in two wadis (W. Tlah & W. Gharaba). In four of the five genera recorded in the study we observed a significant drop in prevalence or/and abundance since 2004, the exception being *Hepatozoon* sp.

Conclusions: During the 12-year-long period of study in the Sinai, we observed dynamic changes and possibly even cycles of prevalence and abundance of infections which differed depending on parasite species. Although the exact reasons cannot be identified at this time, we hypothesize that the effects of a 15-year-long scarcity of rainfall in the local environment and a fall in host densities over the period of study may have been responsible for a drop in transmission rates, possibly by a negative impact on vector survival.

Keywords: *Acomys dimidiatus*, *Acomys russatus,* haemoparasites, *Haemobartonella*, *Bartonella*, *Hepatozoon*, *Trypanosoma*, *Babesia*, Species-richness, Prevalence, Abundance, Sinai, Drought, Between year variation

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Background

Long-term field studies of parasite communities provide a powerful insight into ecological and evolutionary processes shaping host-parasite interactions over time. Small mammals, especially rodents, are good model hosts for such studies because their populations are abundant, heterogenous and highly dependent on environmental factors, including food availability and climatic conditions [1–3]. The high heterogeneity and the dynamic between- and within-year variation of rodent populations allow also investigation of the relative contribution of a range of quantifiable intrinsic and extrinsic factors underlying some of the dominant patterns of variation in parasitic infections observed in the field [4–7]. Each rodent community can be regarded as comprising a set of different functional subgroups including, for example, settled, territorial adults of both sexes and mobile juveniles, which may differ in their exposure and susceptibility to infection [8].

Climatic conditions are believed to play a crucial role in shaping plant or animal communities in different habitats, including those living in arid environments such as the deserts of the Middle East, and are likely also to have a major impact on the parasite communities of the indigenous hosts. However, other than in short-term catastrophic events (e.g. earthquakes, intense storms etc.), the influence of climatic changes is likely to be slow over a prolonged period of time and hence long-term ecological studies are essential to identify links between changing climatic conditions and disease.

In earlier studies we have shown that the haemoparasite communities of *Acomys dimidiatus* vary markedly between subpopulations of spiny mice living in four distinct dry semi-isolated desert valleys (wadis) in the Sinai mountains of Egypt. These four wadies are segregated from each other by natural barriers [9, 10] and they differ in altitude but ecologically they show many similarities although distinct differences have also been recorded [11]. Such isolated or semi-isolated subpopulations of animals may differ in the stresses to which they are subjected in each site, including pathogens, and hence may experience different selection pressures created by the specific conditions in their home range (the geographical mosaic theory of co-evolution; [12]). Haemoparasites in particular are likely to be an important source of selective pressure on hosts because they are often associated with pathogenicity (e.g. acute babesiosis, trypanosomiasis; [13–15]) and hence, resistance/tolerance of such infections confers enormous fitness benefits [16–18]. In this context it is pertinent that in our first survey in 2000, the haemoparasite community of the spiny mice living in these wadies were more diverse [10] than in our concurrent and subsequent studies on the haemoparasites of common or bank voles in Poland [4, 19–21] or in other studies on rodents [22–24].

We continued to monitor the haemoparasites of the same spiny mouse populations in 2004, 2008 and 2012 in order to assess the stability of the epidemiological patterns that were observed in 2000. Here, building on the resources collected in these four expeditions to the Sinai and the resultant database on natural infections in wild spiny mice, we report on the spacio-temporal stability of some haemoparasite species carried by *A. dimidiatus* and on the dynamic changes in others in our study sites. Detailed morphometric data on each animal also allowed the effect of host intrinsic factors on haemoparasites to be assessed. We predicted that the effects of host age and sex would be consistent and repeatedly observable in successive surveys, showing little between-year variation in magnitude of the effect due to co-evolution of the hosts and parasites involved. Haemoparasites are vector-borne pathogens (VBP) and in short-lived host species, such as spiny mice, we would expect a significant increase in the prevalence of VBP with host age, as the probability of being infested with ticks or fleas carrying VBP increases also with host age. However, host immune responses to each of the haemoparasites differ, and where host-protective immunity is generated, we would also expect both prevalence and abundance of infection to decline in the oldest age class [25]. We predicted that extrinsic factors (unique abiotic conditions associated with certain wadi and/or particular years of study) would have a greater influence resulting in repeatable patterns (for between-site differences) or distinct between-survey dynamics (for between-year differences). While abiotic conditions are largely 'unpredictable' for both hosts and parasites, the climatic changes in the Sinai have been well documented [26–28] and in this long-term study of the parasites of spiny mice we had an opportunity to observe the impact of decreasing water availability on host and parasite populations/communities. Egypt is the most arid country on Earth [29], classified as 'hyper-arid' in climatology. Because of the alarming reduction in the water supply in the montane wadies of S. Sinai as a consequence of a long period (15 years) of no or only very low rainfall and the resulting increasing aridity of the local environment with associated loss of arable land (Bedouin gardens), clearly apparent during the expeditions in 2008 and 2012 (compared with the first two expeditions in 2000 and 2004), we expected marked differences in parasite community structure over this period of 12 years. For parasite isolates obtained in 2004–2012 we carried out also preliminary molecular characterization and phylogenetic analyses. Our data provide a novel insight into the ecology of the haemoparasites of rodent hosts living in semi-isolated, hyper-arid habitats, about which little is currently known.

Methods

Field studies in Sinai, Egypt

Fieldwork was conducted over 4–5 -week periods in August-September in 2000, 2004, 2008 and 2012 and was based at the Environmental Research Centre of Suez Canal University (2000, 2004) or at Fox Camp (2008, 2012) in the town of St Katherine, South Sinai, Egypt. Trapping was carried out in four montane wadis (dry valleys) in the vicinity of St Katherine. The local environment and general features of the four study sites (Wadi ElArbaein, W. Gebal, W. Tlah, W. Gharaba), as well as their spatial relationships with one another, have been described elsewhere [9]. At each site, rodents were caught live in Sherman traps, placed selectively among the rocks and boulders around walled gardens and occasionally along the lower slopes of wadis. These were set out at dusk, and inspected in the early morning before exposure to direct sunlight. All traps were brought into the local or main camp, where the animals were removed, identified and processed. Traps were re-set the following evening.

The three most abundant rodent species (*A. dimidiatus, A. russatus, D. dasyurus*) were sampled- sexed, weighed, measured and scrutinized for obvious lesions as described by Behnke et al. [9]. Ectoparasites visible during field examination were removed and placed in 70 % ethanol. Blood and faecal samples were taken and animals were then either fur marked individually or ear clipped and released close to the point of capture, or returned to the main camp at St Katherine for autopsy.

Animals were allocated to three age classes, principally on the basis of body weight and nose-to-anus length. For male and non-pregnant female mice separately, these two measurements were reduced by principal components analysis, and principal component 1 (for males Eigen value = 1.87 and accounted for 93.5 % of variance and for non-pregnant females the Eigen value was 1.83, accounting for 91.4 % of variance) together with observations recorded for each animal in the field (for males whether scrotal or non-scrotal, for females whether lactating, perforate or pregnant), was used to guide allocation of animals to three age classes. Full details of the methods used and statistical verification of this approach are given in Behnke et al. [9] (all means are cited ± one standard error). Age class 1 comprised the youngest animals, mostly weanlings and very young non-reproductively active juveniles (mean weight for males = 17.8 g ± 0.40, $n = 74$; females = 19.5 g ± 0.31, $n = 116$), age class 2 comprised juveniles and young adults (mean weight for males = 28.1 g ± 0.29, $n = 114$, non pregnant females = 27.4 ± 0.30, $n = 104$; pregnant females = 28.8 ± 2.49, $n = 3$), and age class 3 comprised the adult and oldest animals in the study (mean weight for males = 41.5 ± 0.42, $n = 184$; non-pregnant females = 40.6 g ± 0.44, $n = 182$; pregnant females = 47.4 ± 1.09, $n = 58$).

Blood collection and DNA extraction

Thin blood smears were prepared from drops of blood taken from the retro-orbital plexus using heparinized capillary tubes of animals lightly anaesthetized with ether during examination in the field and from the heart of those that were autopsied. Blood smears were air-dried, fixed in absolute methanol and stained for 1 h in Giemsa stain in buffer at pH 7.2. Each smear was examined under oil immersion (×1000 magnification). Parasites were counted in 200 fields of vision. Microscopical observation of stained blood smears was used as the only detection method for study in 2000 and for *Babesia* spp. In subsequent expeditions, in addition to blood smears, molecular techniques were used for species identification of *Bartonella*, *Hepatozoon* and *Trypanosoma* but confined to samples that were positive by microscopical observation in 2004 and 2008, and as the diagnostic method for all samples in 2012. Blood from the tail vein was collected on FTA classic cards (Whatman, UK) for the long-time preservation of DNA. From the culled animals, 200 μl of whole blood were also collected into 0.001 M EDTA and frozen at -20 °C.

Genomic DNA was extracted from whole blood using DNAeasy Blood & Tissue kit (Qiagen, USA) or AxyPrep MiniPrep Blood kit (AxyGen, USA) and stored at a temperature of -20 °C. DNA from FTA cards was cleaned with FTA purification Reagent (Whatman, UK) according to the manufacturer's instructions.

Molecular characterization

The extracted DNA was subjected to specific PCRs as described in detail in Bajer et al. [4]. The primers and cycling conditions used in this study are listed in a table (Additional file 1). Reactions were performed in 1× PCR buffer, 0.2 U *Taq* polymerase, 1 μM of each primer and 2 μl of the extracted DNA sample. Negative controls were conducted in the absence of template DNA. PCR products were subjected to electrophoresis on a 1.5 % agarose gel, stained with Midori Green stain (Nippon Genetics GmbH) and sequenced by a private company (Genomed S.A., Poland).

Genotyping and phylogenetic analysis

Bartonella sp.

One *Bartonella* isolate obtained from *A. dimidiatus* from W. Tlah in 2004 was genotyped by the amplification and sequencing of a 333-bp fragment of the *rpoB* region [30].

Hepatozoon

Thirty nine isolates derived from *A. dimidiatus* and *A. russatus* in 2004, 2008 and 2012 from all sites (Table 1) were investigated by the analysis of a 660 bp 18S rRNA gene fragment [31]. First, all obtained sequences were aligned using MEGA v. 6.0. The phylogenetic analyses

Table 1 *Hepatozoon* isolates/variants by the host, site and year of study

		2004				2008				2012				Total
		Arbaein	Gebal	Gharaba	Tlah	Arbaein	Gebal	Gharaba	Tlah	Arbaein	Gebal	Gharaba	Tlah	
A. dimidiatus	Variant A	0	1	1	0	4	3	3	4	5	0	2	9	32
A. russatus	Variant A	0	0	0	0	1	1	2	0	0	0	0	1	5
	Variant B	0	0	1	0	0	0	0	0	0	0	0	1	2
Total		0	1	2	0	5	4	5	4	5	0	2	11	39

including our sequences (660 bp) and sequences of *Hepatozoon* spp. deposited in the GenBank database were conducted in MEGA v. 6.0 [32]. A representative tree for 18S rDNA sequences was obtained using the Maximum Likelihood method and a Tamura 3-parameter (I + G) model.

Trypanosoma

Forty five isolates derived from *A. dimidiatus* from 2004, 2008 and 2012 from W. Gebal ($n = 1$), W. Gharaba ($n = 15$) and W. Tlah ($n = 29$) were investigated by the analysis of a 520 bp 18S rRNA gene fragment [33]. The phylogenetic analyses including our sequences (520 bp) and other sequences of *Trypanosoma* spp. deposited in the GenBank database were conducted in MEGA v. 6.0 [32]. A representative tree for 18S rDNA sequences was obtained using the Maximum Likelihood method and a Tamura 3-parameter (I + G).

Statistical analysis

Prevalence (percentage of animals infected) was estimated based on microscopical observations and values are reported with the 95 % confidence limits, calculated by bespoke software based on the tables of Rohlf and Sokal [34]. The intensity of infection in each animal was quantified as the number of infected red blood cells (iRBC) [for *Babesia, Bartonella, Haemobartonella* (*Mycoplasma*)] or parasites (for *Trypanosoma, Hepatozoon*) in 200 fields of vision at ×1000 magnification and mean abundance is the average of this measure, including all the sampled animals whether infected or not. Species richness was calculated as the number of different haemoparasite species in each animal. When samples were only positive by PCR (in 2012), an intensity of 1 iRBC/1 parasite in 200 fields of vision was implemented into quantitative statistical analysis.

The statistical approach adopted has been documented comprehensively in our earlier publications [4, 6, 7]. For analysis of prevalence we used maximum likelihood techniques based on log - linear analysis of contingency tables in the software package IBM SPSS (version 21.0.0, IBM Corp). Initially, full factorial models were fitted, incorporating as factors SEX (2 levels, males and females), AGE (3 levels), YEAR of study (4 levels, each of the four surveys), and SITE (4 levels, the four study sites). The presence of parasites was implemented as INFECTION and was considered as a binary factor (2 levels, present or absent). These factors were fitted initially to all models that were evaluated. For each level of analysis in turn, beginning with the most complex model, involving all possible main effects and interactions, those combinations that did not contribute significantly to explaining variation in the data were eliminated in a stepwise fashion beginning with the highest level interaction (backward selection procedure). A minimum sufficient model was then obtained, for which the likelihood ratio of χ^2 was not significant, indicating that the model was sufficient in explaining the data. The importance of each term (i.e. interactions involving INFECTION) in the final model was assessed by the probability that its exclusion would alter the model significantly and these values relating to interactions that included INFECTION are given in the text. The remaining terms in the final model that did not include INFECTION are not given but can be made available from the authors on request.

For analyses of quantitative data we used general linear models (GLM) with normal errors implemented in R version 2.2.1 (R Core Development Team) and the residuals were checked for approximate Gaussian distribution. When the residuals failed to meet the requirements of Gaussian model we explored models based on $\log_{10}(X + 1)$ transformed data and generalised linear models with negative binomial or Poisson error structures. Full factorial models that converged satisfactorily were simplified using the STEP procedure and tested for significance using deletion of terms beginning with the highest order interaction by comparing models with or without that interaction. Changes in deviance (DEV) are given for models based on Poisson errors (interpreted as Chi^2 values), for models based on Gaussian errors we give F and for those based on negative binomial errors the likelihood ratio (LR). Minimum sufficient models were then fitted (all significant interactions and main effects plus any main effects that featured in interactions) and the process was repeated to obtain values for changes in deviance, test statistics and probabilities. Finally, if the data did not meet the assumptions of parametric tests, we employed non-parametric tests (Kruskal- Wallis test and the Mann - Whitney *U*-test).

Ethical issue

Rodents from St Katherine National Protectorate were sampled by agreement with the St Katherine National Protectorate authorities obtained for each set of fieldwork. A maximum of 40 % of the captured rodents from each site were culled by agreement with the St Katherine National Protectorate authorities.

Results

Molecular identification of parasite species (2004, 2008 and 2012)

Bartonella sp.

One *Bartonella* sp. isolate obtained from *A. dimidiatus* from W. Tlah in 2004 was successfully genotyped by the amplification and sequencing of a 333-bp fragment of the *rpoB* region. Comparison with the GenBank database revealed that the isolate showed the highest sequence homology (99.37 %; 317/319 bp) with *Bartonella acomydis* strain KS2-1 obtained from *A. russatus* (AB529942). This reference strain was identified in the golden spiny mouse imported from Egypt to Japan as an exotic pet [35].

Hepatozoon sp.

Thirty nine *Hepatozoon* sp. isolates obtained from 2004, 2008 and 2012 from all sites and two host species, *A. dimidiatus* and *A. russatus* (Table 1), were genotyped by sequencing of a 660 bp fragment of the 18S rRNA gene. Two genetic variants of *Hepatozoon* were identified, variant A and B. Variant A was widespread, identified in 32 *A. dimidiatus* and 5 *A. russatus* at all sites (Table 1). Four representative sequences of this variant were deposited in the GenBank database under the accession numbers KT337467, KT337468, KT337471 and KT337472. Variant B was identified only in 2 *A. russatus* (1 from W. Gharaba, 2004 and 1 from W. Tlah, 2012). Both sequences of this variant were deposited in the GenBank database under the accession numbers KT337469 and KT337470.

