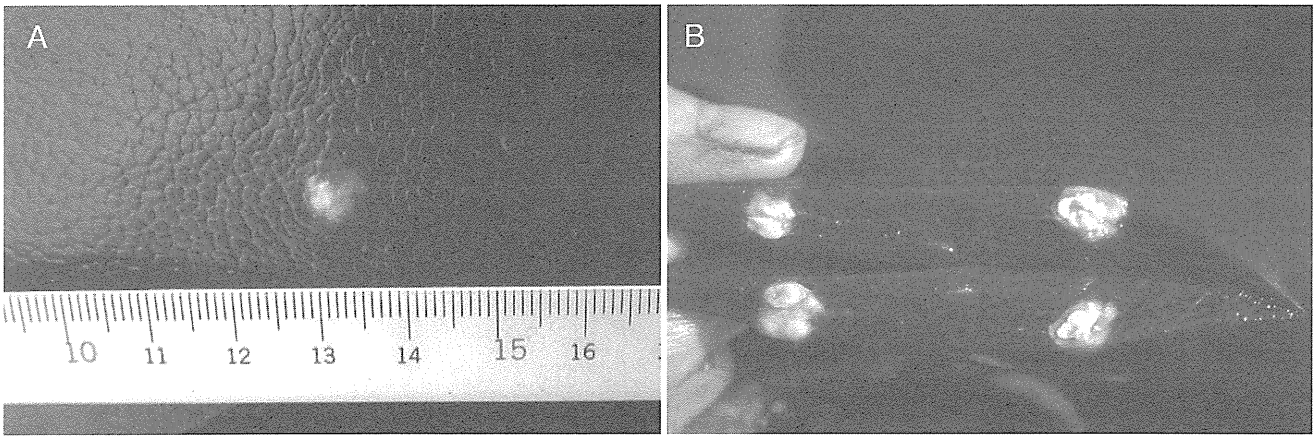

図２．ブタの肝臓に認められた多包虫の肉眼病巣（A：表面からの観察、B：割面の観察）.

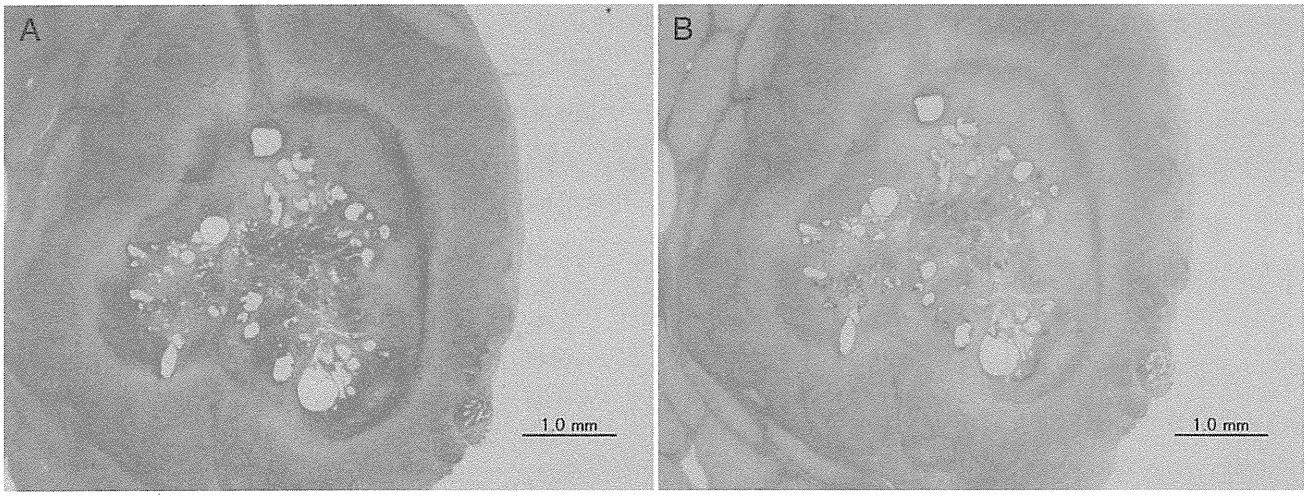

図３．病理組織像（A：HE 染色、B：PAS 染色）.

状で一部乾酪化がみられ、その周囲は灰白色透明感のある被膜で被われている。結節の比較的大きなものでは、割面は蜂窩状を呈している。結節の数は、単発から多発まで様々であり、大きさは、直径 5mm 前後のものが多く、発生部位は、横隔面、臓側面とも左葉に多く認められる。

【組織学的所見】（図 3,4 参照）

結節の中心部は、多数の変性壊死好酸球と少数の好中球の集簇であり、巣内部に多数の小シストで構成された多包虫を認める。シストが幼弱なものでは、胚細胞が、シスト内面に環状に認められる。クチクラ層が形成されたものでは、層が H・E 染色で好酸性に、PAS 染色で強陽性に染まり、概ね、無構造の角皮より成り、胚層は不明瞭である。繁殖胞及び原頭節は認めない。

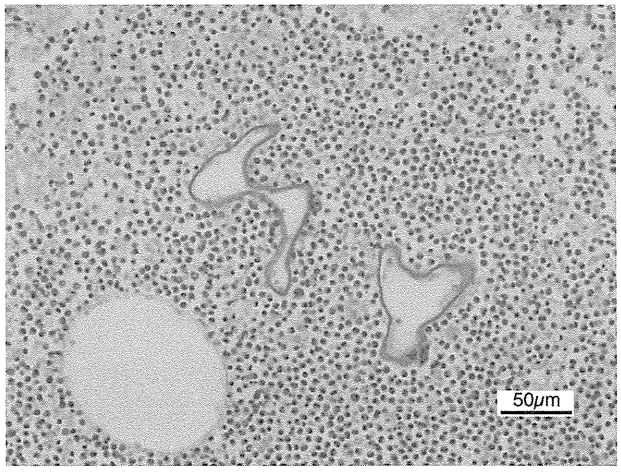

図４　病理組織像(PAS 染色).
PAS 染色で濃染するクチクラ層構造物が写真中央に２つ観察される。

周囲には、強い宿主反応が見られ、類上皮細胞が放射状に並び、時に巨細胞も混在し、最外層は、リンパ球、組織球などによる肉芽組織で、周囲間組織とは菲薄な結合織で隔てられているか又は、そのまま肝組織に移行している。周囲肝組織は、結節により圧排され、小葉間結合織の増幅、好酸球の浸潤、リンパ濾胞形成、ヘモジデリン沈着、小葉中心性うっ血などを認める。

Deplazes et al. [4] は多包条虫卵を実験感染させたブタで、ほぼ同様の病態を観察した。すなわち、感染後7ヶ月の病巣で0.5—8.0mmの白色結節で境界明瞭、内部に壊死巣と石灰化を観察し、PAS陽性の層状の構造物を認めた。また、繁殖胞及び原頭節は観察されず、発育が限定的であることを示した。

3-2. 診断

典型的な病巣については、上記の特徴的な所見を観察することにより、多包虫と判定することが可能である。更に、病巣の組織標本を作成し、PAS陽性のクチクラ層等を確認することにより確定することができる。遺伝子診断（PCR）の有効性については井島ら[10]によって報告されている。井島らは市販のDNA抽出キットを用い抽出したDNAをテンプレートとし、特異的な増幅であるU1 sn RNA遺伝子のPCR [2, 33]およびミトコンドリア12SrRNA遺伝子のPCR [5, 37] により同定を行い、遺伝子診断の有用性を示した。病巣の遺伝子診断が有効であることは実験感染でも証明されている[4]。

3-3. ブタの多包虫検出状況

北海道保健福祉部健康安全局食品衛生課食品・生活衛生事業行政概要によると、北海道で検査されるブタの頭数は年間100万頭前後で、1983年からの統計では、毎年数百頭から二千数百頭の多包虫の感染が報告されている(感染率0.03—0.28%) (表1)。ちなみに2012年度は1,083,758頭の検査を行って1,665頭 (0.15%)の多包虫陽性例を検出している。北海道全域の検出地域に顕著な偏りはない。

3-4. ブタの多包虫感染のプロセス

北海道の養豚場における多包虫の感染は、多包条虫に感染したキツネが豚舎にあるブタの餌、ブタの胎盤や死産の仔豚を餌として利用するために豚舎周辺を徘徊し、虫卵で汚染することが感染源であろう[31]。多くの養豚場が、キツネが生息している郊外にあることも、ブタへの感染が頻繁に起こる要因であると考えられる。養豚農家が飼育しているイヌやネコが感染源となる可能性は否定できないが、北海道ではキツネと養豚場とのコンタクトは強いものと考えられる。

表1. 北海道でのブタの屠畜検査頭数とエキノコックス確認数の年次推移（1983年～2012年）

年	検査頭数	エキノコックス確認頭数	検出率(%)
1983	1,068,629	352	0.03
1984	1,121,771	349	0.03
1985	1,208,569	363	0.03
1986	1,252,947	424	0.03
1987	1,258,779	676	0.05
1988	1,242,066	449	0.04
1989	1,207,273	744	0.06
1990	1,169,435	507	0.04
1991	1,119,149	924	0.08
1992	1,088,040	933	0.09
1993	1,107,280	1,556	0.14
1994	1,070,720	2,332	0.22
1995	1,029,732	2,587	0.25
1996	973,230	2,313	0.24
1997	979,353	1,853	0.19
1998	988,790	1,357	0.14
1999	971,315	1,160	0.12
2000	952,526	928	0.10
2001	929,587	614	0.07
2002	937,188	619	0.07
2003	949,503	1,496	0.16
2004	950,385	1,846	0.19
2005	923,664	1,958	0.21
2006	925,807	1,849	0.20
2007	954,433	1,157	0.12
2008	941,202	1,907	0.20
2009	1,002,856	1,627	0.16
2010	1,071,986	2,951	0.28
2011	1,079,256	1,017	0.09
2012	1,083,758	1,665	0.15