A BLAST search in the GenBank database revealed, that variant A showed the highest sequence homology (96.23 %) to *Hepatozoon* sp. AO5 from the olive grass mouse, *Abrothrix olivaceus,* from Chile (FJ719818) and 96.06 % sequence homology to *H. ayorgbor* from the royal python, *Python regius,* from Ghana (EF157822). Variant B showed the highest sequence homology (96.75 %) to *H. ayorgbor* from *P. regius* (EF157822) and 97.09 % to *Hepatozoon* sp. AO5 from *A. olivaceus* (FJ719818). Both variants showed a lower sequence homology with *Hepatozoon* isolates from jerboas *Jaculus orientalis* and *J. jaculus* (95.5–96.5 %) [36].

Alignment of our two *Hepatozoon* variants revealed a difference of 11 nucleotides between them. Alignment of these two variants with the two most similar sequences of *Hepatozoon* from the GenBank database is given in Additional file 2. Phylogenetic analysis revealed that our *Hepatoozon* sequences grouped together with genotypes/species of *Hepatozoon* derived from other species of rodents from different parts of the world [i.e. *A.olivaceus, A. sanborni, Bandicota indica, Jaculus* spp., *Sciurus vulgaris, Clethrionomys* (*Myodes*) glareolus] and from some species of reptiles, but were distant from *Hepatozoon* genotypes/species found in carnivores (i.e. *H. felis, H. americanum, H. ursi*) (Fig. 1).

Trypanosoma sp.

Forty - five *Trypanosoma* isolates were obtained and genotyped: 13 isolates from 2004, 10 isolates from 2008 and 22 isolates from 2012. All isolates were derived from *A. dimidiatus*, mostly from just two wadis where this parasite was found during the study period: 15 isolates from W. Gharaba and 29 isolates from W. Tlah. Additionally, one isolate from W. Gebal was obtained and genotyped. Isolates were genotyped by the amplification and sequencing of a 520-bp fragment of the 18S rRNA gene. Two genetic variants of *Trypanosoma* were identified, variant A and B. Variant A was widespread, and identified in 44 *A. dimidiatus* from W. Gebal, Gharaba and Tlah (2004, 2008 and 2012). One representative sequence of variant A was deposited in the GenBank database under the accession number KT337473. Variant B was identified only in one isolate from *A. dimidiatus* from W. Tlah in 2012. The sequence of this variant was deposited in the GenBank database under the accession number KT337474.

A BLAST search in the GenBank database revealed, that both variants A and B showed the highest sequence homology to a *Trypanosoma* sp. isolate from *A. dimidiatus* from one of our own earlier expeditions to Egypt (100 %; HQ324793) (direct submission). The next closet matches (95.94 %) of variant A were *Trypanosoma* sp. from Anderson's red-backed vole, *Eothenomys andersoni,* from Japan (AB242276) and *T. microti* from the field vole, *Microtus agrestis,* from the UK (AJ009158). Variant B showed the next highest homology (97.39 %) to *Trypanosoma* sp. B08-471 from a squirrel flea, *Ceratophyllus* (*Monopsyllus*) *sciurorum* from the Czech Republic (KF054111) and to *T. microti* from *M. agrestis* from the UK (AJ009158).

Alignment of our two *Trypanosoma* variants revealed a difference of 7 nucleotides between them. Alignment of these two variants with the most similar sequences of *Trypanosoma* from the GenBank database is presented in Additional file 3. Phylogenetic analysis revealed that our *Trypanosoma* sequences grouped together with species of *Trypanosoma* derived from other species of rodents from different parts of the world (*T. blanchardi, T. evotomys, T. grosi, T. kuseli, T. lewisi, T. microti, T. musculi*) and were distant from the key pathogenic species, *T. brucei* or *T. cruzi* (Fig. 2).

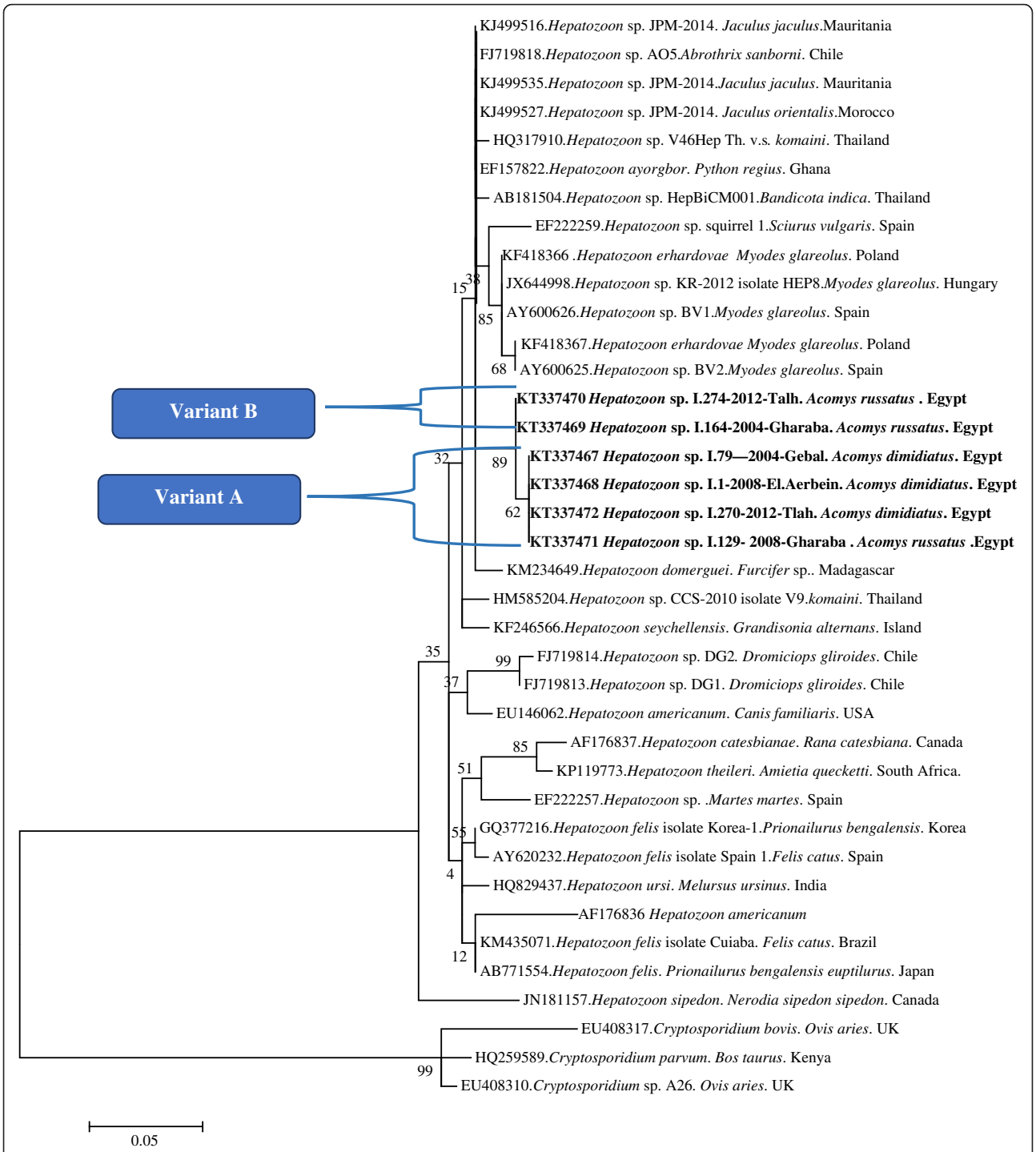

Fig. 1 The evolutionary history of *Hepatozoon,* based on a fragment of the 18S rRNA gene, was inferred using the Maximum Likelihood method and a Tamura 3-parameter (I + G). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The analysis involved 38 nucleotide sequences. All positions containing gaps and missing data were eliminated. The nucleotide sequence of *Cryptosporidium parvum*, *C. ovis* and *C. bovis* were used as outgroups. Evolutionary analyses were conducted in MEGA6.0

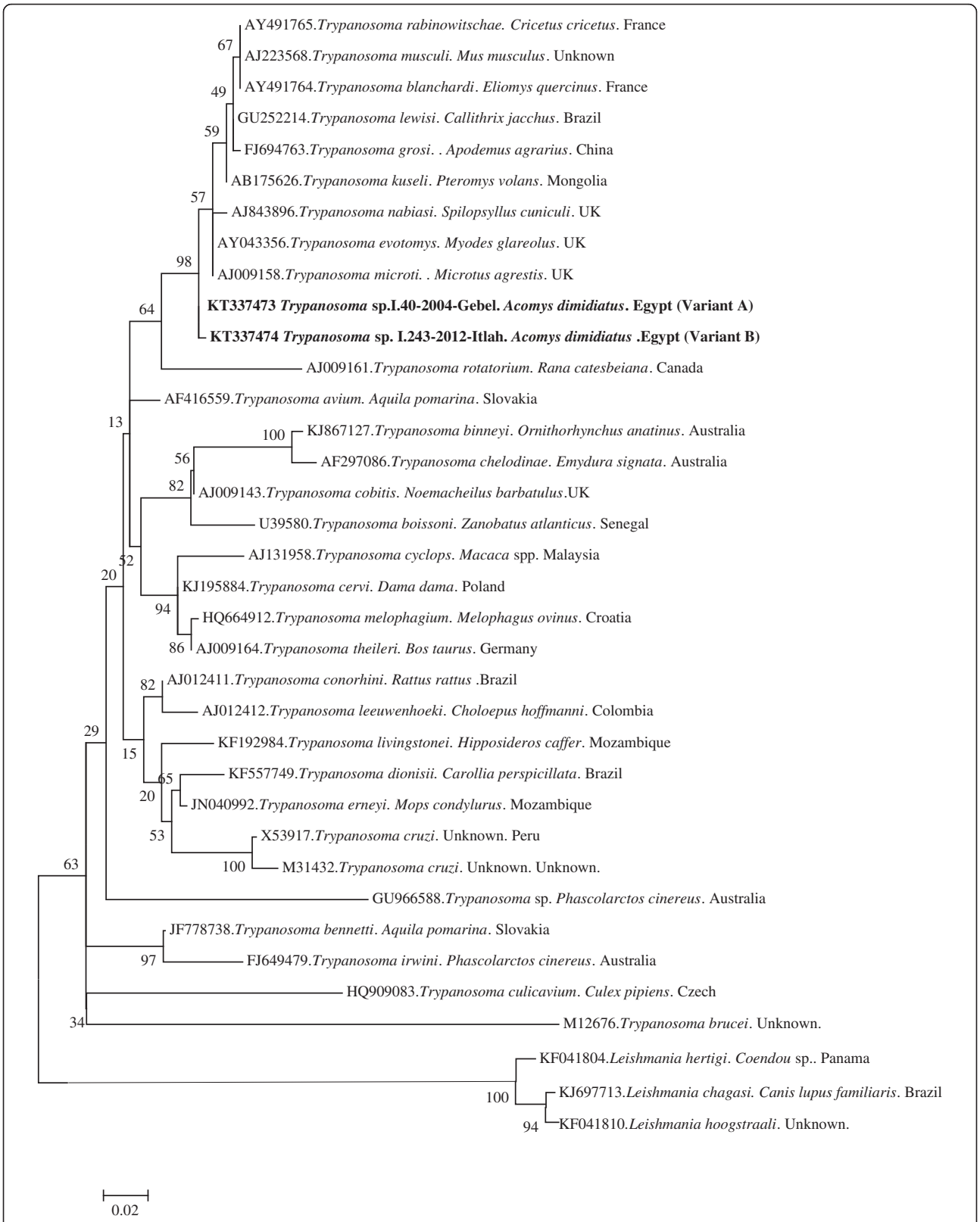

Fig. 2 (See legend on next page.)

(See figure on previous page.)
Fig. 2 The evolutionary history of *Trypanosoma*, based on the fragment of the 18S rRNA gene, was inferred using the Maximum Likelihood method and a Tamura 3-parameter (I + G). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The analysis involved 36 nucleotide sequences. All positions containing gaps and missing data were eliminated. The nucleotide sequence of *Leishmania hertigi, L. chagasi* and *L. hoogstraali* were used as outgroups. Evolutionary analyses were conducted in MEGA6.0

Environmental study in Sinai in the period 2000–2012

Acomys dimidiatus

A total of 857 individual spiny mice were caught over the four surveys (Table 2) and blood samples of sufficient quality to assess microscopically were obtained from 835 (Table 3). Values for the trapping effort and the resulting success in terms of mice caught/100 trap hours (th), and number of mice as a percentage of traps deployed in the field are summarized by wadi and year of survey in Table 2. Analysis of these data by the Kruskal-Wallis test with either YEAR or SITE as the explanatory factor on each of the variables listed in turn, revealed that the only significant effect was that of YEAR on the number of trap hours ($\chi^2_3 = 11.5$, $P = 0.009$). As can be seen in Table 2, the number of trap hours was larger in 2008 and 2012 compared with the two earlier years.

Table 2 Trapping effort and relative population size of *A. dimidiatus*

		Trap Hours	No. of *A. dimidiatus*	Mice/100 th[a]	Trap success (%)[b]
Arbaein	2000	2723	63	2.31	32.0
	2004	3265	43	1.32	16.8
	2008	3714	69	1.86	25.3
	2012	3918	67	1.71	23.4
	Combined	13620	242	1.78	23.9
Gebel	2000	1838	32	1.74	21.3
	2004	2112	43	2.04	27.4
	2008	3831	43	1.12	16.4
	2012	3675	47	1.28	17.2
	Combined	11456	165	1.44	19.6
Gharaba	2000	2136	28	1.31	16.5
	2004	2913	61	2.09	29.2
	2008	4314	54	1.25	16.1
	2012	3989	52	1.30	17.2
	Combined	13352	195	1.46	19.2
Tlah	2000	2199	46	2.09	27.7
	2004	2117	70	3.31	45.2
	2008	5344	80	1.50	20.1
	2012	3988	59	1.48	19.9
	Combined	13648	255	1.87	25.1
Combined	2000	8896	169	1.90	24.7
	2004	10407	217	2.09	27.9
	2008	17203	246	1.43	19.3
	2012	15570	225	1.45	19.4
	Grand total/average	52076	857	1.65	22.0

[a]No of animals caught per 100 trap hours (th)
[b]Trap success = percentage of traps occupied by a mouse after overnight deployment of traps in the study sites

The structure and the sample sizes of each subset of the host population that was assessed for haemoparasites, is shown in Table 3 by site and year of study, host sex and age. The numbers of mice sampled differed significantly between the wadis ($\chi^2_3 = 25.4$, $P < 0.001$), with most from W. Tlah and the least from W. Gebal. The distribution of mice among age classes also varied significantly between the sexes (SEX × AGE, $\chi^2_2 = 6.8$, $P = 0.033$) and between the four surveys (YEAR × AGE, $\chi^2_6 = 15.2$, $P = 0.019$), and these effects are taken into consideration in further analyses.

Haemoparasites - all species combined

Overall 76.2 % (72.61–79.42) of the 835 spiny mice carried at least one of the five haemoparasite genera recorded in the study. Prevalence varied significantly between the sites (Table 4; SITE × INFECTION, $\chi^2_3 = 34.5$, $P < 0.001$) with the highest overall prevalence in W. Tlah and the lowest in W. El Arbaein, and it changed significantly with time (Table 4; YEAR × INFECTION, $\chi^2_3 = 99.1$, $P < 0.001$), although the pattern of change of prevalence with time varied between the sites (Fig. 3a; YEAR × SITE × INFECTION, $\chi^2_9 = 29.7$, $P < 0.001$). In the first two surveys there was little change in prevalence, but in 2008 prevalence began to drift downwards, especially in W. Gharaba and by 2012, prevalence had fallen in all four sites relative to 2000, although the least change was observed in mice from W. Tlah. Age on its own was just the wrong side of significance (AGE × INFECTION $\chi^2_2 = 5.9$, $P = 0.051$) and as can be seen in Table 4, prevalence was lowest among the young age class but only 9 % higher in the intermediate age class and slightly lower among the oldest mice, so overall little change with increasing host age. However, the age of hosts featured also in two interactions, one with year of survey (YEAR × AGE × INFECTION, $\chi^2_6 = 13.5$, $P = 0.036$) and one with location of sampling (SITE × AGE × INFECTION $\chi^2_6 = 19.0$, $P = 0.004$), which we did not explore further. There was no significant difference in prevalence between the two sexes and SEX did not figure in any of the interactions that were detected.

Table 3 Numbers of *Acomys dimidiatus* examined by site, year, host age and sex

Site	Year	Sex	Age class			Totals	
			Class1	Class2	Class3	Row	Site and year
El Arbaein	2000	Males	6	15	7	28	
		Females	11	8	11	30	58
	2004	Males	4	5	12	21	
		Females	7	6	9	22	43
	2008	Males	3	10	17	30	
		Females	11	9	16	36	66
	2012	Males	8	3	18	29	
		Females	9	11	15	35	64
	All years	Total males	21	33	54	108	
		Total females	38	34	51	123	
		Total combined sexes	59	66	105	231	
Gebal	2000	Males	3	7	2	12	
		Females	3	5	8	16	28
	2004	Males	4	2	8	14	
		Females	8	1	20	29	43
	2008	Males	5	2	12	19	
		Females	4	5	15	24	43
	2012	Males	4	7	10	21	
		Females	7	1	16	24	45
	All years	Total males	16	18	32	66	
		Total females	22	12	59	93	
		Total combined sexes	38	30	91	159	
Gharaba	2000	Males	1	5	5	11	
		Females	2	6	9	17	28
	2004	Males	5	8	12	25	
		Females	6	10	19	35	60
	2008	Males	7	3	14	24	
		Females	8	5	15	28	52
	2012	Males	1	4	14	19	
		Females	6	8	19	33	52
	All years	Total males	14	20	45	79	
		Total females	22	29	62	113	
		Total combined sexes	36	49	107	192	
Tlah	2000	Males	4	9	11	24	
		Females	4	5	13	22	46
	2004	Males	2	15	17	34	
		Females	7	8	21	36	70
	2008	Males	13	12	14	39	
		Females	12	12	16	40	79
	2012	Males	4	7	10	21	
		Females	11	8	18	37	58
	All years	Total males	23	43	52	118	
		Total females	34	33	68	135	
		Total combined sexes	57	76	120	253	
Total by year	2000	Males	14	36	25	75	
		Females	20	24	41	85	
		Both sexes	34	60	66	160	
	2004	Males	15	30	49	94	
		Females	28	25	69	122	
		Both sexes	43	55	118	216	
	2008	Males	28	27	57	112	
		Females	35	31	62	128	
		Both sexes	63	58	119	240	
	2012	Males	17	21	52	90	
		Females	33	28	68	129	
		Both sexes	50	49	120	219	
Total by sex		Males	74	114	183	371	
		Females	116	108	240	464	
		Both sexes	190	222	423	835	

The mean species richness was 1.2 ± 0.03. The best fit model was one with a Gaussian error structure (YEAR + SITE + AGE + YEAR x SITE + YEAR x AGE on species richness, adjusted $R^2 = 0.31$). The data in Table 5 show that species richness peaked in 2004 and dropped by more than 50 % to 2012 (main effect of YEAR, $F_{3,826} = 45.9$, $P < 0.0001$), was highest among mice from W. Tlah (main effect of SITE, $F_{3,826} = 38.1$, $P < 0.0001$) and peaked in age class 2 mice (main effect of AGE, $F_{2,826} = 5.5$, $P = 0.004$). While species richness was consistently highest among mice from W. Tlah, there was significant variation in the rank order of species richness among mice for the other wadis from survey to survey (Fig. 4a; 2-way interaction YEAR × SITE, $F_{9,820} = 7.5$, $P < 0.0001$), as illustrated for example among mice from W. Gharaba for which species richness was second highest in 2000 and 2004, but lowest in 2008. The profile of species richness across the three age classes also varied between surveys (Fig. 5a; YEAR × AGE, $F_{6,\ 817} = 3.1$, $P = 0.006$). In three surveys (2004, 2008 and 2012), species richness was highest among

Table 4 Prevalence of haemoparasites by year, site, host sex and age class

	Haemoparasites	*Babesia* spp.	*Bartonella* spp.	*Haemobartonella* spp.	*Trypanosoma acomys*	*Hepatozoon* spp.
Year						
2000	**86.3** (79.11–91.34)	**1.9** (0.44–6.20)	**2.5** (0.76–7.00)	**80.0** (72.16–86.30)	**17.5** (11.68–25.22)	**20.6** (14.26–28.47)
2004	**91.2** (88.25–93.47)	**6.9** (4.91–9.61)	**8.3** (6.12–11.24)	**85.2** (81.69–88.17)	**22.7** (19.08–26.70)	**29.2** (25.18–33.48)
2008	**77.1** (72.86–80.85)	**3.3** (1.97–5.48)	**0.8** (0.31–2.25)	**45.8** (41.09–50.58)	**12.1** (9.28–15.53)	**40.0** (35.42–44.74)
2012	**53.0** (48.40–57.53)	**0** (0–0.82)	**2.7** (1.56–4.65)	**27.9** (23.92–32.10)	**11.9** (9.18–15.16)	**25.6** (21.78–29.76)
Site						
Arbaein	**68.8** (64.36–72.98)	**0.9** (0.33–2.27)	3.5 (2.08–5.59)	**66.2** (61.67–70.55)	**0.4** (0.16–1.57)	**10.4** (7.80–13.61)
Gebal	**72.3** (63.96–79.50)	**8.2** (4.38–14.45)	6.9 (3.61–12.75)	**71.7** (63.24–78.90)	**0.6** (0.07–4.02)	**6.3** (3.17–11.88)
Gharaba	**71.9** (62.51–79.80)	**0.5** (0.04–4.43)	1.6 (0.25–6.27)	**44.8** (35.47–54.56)	**21.9** (14.92–30.98)	**33.9** (25.33–43.58)
Tlah	**88.5** (85.06–91.34)	**4.0** (2.41–6.30)	3.2 (1.81–5.34)	**51.4** (46.52–56.24)	**34.8** (30.27–39.55)	**58.9** (54.04–63.63)
Sex						
Males	76.0 (70.62–80.73)	4.0 (2.21–7.04)	3.0 (1.49–5.70)	56.9 (51.00–62.58)	15.4 (11.55–20.08)	28.3 (23.29–33.90)
Females	76.3 (70.13–81.59)	2.4 (0.97–5.43)	4.1 (2.04–7.60)	58.6 (51.94–65.09)	16.2 (11.75–21.64)	30.8 (25.03–37.24)
Age						
Class 1	69.5 (59.84–77.81)	**6.3** (2.95–12.62)	4.2 (1.56–9.94)	60.0 (50.30–69.17)	**15.3** (9.32–23.40)	**9.5** (5.06–16.61)
Class 2	78.8 (74.85–82.40)	**0.9** (0.35–2.28)	3.2 (1.86–5.17)	57.7 (53.08–62.14)	**26.6** (22.69–30.84)	**36.9** (32.56–41.51)
Class 3	77.8 (72.12–82.65)	**2.8** (1.33–5.81)	3.5 (1.77–6.70)	57.0 (50.64–63.11)	**10.4** (7.03–14.92)	**35.0** (29.14–41.28)

Significant main effects are highlighted in bold

age class 2 mice, but the pattern was different in 2000 with a peak among the oldest animals.

Babesia spp.

The overall prevalence of *Babesia* spp. was just 3.1 % (1.97–4.81). Prevalence varied from 6.9 % in 2004, to zero in 2012, and the difference between the surveys was significant (Table 4; YEAR × INFECTION, $\chi^2_3 = 22.7$, $P < 0.001$), as was that also between sites (Table 4; SITE × INFECTION $\chi^2_3 = 21.9$, $P < 0.001$). Prevalence was highest in W. Gebal and lowest in W. Gharaba, where just one of the 192 spiny mice examined was found to be infected. However, there was also a significant interaction (YEAR × SITE × INFECTION, $\chi^2_9 = 22.7$, $P = 0.007$) and this is illustrated in Fig. 3b. Infections were sporadic in three of the wadis, recorded in only 1 or 2 of the 4 surveys, except in W. Tlah where *Babesia* was detected in three of the four surveys. There was also a marked peak in prevalence in 2004 in mice from W. Gebal, where otherwise in 2000 and 2012 no infections were detected. Prevalence was higher among the youngest age class (Table 4; AGE × INFECTION $\chi^2_2 = 10.8$, $P = 0.004$) compared with the older age classes, but there was no significant difference in prevalence between the sexes.

With just 26 out of 835 animals infected with *Babesia* sp., the overall mean abundance was 0.79 ± 0.600 IN/200FV. Quantitative analysis was problematic with parametric models with Gaussian errors on raw and log transformed data failing to generate acceptable distributions of residuals and to those with negative binomial errors failing to converge (See Methods). Therefore, only the main effects were tested using non-parametric tests, with much the same outputs as those for prevalence (Kruskal-Wallis test for the effects of YEAR, $\chi^2_3 = 18.6$, $P < 0.001$; SITE, $\chi^2_3 = 22.5$, $P < 0.001$ and AGE, χ^2_2 10.1, $P = 0.006$). There was no significant difference between the sexes (Mann-Whitney U test $Z = -1.37$, $P = 0.17$). The mean values are given in Table 5 and the SITE x YEAR effect in Fig. 4b. Mostly mean abundance was less than 0.4 IC/200FV, with the exception of W. Gebal when in

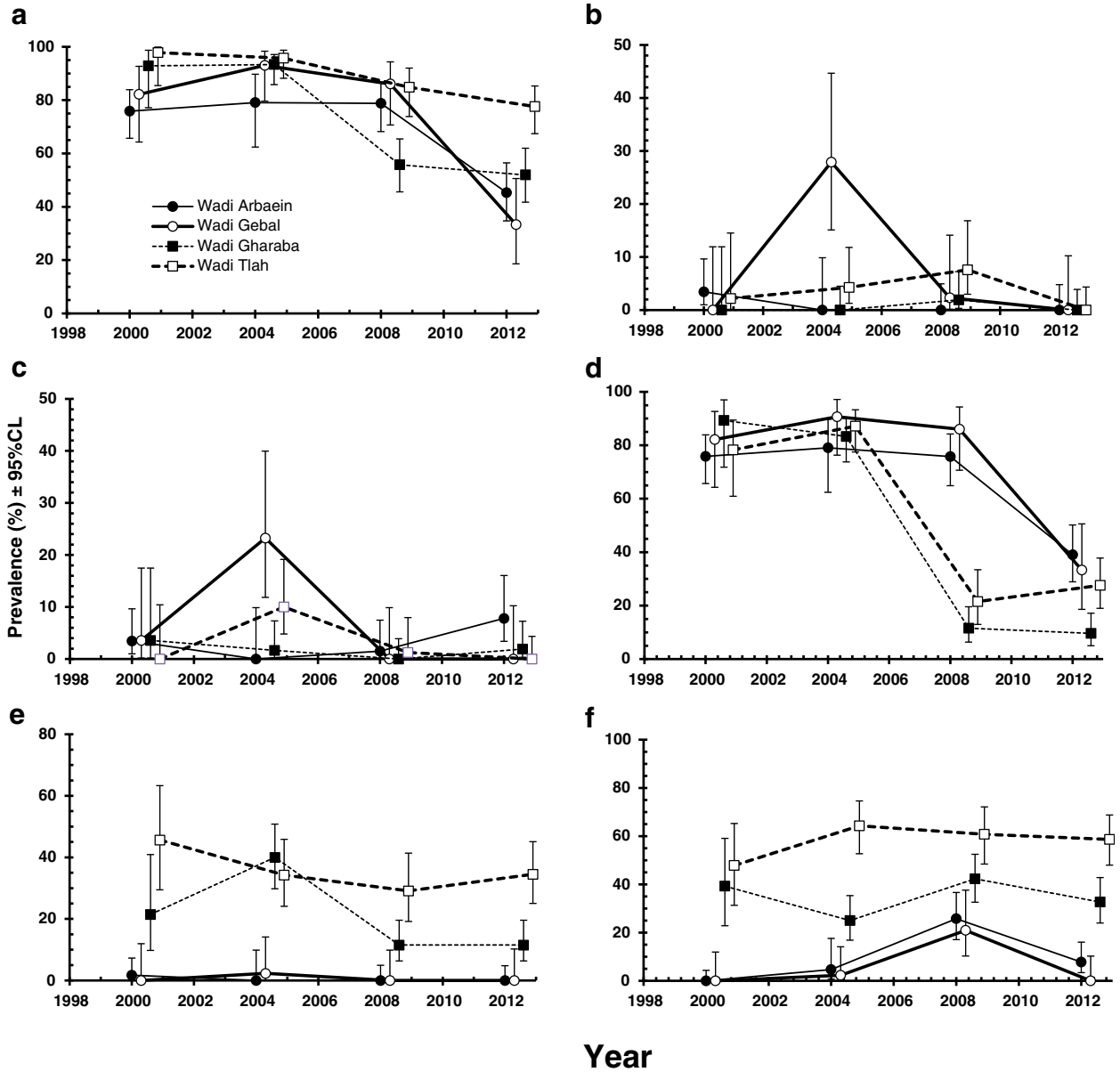

Fig. 3 Prevalence of haemoparasites recorded in spiny mice at the four study sites in the Sinai between 2000 and 2012. **a**, all haemoparasites, combined; **b**, *Babesia* sp.; **c**, *Bartonella* sp.; **d**, *Haemobartonella* sp.; **e**, *Trypanosoma acomys*; **f**, *Hepatozoon* sp. The key to symbols used for the four study sites are given in panel A

2004, twelve mice were infected, and one with 500 IC/200 FV, generating a mean for this site of 13.7 ± 11.60 for that year.

Bartonella spp.

The prevalence of *Bartonella* spp. was also very low (3.6 %, 2.36–5.38). Prevalence varied significantly between years (Table 4; YEAR × INFECTION $\chi^2_3 = 19.0$, $P < 0.001$) with a peak in 2004, mostly accounted for by high values among mice from Wadis Gebal and Tlah. Prevalence also rose in W. El Arbaein in 2012, but otherwise values were very low. There was no independent effect of SITE, but the significant SITE × YEAR × INFECTION interaction ($\chi^2_9 = 28.7$, $P = 0.001$) is illustrated in Fig. 3c. There were no independent effects of host sex or age, but there was a complex interaction involving these factors (SEX × SITE × AGE × INFECTION, $\chi^2_6 = 19.6$, $P = 0.003$) which we did not explore further.

As with *Babesia*, quantitative analysis of abundance of *Bartonella* sp. was problematic because so few animals were infected (30/835). The overall mean abundance was 16.0 ± 10.23 IC/200FV, but this relatively

Table 5 Abundance of haemoparasites by year, site, host sex and age class

	Species richness		*Babesia* sp.		*Bartonella* sp.		*Haemobartonella* sp.		*Trypanosoma acomys*		*Hepatozoon* sp.	
	Mean ± S.E.M.		Mean ± S.E.M.		Mean ± S.E.M.		Mean ± S.E.M.		Mean ± S.E.M.		Mean ± S.E.M.	
Year												
2000	**1.23**	0.063	**0.11**	0.079	**0.04**	0.024	**5.17**	0.502	**4.19**	1.279	**8.65**	1.889
2004	**1.53**	0.058	**2.81**	2.318	**61.72**	39.432	**15.73**	3.435	**5.55**	1.337	**20.29**	4.884
2008	**1.02**	0.047	**0.15**	0.056	**0.07**	0.063	**7.18**	2.159	**0.94**	0.249	**23.98**	4.334
2012	**0.68**	0.050	**0**	0	**0.05**	0.020	**1.30**	0.211	**1.29**	0.330	**15.75**	5.146
Site												
Arbaein	**0.81**	0.042	**0.07**	0.055	**0.05**	0.019	**8.93**	2.543	**0.03**	0.026	**3.84**	1.167
Gebal	**0.94**	0.059	**3.75**	3.147	**69.78**	52.114	**13.09**	3.319	**0.11**	0.113	**1.10**	0.583
Gharaba	**1.03**	0.060	**0.03**	0.026	**0.06**	0.036	**3.28**	0.571	**4.65**	1.438	**15.59**	2.629
Tlah	**1.52**	0.056	**0.17**	0.06	**8.89**	7.929	**5.76**	1.797	**5.77**	0.925	**43.14**	6.835
Sex												
Males	1.08	0.043	0.25	0.073	5.72	5.394	7.68	1.820	1.55	0.262	16.21	2.741
Females	1.12	0.039	1.22	1.079	24.23	17.889	7.29	1.352	3.88	0.766	19.30	3.433
Age												
Class 1	**0.95**	0.057	**0.38**	0.122	63.32	44.54	8.98	3.046	**1.66**	0.419	**2.25**	0.772
Class 2	**1.26**	0.062	**0.13**	0.097	1.09	0.757	6.44	1.897	**3.23**	0.531	**29.32**	6.697
Class 3	**1.09**	0.039	**1.32**	1.183	2.58	2.365	7.32	1.375	**3.18**	0.806	**19.00**	2.664

Significant main effects are highlighted in bold

high value was largely attributable to young mice from Wadis Gebal (mean = 258.0 ± 191.4 with values exceeding 1000 IC/200FV in three mice, maximum =8000) and Tlah (mean = 31.9 ± 28.6; maximum = 2000) in 2004 (Fig. 4c and Table 5). In marked contrast, in the remaining surveys the mean abundance values did not exceed 0.3 IC/200 FV in the other wadis. Analysis was by non-parametric tests which showed that there was a hugely significant difference between surveys (Table 5; Kruskal-Wallis test with YEAR, $\chi^2_3 = 20.9$, $P < 0.001$) and a weaker effect of SITE ($\chi^2_3 = 7.8$, $P = 0.05$). There was no significant difference between the sexes or between the age classes.