（北海道保健福祉部健康安全局食品衛生課資料）

3-5．ブタの多包虫検出の疫学的意義

上述のようにブタでの多包虫の発育は抑制的であり、原頭節の形成も認められない。このことは、ブタがこの寄生虫の流行を維持する役割を持たないことを意味する。ブタは、いかなる動物に対しても感染源とならないことを明確に理解しておく必要がある。しかしながら、ブタでの多包虫検出の疫学的意義は以下の理由から重要である。

1）感染した時期と地域を特定

養豚場のブタの飼育形態にはいくつかのバリエーションがあるが、市場に流通する肥育豚は一般的に仔豚を自家生産もしくは購入し、6—7ヶ月の肥育期間を経て屠畜場に出荷し検査を受けるものである。北海道で検査されるブタの多くは、この形態で飼育されたものである。すなわち、これらの肥育豚で多包虫陽性が確認された場合は、「6—7ヶ月以内」で、「肥育していた場所」に感染源である多包条虫卵が存在していたことを意味する。

2）検査材料を集める必要がない

食肉に供されるすべてのブタの肝臓は、肉眼的な病理学的検査を受けなければならない。すなわち、多包虫検査の材料を集めるためのコストはかからない。確定診断には病理検査や遺伝子検査が必要になるが、肉眼的な検査については通常の検査の範囲内である。

これらのメリットについて、他の感染動物であるヒト患者、キツネ、イヌ、野ネズミ、ウマ等との比較をする。感染時期と感染場所の特定については、ヒト患者は感染から発症するまでの時間経過が数年から十数年と長期にわたること、また、転居や旅行などで移動することから、ヒト患者から時期と場所の推定は困難である。キツネやイヌなどの終宿主動物は、多包条虫の寄生期間が 2—5 ヶ月であることが実験的に明らかにされている[19, 32]。そのため、感染時期をこの範囲で絞り込むことは可能である。感染場所については、キツネの季節による行動圏の変化を理解すれば、ある程度は特定が可能である。イヌについても感染期間が短いことから、飼育履歴が明確であれば、時期と場所を特定することが可能である。野ネズミは、いくつかの実験感染が報告されており[36, 40]、病巣の発育状態から感染時期を推定することは可能である。また、野ネズミの寿命が短いため、感染時期は絞り込まれる[29]。上述のように、最近、北海道以外の屠畜場でウマからの多包虫の報告されている[8, 11]。多くのケースが北海道での飼育期間に感染したものと推定されるが、明確でない症例もある。ウマは長期間飼育されているものが多く、移動も伴うため、感染時期と場所の特定が困難な場合が多い。

検査材料の収集の容易さについては、ヒト患者は、四類感染症に分類されており、診断されれば自動的に報告される。キツネ、イヌなどの終宿主動物については、北海道では、捕獲と解剖検査による調査を続けているが、捕獲も解剖検査もコストがかかる。キツネの感染状況を、糞便中の寄生虫特異抗原や虫卵の遺伝子確認により検出することも試みられていることから[20, 22, 23, 26]、将来的には現場での標準的検査法となるかもしれない。野ネズミの調査は、全体的な感染率が低いこと、感染個体が集中していることなどから[29, 35]、流行を捕捉するためには多数の検査材料を収集する必要がある。

これらのことをまとめると、新しい流行地域の摘発にはブタの多包虫検査が有用であることが明らかである。ブタは飼育期間が短く、移動が少なく、必ずすべての肝臓が食肉検査に従事する屠畜検査員(獣医師)の検査を受ける。検査員が多包虫の知識を有していれば、容易に検出される。北海道での流行の拡大は、ブタにおける多包虫感染の確認抜きでは短期間に明らかにされ得なかった。一方、流行が確認されている地域における感染状況の調査には、キツネの検査が有効である。北海道では、毎年主として農業被害等の有害鳥獣駆除で集められたキツネを譲り受け、400 頭あまりのキツネの解剖検査を行い、北海道での流行状況の把握に努めている。検体の捕獲地域の偏りが無いとすれば、他の動物に比べ、全体の流行状況の変化を把握するには有効な動物といえる。しかしながら、感染防御に配慮し

た解剖施設が必要であることが欠点として指摘できる。キツネの糞便の調査による感染状況の把握は、積極的な調査方法として将来的には有効に機能するであろう。野ネズミの調査は、感染率が低いこと、感染個体が集中して分布していること、また、特定の野ネズミにしか感受性がないこと等が欠点である。しかしながら特殊な解剖施設が必要でないことや特定の地域の能動的な調査が可能なことから、汚染状況を知る手がかりとして利用できる。