Haemobartonella spp.

The overall prevalence of *Haemobartonella* spp. was 57.8 % (53.88–61.73). However, as can be seen in Table 4 and Fig. 3d, initially in the first two surveys prevalence was higher exceeding 75 % in four wadis, but then prevalence fell markedly in two wadis in 2008 (Gharaba and Tlah), and the other two also by 2012 (YEAR × INFECTION, $\chi^2_3 = 205.4$, $P < 0.001$). The difference in prevalence between wadis was also significant (Table 4; SITE × INFECTION, $\chi^2_3 = 37.4$, $P < 0.001$), with the highest overall value in W. Gebal and the lowest in W. Gharaba. Prevalence of this species was not affected by either host age or sex, but there was a significant interaction between all four factors and INFECTION ($\chi^2_{18} = 35.9$, $P = 0.007$), suggesting some differences between age classes and the two sexes in particular data subsets (i.e. in particular years and sites) which we did not explore further.

The mean abundance for *Haemobartonella* sp. was 7.5 ± 1.10 IC/200FV. The best-fit model was a GLM with negative binomial errors (YEAR + SITE + AGE + YEAR x SITE+ SITE x AGE). There were highly significant main effects of YEAR ($LR_{3,826} = 182.1$, $P < 0.0001$) and SITE ($LR_{3,826} = 76.5$, $P < 0.0001$) and these are summarised in Table 5. Abundance peaked in 2004 and was lowest in 2012, and peak abundance was detected in mice from W. Gebal, whilst the lowest value was from those from W. Gharaba. The highly significant interaction between SITE and YEAR ($LR_{9,811} = 120.9$, $P < 0.0001$) is illustrated in Fig. 4d. This shows the dynamic changes that occurred between surveys, with peak abundance among mice from W. Gebal in three surveys (2000, 2008 and 2012) but not in 2004 when peak abundance was among mice from W. El Arbaein. There was no main effect of host age ($LR_{2,826} = 0.78$, $P = 0.96$), but there was a significant interaction between SITE and AGE ($LR_{6,811} = 19.5$, $P = 0.0034$) which is illustrated in Fig. 5b. As can be seen, the age-prevalence profiles were quite different among mice from each of the 4 wadis, although there was some similarity between those from Wadis Gharaba and Tlah where overall prevalence was lowest. Peak prevalence was observed in age class 1 mice in Wadi El Arbaein, in age class 3 in Wadi Gebal and in age class 2 in Wadis Gharbara and Tlah. An alternative

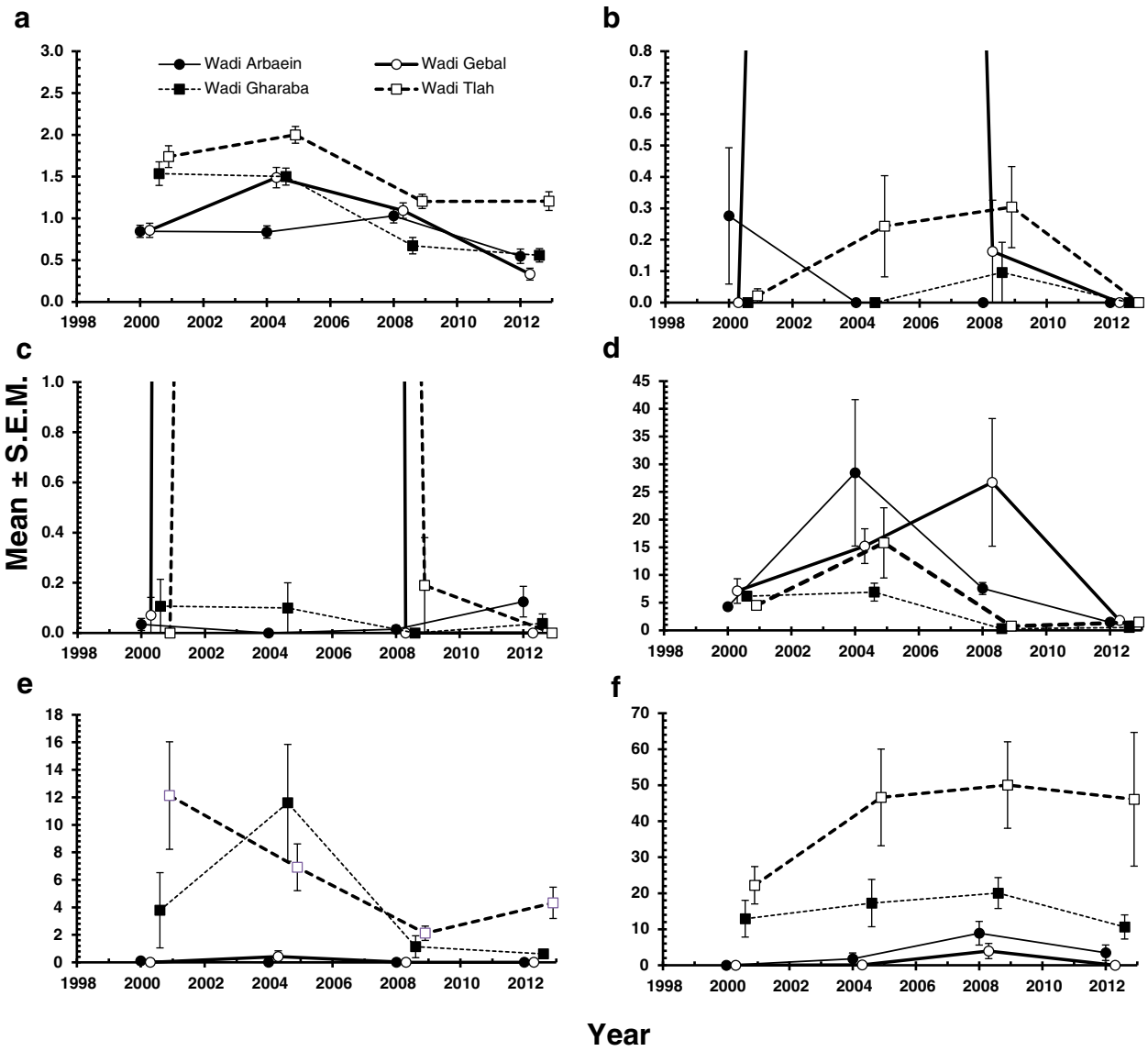

Fig. 4 Abundance of haemoparasites recorded in spiny mice at the four study sites in the Sinai between 2000 and 2012. **a**, species richness; **b**, *Babesia* sp.; **c**, *Bartonella* sp.; **d**, *Haemobartonella* sp.; **e**, *Trypanosoma acomys*; **f**, *Hepatozoon* sp. In b, the abundance of *Babesia* sp. in mice from Wadi Gebal in 2014 was 13.7 ± 11.60, and in c the abundance of *Bartonella* sp. in mice from wadis Gebal and Tlah was 257.98 ± 191.37 and 31.9 ± 28.62, respectively. The key to symbols used for the four study sites are given in panel A

model based on log-transformed abundance and Gaussian errors generated much the same output ($R^2 = 0.34$).

Trypanosoma acomys

The overall prevalence of *T. acomys* was 15.8 % (13.09–18.92). Although prevalence changed significantly over the four surveys, peaking in 2004 (Table 4; YEAR × INFECTION, $\chi^2_3 = 12.5$, $P = 0.006$), there was a huge difference between the wadis (SITE × INFECTION, $\chi^2_3 = 175.3$, $P < 0.001$), with this species being largely confined to two of the four study sites in all four surveys (Gharaba and Tlah; Fig. 3e). There was no difference in prevalence between the two sexes, but there was a significant difference between age classes (AGE × INFECTION, $\chi^2_2 = 26.8$, $P < 0.001$). Prevalence was highest in age class 2 (Table 4), and then dropped by more than 50 % in the oldest age class. There were no significant confounding interactions in this case.

Since this parasite was largely confined to just two of the four wadis, we recalculated the effect of age on prevalence restricting the data to mice from wadis Gharba and Gebal, and this is shown in Fig. 6a. Prevalence in age class 2 spiny

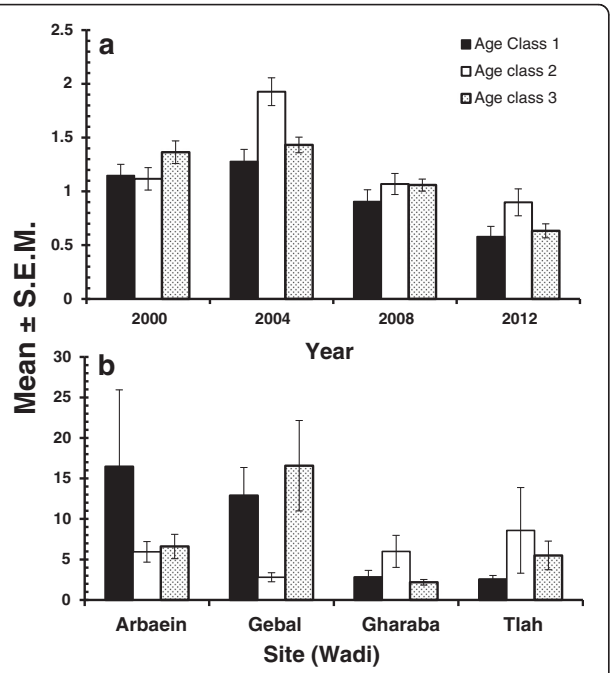

Fig. 5 Variation in haemoparasites in spiny mice sampled by age class. **a**, species richness by year of survey; **b**, abundance of *Haemobartonella* sp. by site

mice was now 46.4 % (38.75–54.16), falling to 18.9 % (15.57–22.85) among the oldest mice, a reduction of 59.2 %.

The mean abundance of *T. acomys* was 2.85 ± 0.443. Parametric models based on raw data with Gaussian or negative binomial errors, and $\log_{10}(X + 1)$ transformed data with Gaussian errors all failed to generate acceptable distributions of residuals. However analysis by 1-way non-parametric tests identified a marked effect of SITE (Table 5; Kruskal-Wallis test, $\chi^2_3 = 141.5$, $P < 0.001$) mean abundance of trypanosomes being higher in mice from W. Gharaba and Tlah. There were also significant changes in abundance between the 4 surveys (Kruskal-Wallis test on effect of YEAR $\chi^2_3 = 16.1$, $P = 0.001$) with mean abundance higher in the first two surveys compared with the latter two. Abundance also increased with host age, especially between the youngest mice and age class 2, and then fell marginally in the oldest mice (Kruskal-Wallis test on effect of AGE $\chi^2_2 = 26.8$, $P < 0.001$). There was no significant difference in abundance between the sexes. Changes of abundance over time in the 4 wadis are illustrated in Fig. 4e, where it can be seen clearly that mean abundance was consistently higher among mice from Wadis Gharaba and Tlah compare to those from Wadis El Arbaein and Gebal.

Hepatozoon sp.

The prevalence of this species was 29.7 % (26.17–33.47). As with *T. acomys*, *Hepatozoon* sp. was largely confined to the same two wadis (Gharaba and Tlah), and not surprisingly there was a huge SITE effect (SITE × INFECTION, $\chi^2_3 = 198.6$, $P < 0.001$). Prevalence also varied between the surveys (YEAR × INFECTION, $\chi^2_3 = 20.2$, $P < 0.001$) and differently among the mice from the four wadis (Fig. 3f; SITE × YEAR × INFECTION, $\chi^2_9 = 36.1$, $P < 0.001$). There was no effect of host sex, but age affected prevalence significantly (Table 4; $\chi^2_2 = 56.7$, $P < 0.001$), and the precise effect of age on prevalence varied between the surveys (Not illustrated, AGE × YEAR × INFECTION, $\chi^2_6 = 17.7$, $P = 0.007$). Very few of the youngest mice were infected, but then prevalence increased and stabilized in mature and older individuals some 3–4 times higher (Table 4). However, since this species, like *T. acomys*, was largely confined to mice from Wadis Gharaba and Tlah, the analysis was repeated excluding individuals from the other two wadis and this is illustrated in Fig. 6b. Prevalence was still very low among the youngest mice (16.1 % [7.95–28.36]), but rose to over 55 % among age classes 2 and 3.

The mean abundance of this species was 17.9 ± 2.26. Parametric models based on raw data with Gaussian or negative binomial errors, and $\log_{10}(X + 1)$ transformed data with Gaussian errors all failed to generate acceptable distributions of residuals. Analysis by 1-way non-parametric tests identified a marked effect of SITE (Table 5; Kruskal-Wallis test, $\chi^2_3 = 190.8$, $P < 0.001$), mean abundance of *Hepatozoon* sp. being highest in W. Tlah and lowest in W. Gebal. There were also significant changes in abundance between the 4 surveys with peak abundance detected in 2008 (Kruskal-Wallis test on effect of YEAR $\chi^2_3 = 21.2$, $P = 0.001$). As with *T. acomys* abundance was lowest in the youngest mice, higher among age class 2 before falling in age class 3 (Kruskal-Wallis test on effect of AGE $\chi^2_2 = 51.1$, $P < 0.001$). There was no significant difference in abundance between the sexes. Changes of abundance over time in the four wadis are illustrated in Fig. 4f, where the consistently high abundance among mice from W. Tlah can be seen. This is followed by a consistent intermediate level of abundance among mice from W. Gharaba and much lower abundance among animals from the remaining two wadis, although there was a small peak in abundance in 2008 among mice from the latter two sites.

Discussion

Molecular identification of parasites

Molecular techniques were used for the identification of parasite species/genotype and for a preliminary study of the diversity of parasites among the four semi-isolated populations of spiny mice. Analyses of the sequences that we obtained did not allow exact identification of species (no 100 % sequence homology was found in the GenBank database) of *Bartonella, Trypanosoma* or *Hepatozoon* as to-date, excepting our own depositions,

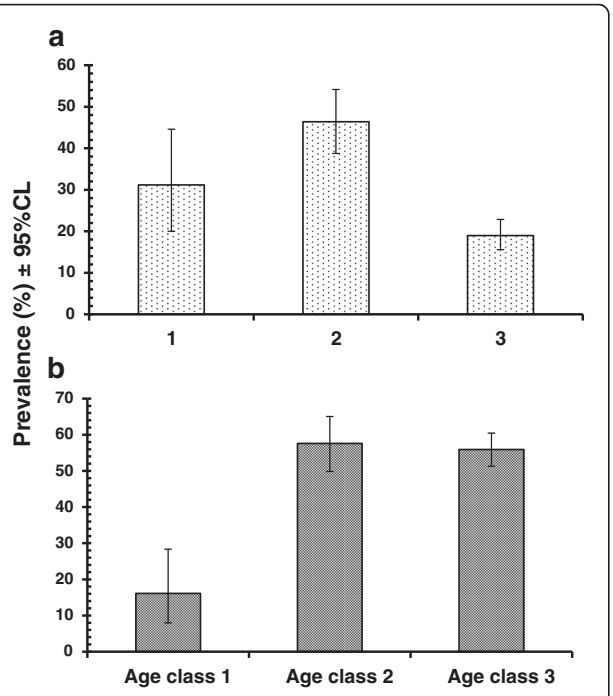

Fig. 6 Prevalence of *T. acomys* **a** and *Hepatozoon* sp. **b** in mice of three age classes, restricted to animals from Wadis Gharaba and Tlah

no data on the haemoparasites specific to *A. dimidiatus* have been deposited. The closest match was for *Bartonella*, as our sequence was >99 % similar to *B. acomydis*, the species described from the golden spiny mouse, *A. russatus* [35]. As *Bartonella* species/genotypes show some host-specificity, further study on the *Bartonella* from *A. dimidiatus* is needed to clarify with certainty the identity of the parasite in the spiny mice in our study sites. Interestingly, in our study more *Bartonella*-positive mice were detected by conventional microscopy of blood smears, but only one PCR product was obtained with the primers that we used. In Inoue et al. [35], the study referred to above, the overall prevalence of *Bartonella* in *A. dimidiatus* imported from Egypt to Japan was 9.7 %, relatively low as in our study, but in three isolates *B. elizabethae* was identified (100 % homology with GenBank reference sequences), a species that is pathogenic for humans. Therefore, it is still possible that other species/strains of *Bartonella* may be found in the future in spiny mice from our S. Sinai study sites.

There is only one species of *Trypanosoma* described from spiny mice, *T. acomys* [37, 38], but no reference sequences for 18S rDNA of this species are available in GenBank. However, almost no diversity was observed in the 45 *Trypanosoma* sequences that we compared and this supports the identification of our isolates as the host-specific *T. acomys*. Interestingly, one of our sequences was different (variant B), but still most closely related to our dominant variant- variant A of *T. acomys*. *T. acomys* was found in mice from two quite distant wadis (W. Tlah and W. Gharaba) which despite the distance between them are connected, W. Gharaba being located at the extreme north end of the continuous valley system leading away from St. Katherine [9], and there was no detectable diversity between the isolates from these two wadis.

The genotyping of *Hepatozoon* isolates from the two *Acomys* species revealed the presence of two variants, A and B, and neither of these could be identified to species level through comparison with data in GenBank (at best homology was 96–97 % with known *Hepatozoon* species). Only one species of *Hepatozoon* has been described from *Acomys* spp. - *H. acomys* (Mohammed and Saound 1972, following [39]) and generally the systematics of this genus are still poorly developed [36, 39]. The majority of identified *Hepatozoon* infections from different groups of animals, including amphibians, reptiles and mammals, are reported simply as '*Hepatozoon* sp.' However, even among the conserved 18S rDNA sequences of those *Hepatozoon* spp. isolates that have been deposited, there are significant differences (Fig. 1) and there is an urgent need to name the different genotypes of *Hepatozoon* that are known to be associated with certain host species. Only one detailed description of *Hepatozoon* from Egyptian rodents is currently available- *H. balfouri* in *J. jaculus* and *J. orientalis* [40]. However, the gamonts of this species were found only in erythrocytes, and never in leucocytes, in contrast to the *Hepatoozon* that we observed in *A. dimidiatus*. Moreover, a recent study of *Hepatozoon* from jerboas revealed sequences that were quite different to our own isolates (Fig. 1; [36]), supporting the existence of a different *Hepatozoon* species in *A. dimidiatus*. Interestingly, our variant B of *Hepatozoon* was found only in one of the two *Acomys* species, *A. russatus*, which is consistent with the idea of host-specificity among parasites in this genus.

Long-term ecological study

Our data show that the haemoparasite communities varied markedly between the four subpopulations of spiny mice living in isolated wadis and displayed long-term trends, most likely associated with the increasing aridity of the environment during the years of our surveys. The haemoparasite communities of *A. dimidiatus* were dynamic, with only some species showing stability across the 12-year-long period. As predicted, external factors (site and year of study) had a much greater influence on the haemoparasite community than the intrinsic factors that we took into account in our analyses (host age or sex).

Long-term dynamic changes of haemoparasites were observed in prevalence, abundance and in mean species richness, as reflected in the spatio-temporal patterns/trends illustrated in Figs. 3 and 4 (the year x site interactions). While the prevalence of haemoparasites was

relatively high and stable during the first two surveys, in 2000 and 2004, there was a 50 % reduction by the last survey in 2012, and this pattern of declining prevalence was observed clearly among the spiny mice in 3 of the 4 wadis in our study (W. El Arbaein, W. Gebal, W. Gharaba), with only slightly lower values for mice from W. Tlah. Mean species richness was the highest in 2004 and then decreased by more than 50 % to 2012. Both of these two parameters (prevalence and mean species richness) were significantly reduced in 2008 and then in 2012, compared with the earlier surveys. The fall in value of both parameters may have two non-mutually exclusive explanations– an exceptionally high prevalence of two 'rare' pathogens, *Babesia* and *Bartonella* sp. in 2004, exceeding 20–30 % in W. Gebal, and the marked decrease in *Haemobartonella* sp., in 2008 in W. Tlah and Gharaba, and then in 2012 in W. El Arbaein and Gebal. Although overall prevalence of *T. acomys* was generally stable over the 12-year-long period, the greatest stability was observed in W. Tlah while in W. Gharaba prevalence of *Trypanosoma* followed the general trend in the prevalence of other haemoparasites – a significant, albeit relatively small, reduction during last two surveys, contributing to an overall drop in haemoparasite prevalence and mean species richness. Interestingly, even for this 'stable' parasite, the abundance and intensity of infection were much lower in 2008 and 2012 in both W. Tlah and Gharaba, compared to the first two surveys. An opposite trend was found for *H. acomys* – the only parasite species displaying an increase in prevalence in 2008 and a spread to new sites.

This fall in the value of three parameters must reflect a drop in transmission of these vector-borne parasites. There have been severe fluctuations in the weather in the Sinai over the past decade (2002–2010). Since 2002 there has been a severe drought with very little (< 50 mm) or no rain every year until March 2010 and therefore one underlying explanation could be the marked decrease in water availability, first noted during the expedition in 2008. This was the first expedition during which we observed a lack of water pipes that are usually employed to deliver water to Bedouins' gardens situated at higher altitudes in the wadis, the lack of water in wells located in the wadis, the abandonment of several gardens in W. Gebal and Gharaba, resulting not only in a lack of ground-cover plants and vegetables but also in desiccation of trees. This drought was broken in May 2010 when there was heavy rainfall. 2011 was again extraordinarily wet, with heavy rainfall and snow in the winter and spring, while 2012 had very little rainfall and was colder than normal. Although almost the wettest place in Egypt (second only to the Mediterranean northwestern coast), according to the best (patchy) data we have, the mountains of South Sinai received only an average of 42.5 mm per year precipitation between 1970 and 1994, and substantially less (15.5 mm) between 2001 and 2009 (data courtesy of the St Katherine Protectorate Management Unit) [28]. These weather conditions have probably caused the marked changes recorded in a parallel long-term study on the Sinai thyme (*Thymus decussatus*) population. Between 2002 and 2010, the number of thyme plants in Farsh Shoeib near St Katherine fell from 1208 to 669, i.e. 44.6 % of the plants disappeared (assumed to have died). Between 2002 and 2010, the condition of plants deteriorated decreasing from 53 % to 25 %; one-third of the surviving plants were <10 % green [28].

Most likely as a result of these local climatic changes, a greater effort had to be made to catch representative numbers of mice for our study - reflected in the significantly higher number of trapping hours in 2008 and 2012, in comparison to 2000 and 2004. Although we are not able to conclude with any degree of certainty if rodent population sizes have actually fallen because of this significant change in their habitats (reduction in water availability and in the acreage of Bedouin gardens), we may nevertheless be certain that at least the population densities of the mouse subpopulations were lower in the latter two surveys. This may have affected the transmission of parasites with consequent lower prevalence - both density-dependent or frequency-dependent, as established convincingly in the long-term studies on cowpox virus transmission in field voles, *Microtus agrestis,* in Kielder forest, UK (reviewed in [3]). Changes in the abiotic factors in the study sites affected not only the host populations but also could have had a direct negative impact on the survival of parasite vectors such as juvenile fleas and ticks, contributing also to overall lower transmission rates.

Generally, the changes observed in the haemoparasite communities of spiny mice in the Sinai were much more pronounced and more diverse than the patterns/trends observed in our other studies on haemoparasites in common and bank voles from central Europe (Poland) [4, 19, 20]. This marked dynamic may reflect a more fragile structure of parasite communities in the hyper-arid environment in Egypt, in comparison to the relatively more predictable abiotic conditions in woodland habitats in central and northern Europe, which show marked seasonal changes but an annual sequence of changes that varies little from year to year.

Although we observed significant temporal changes, nevertheless the site of sampling of the mouse population was always the main factor influencing haemoparasite community structure and many of the differences between the wadis that we observed in 2000 [10] were maintained during the subsequent 12-year-long period of monitoring. Subpopulations from wadis Tlah and Gharaba constituted the main hosts for *T. acomys* and *Hepatozoon* sp., and

spread of the latter species to other mouse populations observed in 2008 was only partially successful. It is apparent therefore that ideal conditions for maintaining the host-vector-parasite relationship exist only in these two sites/habitats. As we reported in our earlier paper [10], fleas (*Parapulex chephrensis)*, the most likely vectors of *Trypanosoma* [41] and *Hepatoozon,* were found mostly on mice from Wadis Tlah and Gharaba (unpublished observations for 2012 confirm the published data from 2000, reported in [10]). In 2000 no fleas were found on mice from W. Gebal, and only two mice from W. El Arbaein were found with fleas. The overall prevalence of *T. acomys* among mice with its likely flea vector was 44.8 % compared with just 12 % among mice without fleas and there was a weak but statistically significant overall association between prevalence of *T. acomys* and flea infestation [10]. Similarly, in 2000 the prevalence of *Hepatozoon* sp. among mice with fleas was 41.4 %, in contrast to 16.8 % among mice without fleas. However, when the site, sex and age effects were controlled for, no significant association was evident between these taxa.

Comparing haemoparasite communities between the host subpopulations from the four wadis, spiny mice from W. Tlah consistently showed the highest mean species richness, total species richness and the highest prevalence of all haemoparasites. This site experienced also the least change in habitat structure over the 12-year period and the lowest loss of arable land. Wadi Tlah consists of a deep, narrow valley with steep cliffs on both sides, with ample shaded areas, well-maintained gardens and few apparent signs of aridification because it drains most of the high-mountain region, so any rainfall will percolate through it. The prevalence of *T. acomys* underwent almost no change in mice from this wadi. In contrast, by the end of our study period mice from the high-altitude W. Gebal showed the lowest mean species richness and lowest prevalence of all haemoparasites. This is in agreement with previous studies on haemoparasites from *A. dimidiatus* from this wadi, and is also consistent with studies on intestinal micro- and macroparasites and ectoparasites [9, 10]. Mice from W. Gebal have consistently revealed an impoverished parasite fauna, lacking both the flea *P. chephrensis* and *T. acomys*, in addition to the absence of the dominant nematode *Protospirura muricola* [9]. However, this wadi constituted the main focus for the transmission of a novel *Babesia* species- *B. behnkei*– discovered in Wagner's gerbil *D. dasyurus* in 2004 and recently described [42]. This high-altitude wadi experiences the most extreme abiotic conditions and greatest degree of aridification/desertification: by 2008 about half of Bedouins' gardens in this wadi had been abandoned and in 2012 even the remaining trees and bushes were extensively desiccated and damaged by grazing feral donkeys. In contrast, mice from the low-level W. Gharaba, which has greater exposure to direct sunlight and is considerably warmer than the other three wadis, were heavily infected with *P. muricola*, *P. chephrensis* and *T. acomys* [9, 10]. As with W. Gebal, this site has experienced severe shortage of water with resulting increased aridity, and this change in climatic conditions is reflected in reduced prevalence and abundance of both *Haemobartonella* sp. and *T. acomys*, but interestingly not *Hepatozoon*. Wadi El Arbaein is the site that is most affected by human activities, with a large town (St Katherine) localized at its mouth, and it experiences extensive exposure to livestock, mainly goats and camels, but also cats and dogs. This is also the wadi with the highest tourist activity. During the 12-year-long period the town has grown and developed (i.e. construction of new paved roads, lighting on the streets) but has also been affected by drought, the level of ground water having fallen alarmingly over this period (from 7 m to about 25 m in Fox Camp). The construction of new water storage tanks and water pipes for the provision of water for the city from the coast had not been successfully completed by 2012. Perhaps not surprisingly therefore, the mice from W. El Arbaein showed the lowest prevalence of haemoparasites in the early surveys and a variable pattern of haemoparasite species richness and prevalence, generally as in the mice from W. Gebal. Interestingly, as in the mice from W. Gebal, *H. acomys* was introduced to the mice in this wadi in 2004 and then increased in prevalence and persisted through to 2012, creating a third location for the occurrence of this parasite among spiny mice in our study sites.

As we had predicted, host sex has no detectable influence on the haemoparasite community, and this is consistent with many earlier studies [3, 22–24], including our work in Egypt and Poland [4, 10, 20, 21]. However, there were two contrasting patterns in relation to host age. For *Babesia* and *Haemobartonella*, the highest prevalence or abundance were observed in young animals, but for *Trypanosoma* and *Hepatozoon* the highest infection parameters were from adults (class 2 or 3). The former pattern of increasing likelihood of carrying infection with increasing host age is consistent with the idea that the longer the mouse lives, the higher the probability of being infested by an infected vector and hence of contracting the infection. Most of the parasites identified in this study typically cause long subclinical infections in their natural hosts [37, 42], and thus the proportion of animal carrying infection increases with host age, peaking among the older animals. The latter pattern of peak prevalence in young animals, as observed in the case of *T. acomys* and *Hepatozoon* sp., can have two explanations. One possibility is vertical transmission resulting in congenital or neonatal (i.e. transmitted by nest ectoparasites) infection and this combined with the high susceptibility of young naïve individuals to infections, and

followed by acquired immunity reducing prevalence among the older sectors of the population. Vertical transmission and congenital infections have been confirmed for *Babesia* spp. [43, 44], *Hepatozoon balfouri* [40] and *Haemobartonella* (*Mycoplasma*) [45].

Rodent trypanosomes are known to generate potent sterilizing immunity. Thus the declining prevalence of *T. acomys* in the oldest age class, was not unexpected given that this species probably generates life-long immunity just like *T. lewisi* and *T. musculi* to which it is closely related. The long patent period explains the high prevalence of infection with *T. acomys* in mice of age class 2, and the decline in the oldest mice (age class 3) suggests either the action of some form of immunity in laterlife or selective mortality of infected older mice. An immune response certainly occurs in *T. lewisi* and *T. musculi*, and is suggested by the data of Abdallah et al. [37] for *T. acomys*, which show clearance from peripheral blood after 150 days, although a mechanism for this has not yet been identified.

Conclusions

Haemoparasite communities varied markedly between four subpopulations of spiny mice living in isolated wadis and displayed long-term trends, most likely associated with a changing environment driven by decade-long drought. As predicted, external factors (site and year of study) had a much greater influence on parasite communities than intrinsic factors (host age or sex).

Additional files

Additional file 1: Nucleotide sequences and annealing temperature of the primers used for polymerase chain reaction (PCR). (DOCX 17 kb)

Additional file 2: Alignment of two 18S rDNA *Hepatozoon* variants with the two most similar sequences of *Hepatozoon* from the GenBank database. (DOCX 74 kb)

Additional file 3: Alignment of two 18S rDNA *Trypanosoma* variants with the most similar sequences of *Trypanosoma* from the GenBank database. (DOCX 104 kb)

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MA and RWF performed microscopical, molecular and phylogenetic studies, AB designed the study and supervised laboratory and field analyses, MB and AK participated in detection of haemoparasites, JMB, FG and SZ organized and supervised field work in Sinai. EJM, JBB and EMEM performed the laboratory and field studies and drafted the manuscript. All authors read and approved the final version of the manuscript.