4．本州における調査

上述のように1998年に青森県のブタで多包虫症が報告され、北海道から本州への流行地域の拡大の懸念から東北における野生動物の疫学調査が行われたが、野生動物の感染は確認されなかった。2005年に埼玉県のイヌで感染が確認された際の周辺地域の野ネズミ調査では陽性例を検出できなかった。この野ネズミの調査では、捕獲された野ネズミの大半が、実験感染で感受性を持たないことが示されたアカネズミ*Apodemus speciosus* であり[32, 40]、感受性を持つハタネズミ*Microtus montebelli* はほとんど捕獲されていない。愛知県で感染が報告されたイヌは、野犬と報告されている。野犬の感染であれば、感染源として幼虫が感染した野ネズミが存在すると考えられる。狭い範囲での少数の野ネズミの調査では、陽性個体を見つけることは困難かもしれない。

5．さいごに

北海道の多包条虫の流行は、一部地域におけるキツネに対する駆虫薬 (ベイト)散布の効果により、感染リスクの軽減に成功している地域もあるが[23]、北海道全域を見ると、キツネの感染率は30-40%の高い水準で推移している (図1)。しかしながら、年間20名程度の患者の発生でとどまっていることは、感染流行状況の把握と啓蒙活動が適切に行われている結果と考えている。1983年のブタでの多包虫の発見により、ネズミやキツネでの調査が促がされ、動物で陽性例が検出された市町村は、新たに流行地域 (当時、北海道では汚染地域と呼ばれた) として指定された[30]。新たな流行確認地域では、人の健康診断や啓蒙活動が行われ、感染予防に努められた。動物での疫学調査が、患者発生に先行して行われ、患者の発生を抑制することが出来たものと理解している。

本州は、感染動物の報告が散発的に見られるように、多包条虫の侵入・定着のリスクがあることを理解しておく必要がある。北海道での多包条虫の流行の拡大を検知する上で、屠畜検査におけるブタからの多包虫検出が果たした役割は大きい。同様に、本州においても、本寄生虫の侵入・定着のモニタリングに有用であることを強調しておきたい。

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Swine alveolar echinococcosis: the significance of its detection

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ABSTRACT

In1982, swine alveolar echinococcosis (AE) was found in a pig farm of Abashiri District, Hokkaido. It was the first report of natural infection of AE in domestic ungulates in the world. At that time, the distribution of *Echinococcus multilocularis*, the causative cestode of AE, had not been confirmed in the region. However, infected wild rodents were subsequently found in the field survey around the pig farm. These results indicated that the detection of swine AE in the meat inspection is useful to map the distribution of *E. multilocularis*. Thereafter, Hokkaido government notified the local meat inspection offices to identify and report swine AE cases. As a result, the expansion of distribution of *E. multilocularis* was confirmed in Hokkaido Island. Recently, it is concerned that *E. multilocularis* could be introduced into Honshu mainland of Japan, and thus the importance of the pathological examination of pig livers at the meat inspection should be emphasized.

Keywords: hydatidosis, *Echinococcus multilocularis,* pig, meat inspection, monitoring of zoonosis.

A comparison of the diet and fine-scale distribution of sympatric Tibetan and red foxes in Qinghai, PR China

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A comparison of the diet and fine-scale distribution of sympatric Tibetan and red foxes in Qinghai, PR China

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We compared the diet and the spatial distribution of the Tibetan fox *Vulpes ferrilata* and the red fox *Vulpes vulpes* in the Tibetan plateau, to elucidate mechanisms of coexistence for these two sympatric canids and to clarify their roles as definitive hosts for zoonotic *Echinococcus* parasites. Diet and fine-scale distribution patterns were assessed by fecal DNA analysis. A total of 45 fecal samples (15 belonging to Tibetan fox, 30 belonging to red fox were collected from 15 sites into three of which contained only Tibetan fox feces, six only red fox feces, and six contained feces of both species. The abundance of pika burrows, a key prey item for both species, did not differ among the sites. Food composition analysis, estimated using a point-frame method, revealed slight but insignificant differences between the two species. Tibetan foxes consumed primarily mammals, whereas red foxes consumed primarily insects. The dietary range of the Tibetan fox was narrower than that of the red fox but there was little dietary overlap between the two species. These findings suggest that the weak partitioning of food resources between Tibetan and red foxes can facilitate their coexistence even within the same habitat where they share the same key prey items, i.e. small mammals such as pikas. These dietary differences between the two fox species also suggest that the Tibetan fox is a more important definitive host for *Echinococcus* on the Tibetan plateau than is the red fox.

On the Tibetan plateau, sympatric carnivores play important roles in maintaining the sylvatic cycle of zoonotic parasites, such as *Echinococcus* spp. As this area is one of the most serious endemic regions for echinococcosis (Jenkins et al. 2005), studies on the definitive fox host species – the red fox *Vulpes vulpes* and Tibetan fox *Vulpes ferrilata* – are necessary to clarify the epidemiological status of each parasite species (Wang et al. 2008). As the life-cycle of *Echinococcus* parasites is maintained through fox predation of intermediate host species such as voles and pikas, then understanding the diet and feeding habits of these two sympatric canid species is particularly important.