Acknowledgements

The molecular study of haemoparasites and Polish staff expedition to Egypt in 2012 were funded by the National Science Center (NCN), Poland, grant OPUS 2011/03/B/NZ6/02090(AB) and by the Ministry of Science and Higher Education through the Faculty of Biology, University of Warsaw intramural grant DSM number 140000/501/86-110101 (MA). We are grateful to the University of Nottingham for providing travel expenses and to the British Council, Cairo, Egypt, for financial support of this study. Anna Bajer's expedition in 2008 was supported financially by KBN-BC Young Scientist Program no. WAW/342/06. We are grateful to all the staff of the Environment Research Centre of Suez Canal University and our Bedouin hosts from Fox Camp at St Katherine for their support and warm hospitality during our stay. We thank Mohammed Shaker and Mohamed Qotb for permission to work in the St Katherine Protectorate, the staff at the Rangers office for providing vehicles and drivers, and skilled Bedouin guides, enabling access to some of the remote locations and for their company and support on each of the expeditions. Mustafa Rashid El-Rafaei's skills in trapping rodents are also gratefully acknowledged.

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Received: 23 October 2015 Accepted: 23 March 2016
Published online: 08 April 2016

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Babesia behnkei sp. nov., a novel Babesia species infecting isolated populations of Wagner's gerbil, *Dipodillus dasyurus*, from the Sinai Mountains, Egypt

STATEMENT OF CO-AUTHORS OF JOINT PUBLICATION

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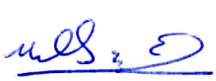
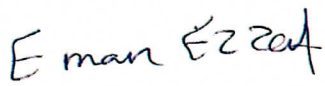
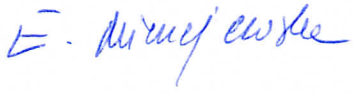
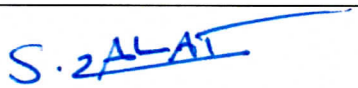
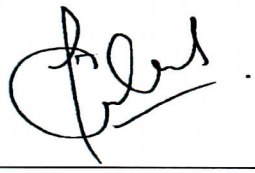
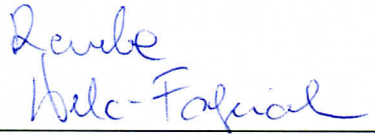
We, the undersigned, co-authors of the above publication, confirm that we are aware the above publication is included to the PhD thesis of Mohmmed Alsarraf.

Anna Bajer	designed the study and supervised laboratory and field analyses	
Mohammed Alsarraf (40%)	performed molecular and phylogenetic studies	
Małgorzata Bednarska	participated in biological characterization of novel *Babesia* and supplied reference *B. microti* strain	
Eman ME Mohallal	performed the laboratory and field studies and drafted the manuscript	
Ewa J. Mierzejewska	performed the laboratory and field studies and drafted the manuscript	
Jolanta Behnke-Borowczyk	performed the laboratory and field studies and drafted the manuscript	

Sammy Zalat	organized and supervised field work in Sinai	
Francis Gilbert	organized and supervised field work in Sinai	
Renata Welc-Falęciak	performed molecular and phylogenetic studies	

Parasites & Vectors

RESEARCH Open Access

Babesia behnkei sp. nov., a novel *Babesia* species infecting isolated populations of Wagner's gerbil, *Dipodillus dasyurus,* from the Sinai Mountains, Egypt

Anna Bajer[1*], Mohammed Alsarraf[1], Małgorzata Bednarska[1], Eman ME Mohallal[2], Ewa J Mierzejewska[1], Jolanta Behnke-Borowczyk[3], Sammy Zalat[4], Francis Gilbert[5] and Renata Welc-Falęciak[1]

Abstract

Background: Although a number of new species of *Babesia/Theileria* have been described recently, there are still relatively few reports of species from Africa. In this study based on the evaluation of morphology and phylogenetic relationships, we describe a novel species from Wagner's gerbil, *Babesia behnkei* n. sp.

Methods: Rodents (n = 1021) were sampled in four montane valleys (wadies) in 2000, 2004, 2008 and 2012 in the Sinai Mountains, Egypt. The overall prevalence of *Babesia* spp. was highest in the Wagner's gerbil (*Dipodillus dasyurus*; 38.7%) in comparison to the prevalence in the spiny mice species, *Acomys dimidiatus* and *A. russatus*. Morphological investigations were conducted for the comparison of trophozoites of the novel species of *Babesia* with the *B. microti* King's 67 reference strain. Thirty-two isolates derived from *D. dasyurus* over a 9 year period (2004-2012) from two wadies (29 isolates from Wadi Gebel and 3 from Wadi El-Arbaein) were investigated by microscopic, molecular and phylogenetic analysis. A near-full-length sequence of the 18S rRNA gene and the second internal transcribed spacer (ITS2) region were amplified, sequenced and used for the construction of phylogenetic trees.

Results: A novel species of *Babesia* was identified in two isolated populations of *D. dasyurus*. Phylogenetic analysis of 18S rDNA and ITS2 sequences revealed that *B. behnkei* n. sp. is most closely related to *B. lengau* from cheetahs from South Africa and to Nearctic species found only in North America (the pathogenic *B. duncani* and *B. conradae*) and that it is more distant to the cosmopolitan rodent parasite *B. microti*. Trophozoites of *B. behnkei* were smaller and less polymorphic than trophozoites of *B. microti*.

Conclusion: *Babesia behnkei* n. sp. is a novel species of the 'Duncani group' maintained in isolated populations of *Dipodillus dasyurus* occurring in the Sinai Mountains of Egypt.

Keywords: *Babesia*, *Dipodillus dasyurus*, 18S rDNA, ITS2, Phylogenetic analysis, Sinai, Egypt

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Background

The genus *Babesia* comprises tick-transmitted, intraerythrocytic protozoan parasites of many different vertebrates including humans [1,2]. Currently there are over 120 recognized species of *Babesia* described from various parts of the world. Even in the last two decades new species have been added to the list, e.g. *B. venatorum* in humans in Europe, *B. benneti* in the yellow-legged gull [3], *B. hongkongensis* in feral cats in Hongkong [4] and a novel *Babesia/Theileria* species from marsupials in Australia [5].

In contrast to the rest of the world, relatively few new species have been described from African hosts in recent years, including for example *B. lengau* from cheetahs [6], *B. bicornis* from black rhinoceros [7], *B. ugwidiensis* from cormorants [8] and *B. leo* from lions [9]. Additionally, putative new species of *Babesia/Theileria* have been reported from sable antelopes [10] and wild felids from Kenya [11]. It is pertinent that new species of *Babesia* (and presumably also other haemoparasites) are often discovered at post-mortem examinations, especially in the case of endangered host species such as the sable antelope and the black rhinoceros.

The diversity of *Babesia* spp. depends on many factors, including host-parasite or vector-parasite specificities, well reflected in the geographically restricted distribution of some species. Cosmopolitan species include parasites of livestock and horses (*B. bovis, B. divergens, B. equi, B. ovis*), dogs or cats (*B. canis, B. rossi, B. vogeli, B. felis*) or rodents (*B. microti*). Other species are specific to particular hosts whose distribution is restricted to continents, as for example with *B. conradae, B. duncani* or *B. odocoilei* found in North America, *B. benetti, B. capreoli, B. venatorum* found in Europe, and *B. crassa, B. hongkongensis, B. motasi, B. orientalis* found only in Asia. In this last Asian group of species, some have been identified to date only in a single host species. However, it is also well established that some hosts are susceptible to, and can carry concurrently, more than a single species of *Babesia/Theileria*; often these species are indistinguishable by conventional microscopy. For example, cats are susceptible to infection with *B. felis* but also with *B. leo*, *B. hongkongensis* and *B. lengau* [4,12,13]. In view of this complexity, it is highly likely that many *Babesia* spp. remain still unrecognized, especially those infecting rarely studied wild species of hosts in isolated regions of the world.

Conventionally and historically, new species of *Babesia* have been erected based on their hosts and on morphological criteria. However, the trophozoites of different species of 'small' *Babesia* and *Theileria* spp. in erythrocytes appear very similar under light microscopy and their differentiation is difficult. In recent decades however, the use of molecular tools have made a significant impact on the field and the sequencing of selected gene fragments has greatly improved the accuracy and reliability of species identification. However, because of the morphological similarities, the systematics of *Babesia/Theileria* spp. are still not fully resolved and in urgent need of revision in view of the many recently conducted molecular phylogenetic studies [14-16]. Based on these, the distant clades of '*Babesia*', including some species that were misidentified as '*Theileria*' [14], require revision of their generic status and new nomenclature. Thus the use of molecular tools, which are clearly more sensitive than conventional morphology based on light microscopy, remains crucial for distinguishing between and for the identification of *Babesia/Theileria* spp. and for their assignment to particular clades.

One cosmopolitan species of public health concern is *B. microti*, the main cause of human babesiosis in the United States of America [2,17,18] but also identified recently in humans in Europe [19,20], China and Japan [21]. This species had been originally described as *Smithia microti* in Portugal from the vole *Microtus incertus* [22]; voles of the genus *Microtus* are still considered to be the main reservoir of this parasite worldwide [22,23]. Surprisingly *B. microti* has subsequently been found in a wide variety of rodent species worldwide [21,22,24-34].

In Eurasia and North America the main rodent hosts of *B. microti* are different species of voles, *Microtus* spp., *Myodes (Clethrionomys*) spp. and mice, *Apodemus* spp. At least 8 species of *Microtus* have been reported as commonly infected with *B. microti*: *M. arvalis*, *M. agrestis* and *M. oeconomus* from Europe [22,32,35,36], *M. montebelli* from Japan and *M. miurus*, *M. montanus*, *M. ochrogaster* and *M. pennsylvanicus* from North America [21,33,34,37]. Additionally, 4 species of *Myodes* (*Clethrionomys*) (*M. glareolus*, *M. gapperi*, *M. rufocanus* and *M. rutilus*) and 5 species of *Apodemus (A. agrarius*, *A. argenteus*, *A. flavicollis*, *A. speciosus* and *A. sylvaticus)* have been reported as hosts of *B. microti* worldwide. *Peromyscus leucopus* has recently been shown to act as a competent host in North America [27] and infected *P. keeni* have been reported from Alaska [37]. Other species of rodents reported to host *B. microti* include eastern chipmunks *Tamias striatus* [27]. However, carnivores (i.e. foxes, raccoons) and insectivores such as shrews (at least 5 species of *Sorex*, *Blarina* and others) may also serve as hosts of *B. microti* [21,25,27,33-35]. On the basis of the above, *B. microti* appears to be the most widely distributed species worldwide evidently lacking tight host-specificity, but caution is warranted. Among the many studies on rodent haemoparasites reporting the presence of infections with *B. microti* [38-42], it is suspected that few have appropriately and critically assessed the species identity; rather it has been merely assumed that the parasite is *B. microti* because it was detected in a rodent host. In fact, recent studies have shown that at least three

distinct clades, differing in their host-specificity, exist among isolates of *B. microti* that have been genotyped [43,44]. Another species of *Babesia* infecting rodents, *B. rodhaini*, has been used worldwide as a laboratory model in mice and rats; this species seems to be closely related to the 'Microti group' according to phylogentic analysis [14].

Our research on the parasite fauna of wild rodents from the Sinai Mountains began in 2000, when we were invited by Professor Jerzy M. Behnke from the University of Nottingham to join an expedition of the university assessing the helminth communities of wild rodents in four isolated montane valleys. Initially, the study focused on gastrointestinal parasites of *Acomys dimidiatus*, the most abundant rodent species inhabiting Bedouins' gardens [45,46]. Subsequently, intestinal protozoa and haemoparasites were incorporated into the study [38] and the host range extended. The highest prevalence of infections with *Babesia* and *Bartonella* spp. were found in the Wagner's gerbil, *Dipodillus dasyurus*, one of the three most numerous rodent species in the study sites (unpublished data). Primary molecular research on the *Babesia*-positive samples revealed surprisingly low homology (approx. 96%) of partial (550 bp) 18S rDNA sequence to those for *B. microti* and other named species. Therefore, exploiting material collected in the latter expeditions to the study sites, we characterized this novel species of *Babesia* by light microscopy study and molecular and phylogenetic analyses.

Methods

Field studies in Sinai, Egypt

Fieldwork was conducted over 4-5-week periods in August-September 2000, 2004, 2008 and 2012 and was based at the Environmental Research Centre of Suez Canal University (2000, 2004) or at Fox Camp (2008, 2012) in the town of St Katherine, South Sinai, Egypt. Trapping was carried out in four montane wadis (dry valleys) in the vicinity of St Katherine. The local environment and general features of the four study sites (Wadi El-Arbaein, Wadi Gebel, Wadi Itlah and Wadi Gharaba), as well as their spatial relationships with one another, have been described elsewhere [46]. At each site, rodents were caught live in Sherman traps, placed selectively among the rocks and boulders around walled gardens and occasionally along the lower slopes of wadis. These were set out at dusk, and inspected in the early morning before exposure to direct sunlight. All traps were brought to the local or main camp, where the animals were identified and processed. Traps were re-set the following evening.

The three most abundant rodent species (*A. dimidiatus*, *A. russatus* and *D. dasyurus*) (Table 1) were sampled-sexed, weighed, measured and scrutinized for obvious lesions as described by [46]. Ectoparasites observed during field examination were removed and placed in 70% ethanol. Blood and faecal samples were taken and animals were then either fur marked individually and released close to the point of capture (Figure 1A), or returned to the main camp at St Katherine for autopsy. A maximum

Table 1 Structure of the rodent communities sampled and numbers of hosts studied during 2000-2012

Year of study	Host species	Site (wadi)				No. of rodents	
		W. El Arbaein	W. Gebel	W. Gharaba	W. Itlah	Total by species	Total by year
2000	*Acomys dimidiatus*	58	28	28	46	160	
	Acomys russatus	4	4	1	6	15	
	Dipodillus dasyurus	3	6	2	2	13	188
2004	*Acomys dimidiatus*	43	43	60	70	216	
	Acomys russatus	1	8	3	8	20	
	Dipodillus dasyurus	4	16	7	0	27	263
2008	*Acomys dimidiatus*	66	43	52	80	241	
	Acomys russatus	3	6	3	8	20	
	Dipodillus dasyurus	2	15	2	0	19	280
2012	*Acomys dimidiatus*	64	46	52	58	220	
	Acomys russatus	0	7	2	9	18	
	Dipodillus dasyurus	14	22	16	0	52	290
Total by site	*Acomys dimidiatus*	231	160	192	254	837	
	Acomys russatus	8	25	9	31	73	
	Dipodillus dasyurus	23	59	27	2	111	
Overall	Total no. of rodents	262	244	228	287		1021