Previous studies have shown that the red fox is largely an opportunistic forager (Schaller 1998, Lin et al. 2010, Murdoch et al. 2010) while the Tibetan fox is a specialist forager of small mammals such as pikas (Zheng 1985, Schaller 1998, Clark et al. 2008, Liu et al. 2010). Identifying the origins (host species) of fecal samples deposited by sympatric carnivores of similar body size is difficult (Heinemeyer et al. 2008). In the majority of dietary studies on carnivores, field-collected fecal samples have been assigned to host species either by the morphological characteristics of the feces or by virtue of being collected at known locations (e.g. near occupied dens or tracks; Zheng 1985, Lin et al. 2010, Liu et al. 2010, Murdoch et al. 2010). Hence, in the absence of prior information or of species-specific differences in scats, identifying the origin of fecal samples becomes increasingly unreliable. Recently, however, a noninvasive genetic method has been developed that enables accurate identification of species from fecal samples (Nonaka et al. 2009, Jiang et al. 2012). Importantly, such fecal DNA analysis enables more precise comparisons of diet among sympatric carnivores. DNA analysis of field-collected fecal samples can also be used to infer the spatial distributions of sympatric carnivores (Ruiz-González et al. 2008). Although the broad geographical ranges of Tibetan and red foxes are considered to overlap (Schaller and Ginsberg 2004, Clark et al. 2008, Wozencraft 2008), the fine-scale spatial distributions of these species (e.g. home ranges) and their spatial relationship has yet to be determined. In this study, we used fecal DNA analysis

to determine the diet and fine-scale spatial distribution of the Tibetan fox and the red fox where they exist in sympatry on the Tibetan plateau, in Qinghai province, PR China. In addition, we used this information to evaluate the relative roles of these definitive host species in the life-cycle of *Echinococcus* spp. on the Tibetan plateau.

Material and methods

Study site

Feces were collected from grassland within 100 km of the town of Heka in Xinghai county, Qinghai province, PR China (35°19′N, 99°05′E – 36°06′N, 100°39′E, Fig. 1). The study area lies on the Tibetan plateau at an altitude of 3000–4500 m above sea level, within the eastern part of the geographical distribution of the Tibetan fox (Schaller and Ginsberg 2004, Wozencraft 2008). The site serves as summer and winter grazing areas for yak and domestic sheep of Tibetan pastoralists.

Fecal sampling

We collected fox feces in September 2010, August 2011 and August 2012. Sampling sites were selected along roads within a radius of about 100 km from Heka town. Four line transects (about 200 m long and 2 m wide, measured by counting the steps of each investigator) were placed at each sampling site (Fig. 1). Sampling sites were placed at least 4 km apart, as this distance was longer than the length of an individual home range for both Tibetan and red foxes (red foxes = 2.28–8.71 km^2; Zhou et al. 1995; Tibetan foxes = 5.2–7.2 km^2; Liu et al. 2007). Within each sampling site we sampled along four line transects that covered approximately the area of an individual home range for both Tibetan and red foxes (~400 m diameter). Feces were labeled and held separately in plastic bags in the field, before being stored at −80°C for at least 10 days to kill any *Echinococcus* eggs (Veit et al. 1995). Feces were then stored at −20°C until use.

Fecal DNA analysis

Fecal DNA was extracted from washings of the frozen feces using QIAamp DNA Stool Mini Kits according to methods described by Nonaka et al. (2009). Briefly, ASL buffer from the kit was added directly to the frozen fecal samples and used to 'wash' the sample, by shaking the plastic bag vigorously 50 times. After removing the feces, we then collected approximately 1.4 ml of the liquid in a tube, to which an EX inhibiting tablet was added. We mixed the mixture vigorously for 1 min and then incubated it at room temperature for 1 min. We centrifuged the sample at 20 000 × *g* for 3 min and transferred 600 µl of the supernatant to a fresh tube to which 15 mAU of Proteinase K was added. The remaining extraction procedures followed the manufacturer's instructions, extracting DNA with 50 µl AE buffer. We performed polymerase chain reaction (PCR) amplification for the partial sequence of the D-loop region of the DNA with primers: prL (5′–CACCATTAGCACCCAAAGCT–3′) and prH (5′–CCTGAAGTAGGAACCAGATG–3′). The sequences of the PCR products were read with a DNA sequencer using Big-Dye terminator cycle sequencing kits ver. 3.1. Sequences were identified to species by alignment to known sequences using the Basic Local Alignment Search Tool (BLAST; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

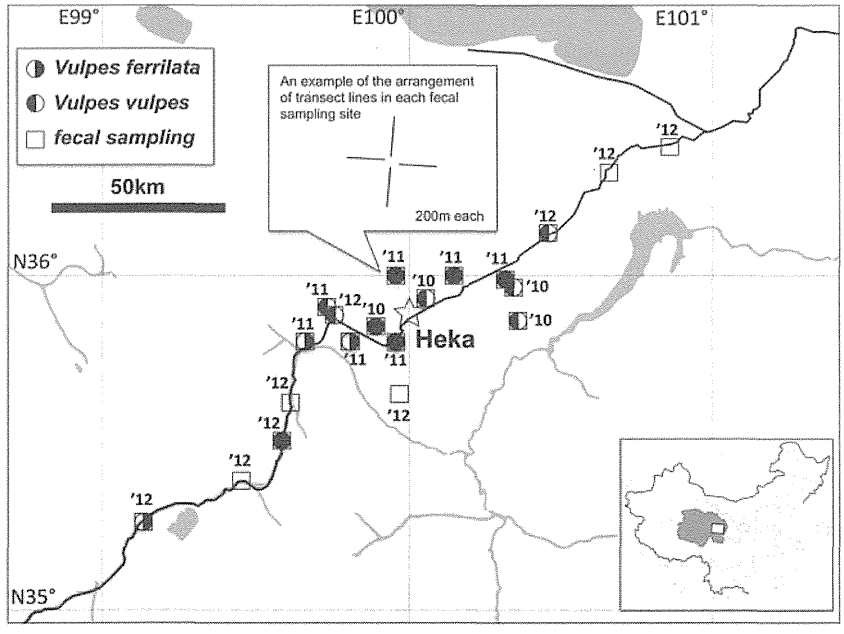

Figure 1. Distribution of Tibetan and red fox feces collected around Heka town, Qinghai Province, PR China, in September 2010 ('10), August 2011 ('11), and August 2012 ('12). The locations of of Tibetan and red fox feces collected are shown as right-half-filled circles and left-half-filled circles, respectively. The filled circles indicate the locations where both foxes's feces could be gathered. The squares indicate the locations where fecal sampling were performed. Black and gray lines show a national road and rivers or lakes, respectively.

Fecal dietary analysis

All but 0.5–1.0 g of each fecal sample that was successfully identified to species was used in the dietary analysis (0.5–1.0 g of each fecal sample was used in concurrent study of fecal parasite load; Li et al. 2013). Feces were washed with tap-water through 1 and 0.5 mm mesh sieves and the fragments remaining on the sieves sorted into broad food categories (mammals, birds, insects and plants). The mammal category was further divided into large or small species according to the thickness of any hairs (<1 or >1 cm) and bones (<2 or >2 mm) found within the sample. In addition, samples containing pika teeth were classed as '*Ochotona* sp'. The insect category was divided into Coleoptera, Orthoptera or larvae.

Dietary composition, breadth and similarity

We evaluated diet composition by tallying the frequency of occurrence of food items and by using the point-frame method (Takatsuki et al. 2007). In the point-frame method, the food remains were spread over a petri dish that was placed on a sheet with a 2 mm grid. All items (i.e. bones, teeth and hairs) that covered any of the crossing points of grids were tallied until 200 items of each food had been counted (Takatsuki and Tatewaki 2012). We defined the proportion of each food item in the fecal sample as:

$$\%P_i = \sum_{j=1}^{n} \%P_{ij} / n, \%P_{ij} = P_{ij} \Big/ \sum_{i=1}^{t} P_{ij} \times 100$$

where $\%P_{ij}$ is the proportion of total crossing points covered by food item i in the fecal sample j, P_{ij} is the total number of crossing points covered by food item i in feces j, and t is the number of food items. We compared the dietary composition estimated by the point-frame method between Tibetan and red foxes nested within year using a permutational, non-parametric, multivariate analysis of variance (MANOVA; Anderson 2001, McArdle and Anderson 2001), that can compare groups without calculating the central locations of these groups, does not require specific assumption concerning the number of variables or the nature of their individual distributions or correlations, and is robust under an unbalanced experimental design. For each food item, we compared the difference between the two fox species using a generalized linear model (GLM), with $\%P_{ij}$ as the response variable and fox species, year of sampling as explanatory variables, and a binomial error structure. We calculated dietary breadth (B) in each species according to Levin's measure (Krebs 1999):

$$B = 1 \Big/ \sum P_i^2$$

where P_i is the proportion of food item i. The scores potentially ranged from 1 (only one item consumed) to the maximum number of food categories (nine in this case, when all food categories were consumed evenly). We used Schoener's (1970) index of overlap, C_{xy}, to assess the dietary similarity between two fox species:

$$C_{xy} = 100\left(1 - 1/2 \sum \left| P_{x,i} - P_{y,i} \right|\right)$$

where $P_{x,i}$ and $P_{y,i}$ are the proportions of food item i of species x and y obtained by the point-frame method.

Prey abundance

The relative abundance of pikas at each of the sampling sites was evaluated by counting the number of new pika burrows (those with fresh soil and feces) within the line transects. This metric has been shown to be correlated with population density of these pikas (Liu et al. 2003). We used a generalized linear mixed model (GLMM) to compare the total number of new pika burrows among three broad 'types' of sampling sites (those where only Tibetan fox feces were collected (T), those where only red fox feces were collected (R), and those where feces of both species were collected (B)). We included 'fecal sampling site' as a random effect in the GLMM. We also analyzed the result of the GLMM by Tukey's multiple comparison test.