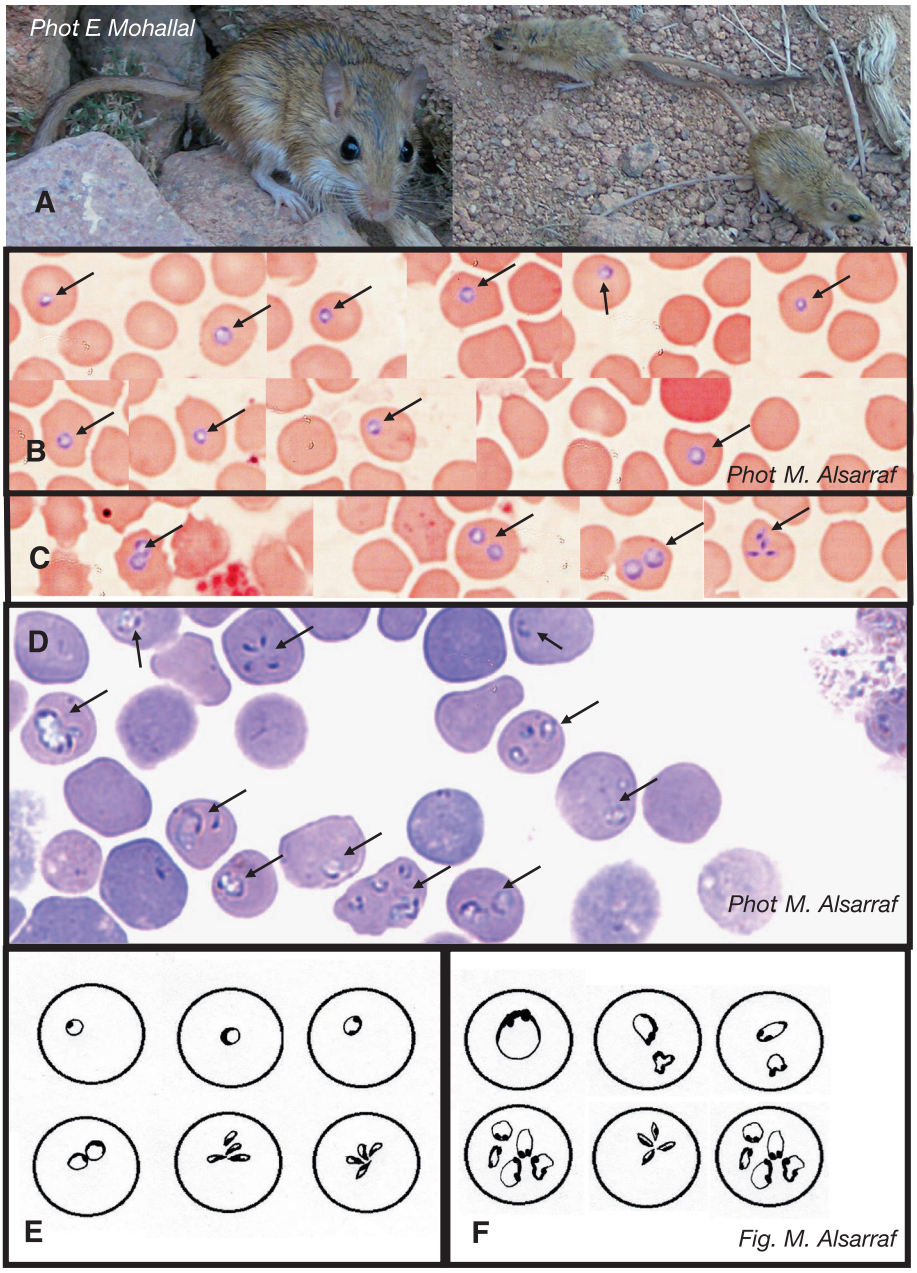

Figure 1 The type-host, Wagner's gerbil *Dipodillus dasyurus* (W. Gebel) trapped in Sinai, Egypt, and type-forms of *Babesia behnkei* n. sp. A. Type host: Wagner's gerbil, *Dipodillus dasyurus* (W. Gebel, Sinai, Egypt). **B**. Type-forms of *Babesia behnkei* n. sp. ex Wagner's gerbil *Dipodillus dasyurus* (W. Gebel) collected in Sinai, Egypt. Typical forms - single rounded trophozoites in erythrocytes. **C**. Double trophozoites and dividing form (tetrad) of *Babesia behnkei* n. sp. ex Wagner's gerbil *Dipodillus dasyurus* in erythrocytes. **D**. Trophozoites of *Babesia microti* King's 67 in erythrocytes of BALB/c mice (acute phase, on the 8th day post infection). **E**. Different forms of *Babesia behnkei* n. sp. ex *D. dasyurus*. **F**. Different forms of *Babesia microti* from BALB/c mice.

of 40% of the captured rodents from each site were culled (by agreement with the St Katherine National Protectorate authorities).

Blood collection and DNA extraction

Thin blood smears were prepared from drops of blood taken from the heart or tail tip. Blood smears were air-dried, fixed in absolute methanol and stained for 1 h in Giemsa stain in buffer at pH 7.2. In 2004, 2008 and 2012, in addition to blood smears, molecular techniques were used for the detection of *Babesia* spp. Blood from the tail vein was collected on FTA classic cards (Whatman, UK) for the long-time preservation of DNA. From the culled animals, 200 μl of whole blood were also collected into

0.001 M EDTA and frozen at -20°C. Genomic DNA was extracted from whole blood using DNAeasy Blood & Tissue kit (Qiagen, USA) or AxyPrep MiniPrep Blood kit (AxyGen, USA) and stored at -20°C. DNA from FTA cards was cleaned with FTA purification Reagent (Whatman, UK) accordingly to manufacturer's instructions.

Molecular characterization

Detection and genotyping of 32 *Babesia* isolates (Table 2) were performed by amplification and sequencing of ITS1, ITS2 and 18S rRNA regions/genes. The primers and thermal profiles used in this study have been described previously [7,10,47-50]. Reactions were performed in 1× PCR buffer, 1 U Taq polymerase, 1 μM of each primer and 2–5 μl of the extracted DNA sample. Negative controls were performed in the absence of template DNA. Primers GF (5'-G(C/T) (C/T)T TGT AAT TGG AAT GAT GG-3') and GR (5'-CCA AAG ACT TTG ATT TCT CTC-3') were used for the detection of *Babesia/ Theileria* spp. by the amplification of a 559 bp fragment of the 18S rDNA [48,49]. Primers Nbab_1F (5'-AGC CAT GCA TGT CTA AGT ATA AGC TTT T-3') [10] and TB Rev (5'-AAT AAT TCA CCG GAT CAC TCG-3') [50] were used for the genetic characterization of positive isolates by the amplification of a 1,700 bp near-full-length sequence of the 18S rRNA gene. As a second genetic marker, the 315 bp of the ITS2 region were amplified using the primers ITS2-F (5'-GGC TCA CAC AAC GAT GAA GG-3') and ITS2-R (5'-CTC GCC GTT ACT AAG GGA ATC-3') [7,47]. Additionally, a 615 bp sequence of the 18S-ITS1-5.8S region was amplified using the primers ITS1-F (5'-CGA GTG ATC CGG TGA ATT ATT C-3') and ITS1-R (5'-CCT TCA TCG TTG TGT GAG CC-3') [7,47]. PCR products were subjected to electrophoresis on a 1.5% agarose gel, stained with Midori Green stain (Nippon Genetics, GmbH) and sequenced by a private company (Genomed S.A., Poland).

Sequence analysis

DNA sequence alignments and phylogenetic analyses were conducted using MEGA v. 6.0 [51]. Akaike information criterion was used in jModel Test to identify the most appropriate model of nucleotide substitution. Tamura 3-parameter (I + G) model was chosen as the most appropriate for the Maximum Likelihood analysis of the 18S rDNA alignment. Neighbor-Joining method was used as the tree construction method for ITS2 (MEGA v. 6.0), with Kimura 2-parameter model.

Sequences of species/strains of *Babesia*, *Theileria* and *Cytauxzoon* obtained from GenBank (www.ncbi.nlm.nih.gov) were used in the sequence alignment. The stability of inferred phylogenies was assessed by bootstrap analysis of 1,000 randomly generated sample trees.

Morphology by light microscopy

Giemsa stained blood smears were examined under oil immersion (at × 1000 magnification). Parasites (*Babesia* spp., *Bartonella* spp., *Haemobartonella* (*Mycoplasma)* spp., *Hepatozoon* spp. and *Trypanosoma* spp.) were counted in 200 fields of vision. For comparison, stained blood smears prepared from BALB/c mice infected with *B. microti* King's 67 strain were also examined [52]. Trophozoites of *Babesia* spp. were measured with a Nikon screw micrometer calibrated against a standard stage micrometer. Images of the novel *Babesia* forms were made with a digital camera integrated with Nikon Eclipse E600. Typical forms, characteristic of the isolates were drawn on the basis of more than 100 images.

Statistical analysis

Quantitative data reflecting the mean diameter of trophozoites were compared between *B. behnkei* n. sp. and *B. microti* King's 67 strain. The mean diameters were analyzed by multifactorial ANOVA with SPSS v. 21 using models with normal errors.

Ethical issue

Rodents from St Katherine National Protectorate were sampled by agreement with the St Katherine National Protectorate authorities obtained for each set of field work. *B. microti* strain King's 67, originally obtained from Dr. S. Randolph (Oxford University) is maintained in our laboratory by weekly blood passage in adult BALB/c females. Blood sampling was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of National Ethics Committee for Animal Experimentation, Poland. The protocol no

Table 2 Origin of the isolates of *B. behnkei* n. sp. from *Dipodillus dasyurus* used for genotyping and phylogenetic analysis

Year of study	W. El-Arbaein	W. Gebel	W. Gharaba	W. Itlah	All sites
2004	0 isolates	12 isolates	nd	nd	12 isolates (W. Gebel)
2008	1 isolate	3 isolates	nd	nd	4 isolates (1 isolate W. El Arbaein, 3 isolates W. Gebel)
2012	2 isolates	14 isolates	nd	nd	16 isolates (2 isolates W. El Arbaein, 14 isolates W. Gebel)
Total	3 isolates	29 isolates	nd	nd	32 (3 isolates W. El Arbaein, 29 isolates W. Gebel)

Nd- not done, no isolates available.

214/2011 was approved by First Warsaw Local Ethics Committee for Animal Experimentation.

Results

Taxonomic review

Babesia behnkei n. sp.

Type-host: Wagner's gerbil, *Dipodillus dasyurus* (Rodentia, Muridae, Gerbillinae).

Type-locality: Wadi Gebel in Sinai Mountains, Egypt.

Other localities: Wadi El-Arbaein in Sinai Mountains, Egypt.

Type-material: Hapanotype. Eg085 from *Dipodillus dasyurus*, sampled on 22 August 2004 in Wadi Gebel, Sinai Mountains, Egypt, deposited at the Natural History Museum, London, UK (NHMUK 2014.8.26.1).

Parahapanotypes. Eg083 (NHMUK 2014.8.26.2), Eg084 (NHMUK 2014.8.26.3) from *D. dasyurus*, sampled on 22 August 2004 in Wadi Gebel, Sinai Mountains, Egypt; Eg041(NHMUK 2014.8.26.4) from *D. dasyurus*, sampled on 17 August 2008, W. El-Arbaein, Sinai Mountains, Egypt; Eg026 (NHMUK 2014.8.26.5), Eg028 (NHMUK 2014.8.26.6) from *D. dasyurus* sampled on 17 August 2012, W. El-Arbaein, Eg089 (NHMUK 2014.8.26.7), Eg091 (NHMUK 2014.8.26.8) from *D. dasyurus*, sampled on 21 August 2012 W. Gebel, Sinai Mountains, Egypt; all deposited at the Natural History Museum, London, UK.

Vector: currently unknown, but assumed to be a local species of ixodid tick.

Representative sequences: GenBank KJ908691 (18S rRNA gene); KJ908692 (ITS2 region); KM067276 (ITS1 region).

Etymology: The species is named for Professor Jerzy M. Behnke, the pioneer and the leader of studies on rodent parasites from isolated wadis in the Sinai Mountains of Egypt.

ZooBank reference numbers: pub: D3D8C6F4-796B-4E93-9DE4-CD6B7897E169

act: 7491E249-3966-4170-AC52-6D521D988672

Description

The organism is a typical small species of *Babesia*, with trophozoites occupying central to subcentral position within host erythrocytes (Figures 1B, E). On Giemsa stained slides, the cytoplasm is pale with a purple-staining nucleus around the periphery (Figure 1B, C). Trophozoites are mainly rounded, rarely slightly ovoid, less polymorphic than trophozoites of *B. microti* King's 67 observed in BALB/c mice (Figure 1B–F). Trophozoite dimensions (diameter) of *B. behnkei* n. sp. were significantly smaller than those of *B. microti* King's 67 [range 0.5-2.2 μm, mean ± SD 1.26 ± 0.35 μm (n = 212) *vs* range 0.6-3.0 μm, mean 1.46 ± 0.56 μm (n = 50); $F_{1,261} = 8.48$, $P = 0.004$, respectively]. Dividing forms, tetrads (resembling the Maltese cross) were observed and sometimes two forms in one red cell were recorded (Figure 1C).

Field studies: ecology of *Babesia behnkei* n. sp.

Altogether, 1,021 rodents from the Sinai Mountains, Egypt, were sampled in four montane valleys (wadies) in 2000, 2004, 2008 and 2012, including 837 individuals of the spiny mouse *Acomys dimidiatus*, 73 *A. russatus* and 111 Wagner's gerbils *Dipodillus dasyurus* (Table 1). Overall prevalence of *Babesia* spp. was the highest in Wagner's gerbil (38.7%, Table 3) in comparison with *A. dimidiatus* or *A. russatus* (<10%, data not presented). Infections with *B. behnkei* were identified only in two isolated populations of *D. dasyurus*, from Wadi Gebel (66.1%) and from W. El-Arbaein (17.4%). Parasites were maintained in these populations over a period of at least 9 years, 2004-2012 (Table 3).

Genotyping and phylogenetic analysis

Thirty two isolates derived from *D. dasyurus* obtained over a 9 year period from two wadies (29 isolates from Wadi Gebel and 3 from Wadi El-Arbaein) (Table 2) were investigated by the analysis of near-full-length sequence of the 18S rRNA gene. All sequences were identical, indicating the presence of a single parasite species. A BLAST search in GenBank revealed no identical sequences in the database, therefore this new species was designated as *Babesia behnkei* n. sp. The highest homology (about 96%) found was with *B. lengau* from cheetahs [6] and with *B. vesperuginis* from bats *Pipistrellus* spp. in Cornwall, UK [53]. The 18S rRNA sequence for *Babesia behnkei* n. sp. differed from that for *B. lengau* by 43 nucleotides and from that for *B. microti* by 55 nucleotides (Additional file 1).

The phylogenetic analyses including sequences for *Babesia behnkei* n. sp. and for other species of *Babesia/Theileria* were conducted in MEGA v. 6.0 as detailed in

Table 3 Prevalence of *B. behnkei* n. sp. in Wagner's gerbils: no. of infected/examined hosts (prevalence in %)

Year of study	W. El-Arbaein	W. Gebel	W. Gharaba	W. Itlah	All sites
2000	0/3 (0)*	0/6 (0)*	0/2 (0)*	0/2 (0)*	0/13 (0)*
2004	1/4 (25)	15/16 (93.8)	0/7 (0)	0/0	16/27 (59.3)
2008	1/2 (50)	6/15 (40)	0/2 (0)	0/0	7/19 (36.8)
2012	2/14 (14.3)	18/22 (81.8)	0/16 (0)	0/0	20/52 (38.5)
Total by site	4/23 (17.4)	39/59 (66.1)	0/27 (0)	0/2 (0)	43/111 (38.7)

*Prevalence only on the basis of microscopy; no DNA samples available for PCR.

the Methods section [51]. A representative tree for 18S rDNA sequences, obtained using the Maximum Likelihood method and a Tamura 3-parameter (I + G) model is presented in Figure 2. *Babesia behnkei* n. sp. clustered in a monophyletic group/clade with the African species *B. lengau* and with American zoonotic species *B. duncani* (*Babesia* WA1) and canine parasite *B. conradae* ('Duncani group'[14]). This clade was distinct from *Babesia* spp. (*sensu stricto*), i.e. *B. bovis*, *B. canis*, *B. gibsoni*, *B. venatorum* [EU1] and *B. divergens*, as well as from the main *Theileria* spp. clade including *T. annulata*, and from the zoonotic and non-zoonotic *B. microti* strains (Figure 2).

Phylogenetic analysis of an approximately 315 bp region of ITS2 of four isolates by the neighbour-joining method with the Kimura two-parameter distance calculation revealed very similar results (Figure 3). *Babesia behnkei* n. sp. formed a monophyletic group with the American species and strains, *B. duncani* (*Babesia* WA1), *B. conradae* and others ('Duncani group'[14]), and with the African *B. lengau.* Again, this clade was distant from *Babesia* spp. (*sensu stricto*), i.e. *B. divergens*, *B. major* and *B. gibsoni*, as well as from the main *Theileria* clade with *T. parva*, and from *B. microti* and related species (*B. rodhaini* and *B. felis*) (Figure 3).

Comparison of the ITS2 sequences for *Babesia behnkei* n. sp. with those for other species (*B. lengau*, *B. duncani* and *B. microti*) revealed low homology (Additional file 2). Similarly, the ITS1 sequence displayed low homology with a few known sequences for *Babesia* spp., including *B. microti* (Additional file 3).

Discussion

Microscopic, molecular and phylogenetic analysis of the *Babesia* sp. infecting Wagner's gerbil from the Sinai Mountains supported its differentiation from all known species and consequently the naming of a novel rodent species of piroplasms was justified. Infections with *Babesia behnkei* n. sp. were found in two isolated populations of *D. dasyurus* during a 9 year period (2004–2012). This novel species belongs to the 'Duncani group' (Clade VI) and is closely related to *B. lengau* and the human-infecting parasite *B. duncani* from North America.