Statistical analysis

We conducted all statistical analyses using statistical software R (ver. 2.15.1, <www.R-project.org/>). We used the Adonis function of package 'vegan' (Oksanen et al. 2013) for permutational MANOVA and the car package for conducting likelihood-ratio type 2 test (Fox and Weisberg 2011). We used the lmer function of package 'lmer4' for GLMM (Bates et al. 2014) and used the glht function of package 'multcomp' for conducting Tukey's multicomp comparison test (Fox and Weisberg 2011).

Results

We collected 70 fecal samples from 19 sites, but only successfully determined species in 45 of those samples from 15 sites (Fig. 1). In total, 199 to 370 bp sequences obtained from 30 field-collected feces were matched to published sequences of *Vulpes vulpes* (GenBank accession number AB292754), and 222 to 360 bp sequences from 15 field-collected feces were matched to published sequences of *Vulpes ferrilata* (JF520840). We collected feces of Tibetan and red foxes exclusively at three and six sites, respectively, with feces of both species present together in the same year at a further six sites (Fig. 1). We found red fox feces predominately in the eastern portion of our study area, while Tibetan fox feces were collected predominately in the western portion of the study area, with both feces present together in the central area of the study site (around Heka town; Fig. 1). The abundance of new pika burrows did not differ significantly among these three site 'types' (Tukey's multiple comparisons test, T–R, $z = -0.94$, $p = 0.62$; T–B, $z = -0.13$, $p = 0.99$; R–B, $z = -1.26$, $p = 0.42$). However, there was large variation in the average number of burrows they contained: red fox only sites (R) = 1.0 ± 2.1 SE; Tibetan fox only sites (T) = 2.8 ± 3.6; and sites with both species (B) = 31.7 ± 11.4).

Via fecal analysis, we identified nine food categories consumed by both fox species (Table 1). The species differed in the dominant food items they consumed, with Tibetan foxes most frequently consuming mammals, and red foxes most frequently consuming insects. Over the whole study period, the highest $\%P$ and $\%F$ value for Tibetan foxes was for small mammals, whereas in red foxes, the highest $\%P$ and

Table 1. Frequency of occurrence (%*F*) and point-frame scores (%*P*) of food items in Tibetan fox (TF) and red fox (RF) feces in Xinghai country, Qinghai Province, P. R. China in September 2010, August 2011, and August 2012.

	2010–2012				2012				2011				2010			
	%*P*		%*F*		%*P*		%*F*		%*P*		%*F*		%*P*		%*F*	
Food items	TF N 15	RF N 30	TF N 15	RF N 30	TF N 3	RF N 4	TF N 3	RF N 4	TF N 12	RF N 9	TF N 12	RF N 9	TF	RF N 17	TF	RF N 17
Mammals	79.5	32.9	80.0	50.0	99.9	42.9	75.0	75.0	74.4	53.6	100.0	66.7	–	19.5	–	35.3
large mammals	0.0	3.6	0.0	6.7	0.0	2.3	0.0	25.0	0.0	0.0	0.0	0.0	–	5.9	–	5.9
small mammals	59.5	23.4	60.0	41.4	99.9	40.5	75.0	75.0	49.4	34.1	66.7	44.4	–	13.6	–	29.4
Ochotona sp.	20.0	5.9	20.0	6.9	0.0	0.0	0.0	0.0	25.0	19.6	33.3	22.2	–	0.0	–	0.0
Insects	1.2	47.4	53.3	76.7	0.0	23.8	0.0	75.0	1.5	15.6	88.9	55.6	–	69.7	–	88.2
Coleoptera	1.2	13.7	53.3	41.4	0.0	23.8	0.0	75.0	1.5	12.9	88.9	44.4	–	11.6	–	29.4
Orthoptera	0.0	33.7	0.0	44.8	0.0	0.0	0.0	0.0	0.0	2.6	0.0	11.1	–	58.0	–	70.6
Larva	0.0	0.1	0.0	6.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	–	0.1	–	11.8
Birds	6.5	7.4	6.7	13.8	0.0	32.7	0.0	50.0	8.1	1.1	11.1	11.1	–	4.7	–	5.9
earthworm	6.1	3.2	6.7	3.4	0.0	0.0	0.0	0.0	7.6	10.7	11.1	11.1	–	0.0	–	0.0
Plants	6.8	9.2	40.0	41.4	0.1	0.7	25.0	50.0	8.4	19.0	55.6	66.7	–	6.0	–	23.5
Diet breadth (*B*)	2.46	4.51	4.08	5.52	–	–	–	–	–	–	–	–	–	–	–	–
Diet overlap (C_{xy})	50.2		70.3		–		–		–		–		–		–	

%*F* value was for Orthopteran insects. Overall food composition did not differ significantly between the two fox species (permutational MANOVA, $F_{1,44} = 3.78$, $p = 0.074$). However, for each food item, the %*P* values of pika, Coleoptera, birds and earthworms did differ significantly between the two species (pika LR test, $\chi^2 = 46.6$, DF = 1, $p < 0.001$; Coleoptera LR test, $\chi^2 = 323.7$, DF = 1, $p < 0.001$; birds LR test, $\chi^2 = 63.4$, DF = 1, $p < 0.001$; earthworm LR test, $\chi^2 = 182.6$, DF = 1, $p < 0.001$). Our results showed that the dietary breadth of the Tibetan fox was narrower ($B = 2.46$ in %*P*, 4.08 in %*F*) than that of the red fox ($B = 4.51$ in %*P*, 5.52 in %*F*), particularly in terms of the %*P* value. Interestingly, dietary overlap between the two fox species was low ($C_{x,y} = 50.2$ in %*P*, 70.3 in %*F*).