Morphologically, *Babesia behnkei* n. sp. is indistinguishable from other small *Babesia* spp. but seems less polymorphic than *B. microti*. Dividing forms were observed rarely and parasitaemia exceeded 100 parasites per 200 fields of vision at × 1000 magnification in only 5 individuals. The majority of parasite trophozoites were regular and rounded.

Molecular and phylogenetic analysis of two widely used molecular markers (18S rDNA and ITS2) revealed that *Babesia behnkei* n. sp. is distinct from other known rodent *Babesia* spp. (*B. microti* and *B. rodhaini*), *Babesia* (*sensu stricto*) and *Theileria* spp. Analysis of both loci placed the new species in a recently distinguished 'Duncani group' (Clade VI [14]). This group is interesting because it consists of only a few named species and several unnamed piroplasms, including some pathogenic for humans [6,14]. Among the established species, there are two from North America, *B. duncani* (previously *Babesia* WA1), identified as an etiologic agent in human cases of babesiosis in western states of the USA [54], and *B. conradae*, described from a dog in California [55,56]. Among the parasites of the 'Duncani group', there is only one species from Africa, *B. lengau*, identified recently in cheetahs from South Africa [6]. However, another new strain/species of *Babesia* related to *B. lengau*, has been found recently in spotted hyenas from South Africa [57]; the latter still requires formal description. It is highly likely that this clade of piroplasms will be expanded in the future with new molecular studies on parasites from host species that have yet to be examined in Africa, America and elsewhere.

The pathogenicity of known and new *Babesia* species/strains differs extensively even among species from a single phylogenetic group. *Babesia lengau* appears to be nonpathogenic for cheetahs but is pathogenic for cats [6,12]. *Babesia conradae* causes haemolytic anaemia in dogs in California [55,56] and *B. duncani* may infect humans with an intact spleen or asplenic individuals, and infections in humans were reported to be subclinical or severe [17]. We have not observed any obvious symptoms of babesiosis in the Wagner's gerbil (i.e. brown colored urine, chills, apathy).

Cases of human babesiosis have been recorded in Egypt [58-60] and interestingly, both North and South Sinai (our study site) governorates are considered to be endemic regions for babesiosis in Egypt [61]. The number of reported cases differs [33,61] and so far no molecular identification of the *Babesia* spp. involved in human cases has been carried out. The number of molecular studies on *Babesia* spp. infections in Egyptian ticks is also extremely limited and the results of the few published studies certainly need verification, i.e. the presence of *B. venatorum* (EU1) in ticks *Ixodes ricinus* or of *B. microti*, *B. venatorum* (EU1) and *B. bigemina* in rats/gerbils from Sinai Peninsula [62]. Because of the occurrence of human babesiosis in South Sinai, the high prevalence of *B. behnkei* n. sp. in a common rodent species from the region, the Wagner's gerbil, and the close relationship between *B. behnkei* and the pathogenic *B. duncani*, the possibility of human infection with this novel species should be considered. Our as yet unpublished data indicate that the most common tick in the studied area is the camel tick, *Hyalomma dromedarii*, which also attaches to and feeds on humans. This tick species is certainly the main candidate for a possible vector of the new species of *Babesia*, especially because its

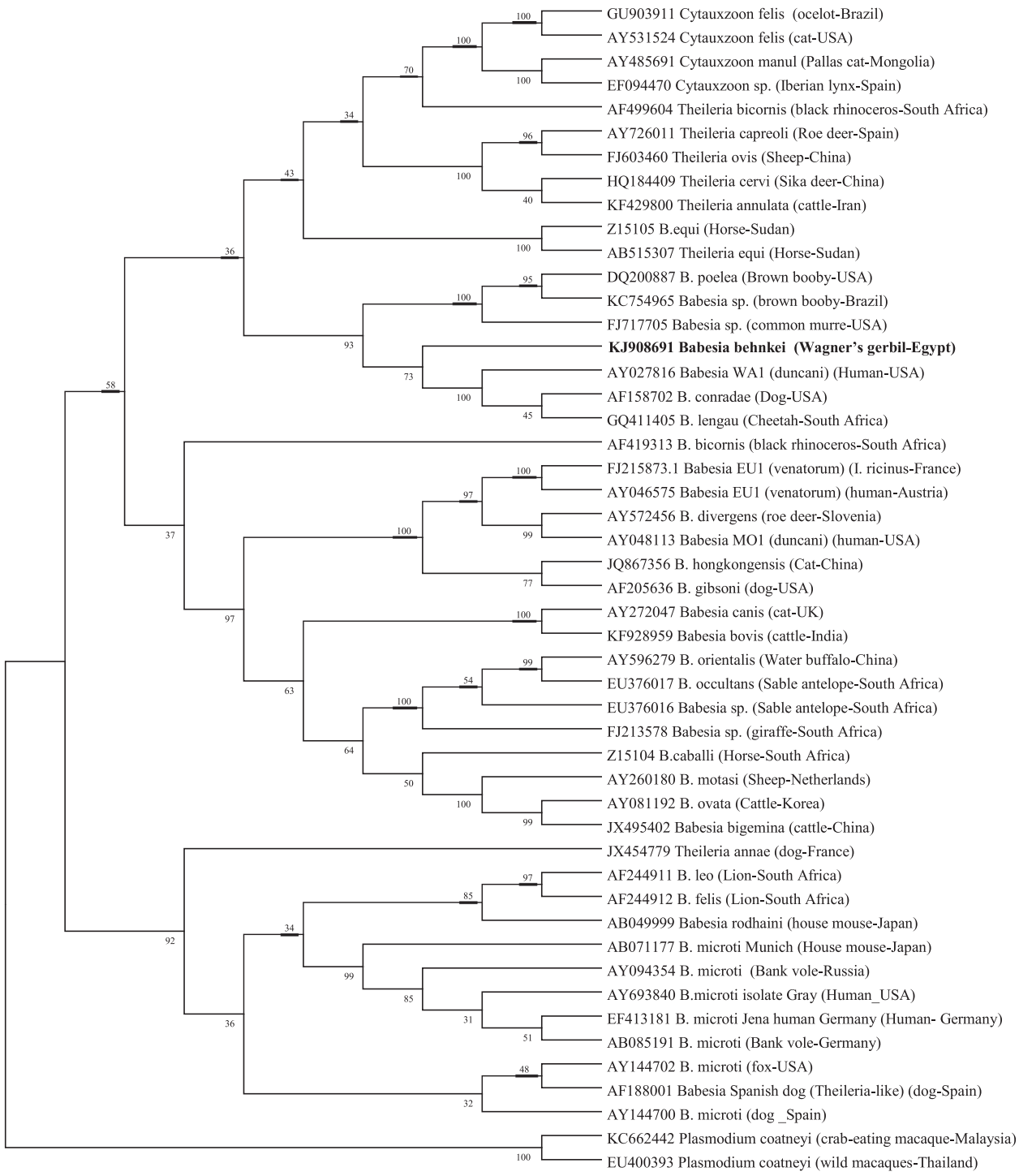

Figure 2 Phylogenetic analysis of 18S rRNA sequences by the Maximum Likelihood method. The evolutionary history was inferred based on the Tam3 (*I* + *G*) model. The tree with the highest log likelihood (0.0000) is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches [67]. The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. The analysis involved 49 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA v. 6.0 [51].

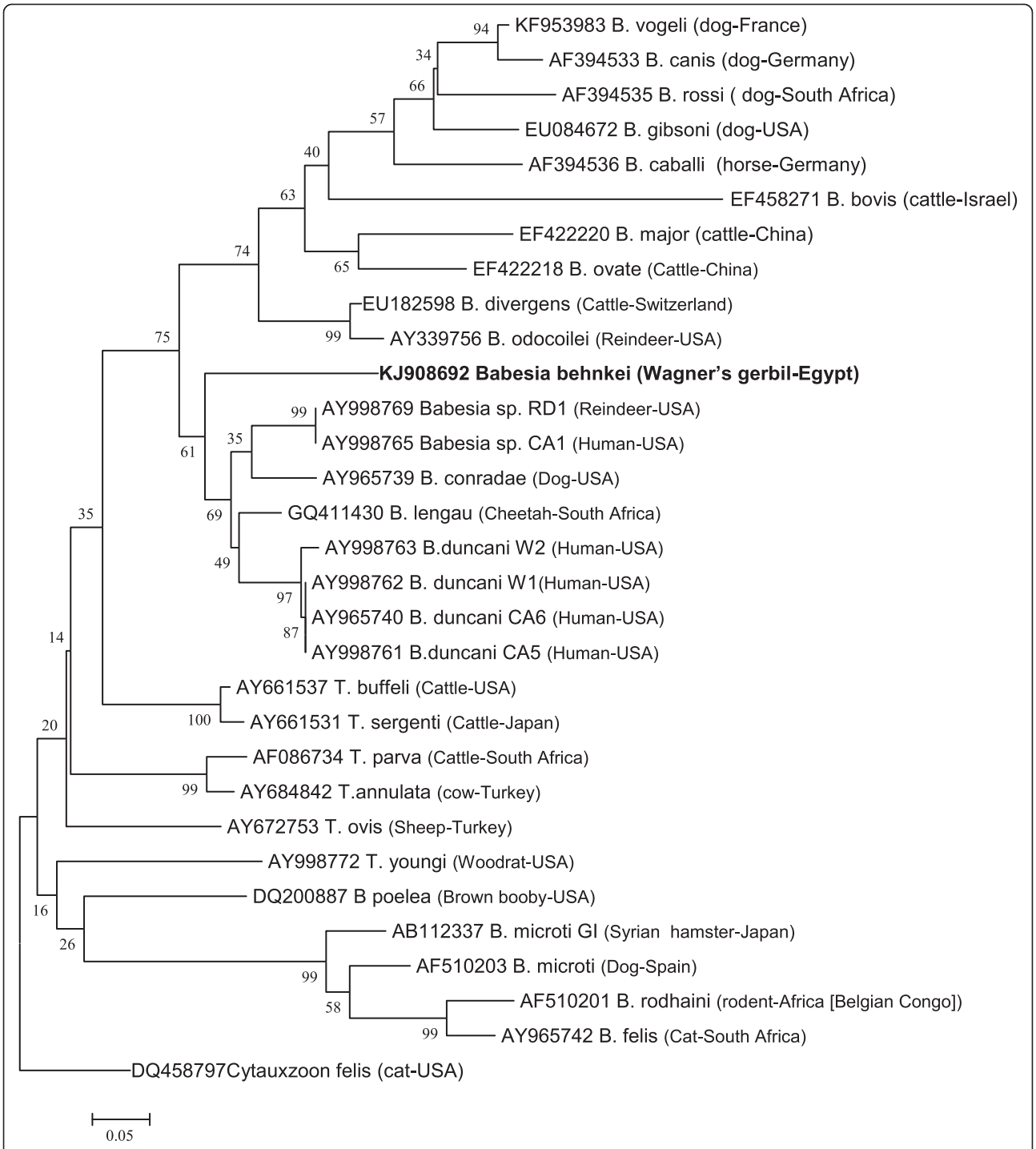

Figure 3 Evolutionary relationships of the taxa based on ITS2 sequences. The evolutionary history was inferred using the Neighbor-Joining method [68]. The optimal tree with the sum of branch length = 3.34829688 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches [67]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method [69] and are in the units of the number of base substitutions per site. The analysis involved 31 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 107 positions in the final dataset. Evolutionary analyses were conducted in MEGA v. 6.0 [51].

juvenile stages were found feeding on rodents in Egypt [63]. Juvenile ticks were also collected from rodents in our study and we plan to screen these for the presence of the diagnostic marker for *B. behnkei* and hence to determine their role as a vector of *B. behnkei* in the region.

Dipodillus (Gerbillus) dasyurus was the second most numerous rodent species sampled throughout the 13 years of field work in Sinai. This solitary, burrowing species occurs in a variety of arid habitats, including desert, semi-desert and rocky habitats in hill country [64]. It is a common species, distributed mainly in the Nile Delta, the Sinai, Syria, Iraq and the Arabian Peninsula and it is listed as of Least Concern in the IUCN Red List of Threatened Species. Interestingly, we were able to amplify *Babesia* spp. DNA only from this host species, so it is likely that host specificity of *B. behnkei* n. sp. is high and that despite the concerns expressed above, it may not constitute a zoonotic treat to people in the region. The wide geographic range of *D. dasyurus* represents a particular challenge for the study of the distribution of the novel species of *Babesia*. On the other hand, the high rate of infection in gerbils registered in only two isolated wadis and the absence of the parasite throughout the period of study in other neighboring wadis, support the idea that *B. behnkei* might have evolved locally in these semi-isolated mountain populations of Wagner's gerbils. In our studies on helminth communities in the same study sites, marked differences in community structure were noted between wadis [46]. Similarly, the prevalence of the intestinal protozoa and other haemoparasites (*Trypanosoma* spp., *Hepatozoon* spp.) differed markedly between rodent populations inhabiting these four sites [38]. Each wadi thus presents its own particular challenges for the animals that live there and local adaptation of parasites to their hosts and vice versa is to be expected [65,66]. In this particular case, *B. behnkei* n. sp. showed generally high prevalence (25–90%) in two wadis and was not detected at all in the other two of the four study sites monitored. Where present, it occurred in Wagner's gerbils in each of the three surveys conducted over a period of 9 years (2004, 2008 and 2012). The Sinai Massif and its associated deep wadis constitute therefore an ideal location for studies of this type, testing the idea that parasites evolve and adapt locally to their hosts and assessing the role of gene flow and metapopulation structure for both hosts and parasites. In future work we hope to unravel further the intricacies of these relationships in the region, notably for haemoparasites such as *B. behnkei*.

Conclusion

In conclusion, both ecological, phenotypic and phylogenetic analyses reported in this paper support the recognition of a new piroplasm, *B. behnkei* n. sp., infecting isolated populations of Wagner's gerbil in Sinai as a distinct species.

Additional files

Additional file 1: Alignment of 18S rRNA sequences.
Additional file 2: Alignment of the ITS2 region.
Additional file 3: Alignment of the ITS1 region.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AB designed the study and supervised laboratory and field analyses, MA and RWF performed molecular and phylogenetic studies, MB participated in biological characterization of novel *Babesia* and supplied reference *B. microti* strain, FG and SZ organized and supervised field work in Sinai, EJM, JBB, EMEM performed the laboratory and field studies and drafted the manuscript. All authors read and approved the final version of the manuscript.

Acknowledgements

This study was funded by the National Science Center (NCN), Poland, grant OPUS 2011/03/B/NZ6/02090. Each of the surveys was contributed to by undergraduate students from the University of Nottingham and travel and accommodation funds were generously provided by the University, the British Ecological Society (2010) and the Royal Society (2006 and 2010 to Professor Jerzy M. Behnke) for which we are most grateful. We also acknowledge the support and hospitality shown us by St Katherine protectorate workers and local Bedouins. We would like to acknowledge Professor Jan Kwiatowski, University of Warsaw, for his expert advice on the phylogenetic analyses.

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Received: 31 July 2014 Accepted: 25 November 2014

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Cite this article as: Bajer *et al.*: *Babesia behnkei* sp. nov., a novel *Babesia* species infecting isolated populations of Wagner's gerbil, *Dipodillus dasyurus,* from the Sinai Mountains, Egypt. *Parasites & Vectors* 2014 **7**:572.

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Granty i stypendia

Dotacja Statutowa Młodych (DSM) 2013, nr. 140000/501-86/104917 „Różnorodność genetyczna pierwotniaków *Hepatozoon erhardovae* u nornicy rudej z różnych siedlisk". Kierownik projektu (**Wyróżnienie**).

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Projekt OPUS NCN 2011/03/B/NZ6/02090 „Badania środowiskowe, opis i charakterystyka biologiczna nowego gatunku *Babesia*, odkrytego u gryzoni z masywu Synaju (Egipt)". Wykonawca.

Nagroda Zespołowa JM Rektora Uniwersytetu Warszawskiego za osiągnięci naukowe w latach 2014, 2015 i 2016.

Wyjazdy zagraniczne

Dwumiesięczny staż naukowy dydaktyczny na University of Nottingham, School of Life Science pod opieką Profesora Jerzego M. Behnke.

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