Discussion

In this study, we have shown that the distribution of feces of red and Tibetan foxes overlapped at a scale of 400 m (i.e. twice the length of the 200 m line transect used in this study), which is less than the average home range size for either species. Although it is known that the geographical distributions of Tibetan and red fox overlap at a broad scale throughout China (Schaller and Ginsberg 2004, Clark et al. 2008, Wozencraft 2008), our results provide evidence that the two species occur sympatrically even at a fine scale. Indeed, we found that these two species apparently share the same defecating places within their home ranges in the same year. These shared defecating places may be a by-product of the foraging or scavenging behavior of red foxes because red foxes are known to defecate on food remnants (Henry 1977), around the carcasses of large mammals (Macdonald 1985) and where prey are abundant (Monclús et al. 2009). Previous studies have shown that the Tibetan fox is a specialist predator on small mammals, and especially pikas (Schaller 1998, Liu et al. 2010). Nevertheless, the abundance of pikas (based on a count of new burrows) did not differ significantly among site types, though burrows were most numerous at sites where we found feces of both species together. The plateau pika *Ochotona curzoniae* is the dominant small mammalian herbivore on the Tibetan plateau and is regarded as a keystone species in the ecosystem (Smith and Foggin 1999). Additionally to the red fox (Schaller 1998) several other carnivores, including the steppe polecat *Mustera eversmanni*, the weasel *Mustera altaica, M. eversmanni*, and Pallas' cat *Otocolobus manul* also rely hevily on pika (Smith et al. 1990, Schaller 1998, Smith and Foggin 1999). Our finding that there was likely a relatively high abundance of pikas in areas where both foxes were present suggests that the Tibetan and red fox are able to share foraging areas without excluding each other.

Nevertheless, the distributions of the Tibetan and the red fox showed some dissimilarities that are likely to be correlated with geoenvironmental differences that occur on a larger regional scale. We found red fox feces predominately in low altitude areas in the eastern portion of our study area and Tibetan fox feces in the higher altitude western areas, which suggests key differences in habitat preferences between these two species. Fecal collections over a much larger area will be required to more fully understand the relationship between altitude and the density of each fox species.

Our results showed that while the food items comsumed by the two species did not differ significantly, the dietary overlap between them was low (in terms of %*P*). We found that Tibetan foxes ate more small mammals and fewer orthopteran insects than did red foxes, and showed a more restricted dietary breadth. These results support those of previous feeding studies (Zheng 1985, Schaller 1998, Liu et al. 2010). For example, previous studies have shown that while the diet of the red fox in China and Mongolia varies among regions, small mammals constitute their principal food items, with Coleopteran and Orthopteran insects also an important resource (Schaller 1998, Lin et al. 2010, Murdoch et al. 2010). Interestingly, we found that the proportions of each food item consumed by red foxes changed among years. This also supports previous work showing that that the diet of red foxes varies both seasonally and regionally (Schaller 1998, Lin et al. 2010, Murdoch et al. 2010, Xuanlong et al. 2010).

Interspecies competition among sympatric canids can be greatly reduced via partitioning the use of shared food resources, as has been reported to occur for corsac and red foxes (Murdoch et al. 2010), and for San Joaquin kit foxes and coyotes (Cypher and Spencer 1998). Our results support these previous findings and suggest that the weak partitioning of food resources we observed between these two species can facilitate their coexistence within the same habitats on the Tibetan plateau. Differences in activity patterns may further facilitate coexistence between these two canid species. The Tibetan fox is relatively diurnal, corresponding to the activity of pikas (Schaller 1998, Wang et al. 2004), while the red fox is largely nocturnal (Ables 1969, Eguchi and Nakazono 1980, Weber et al. 1994, Zhou et al. 1995, Doncaster and Macdonald 1997). Indeed, we have observed Tibetan foxes being active in the daytime both directly and using camera traps (Tsukuda et al. unpubl.). Such temporal segregation between Tibetan and red foxes might further facilitate sympatric coexistence between the two fox species.

In this ecosystem, wild foxes are known to be important definitive hosts of *Echinococcus multilocularis* and *E. shiquicus* (Jenkins et al. 2005). *Echinococcus shiquicus*, which exclusively uses the plateau pika *O. curzoniae* as an intermediate host, has been found solely in the Tibetan fox (Xiao et al. 2005). *Echinococcus multilocularis*, meanwhile, can use many small mammalian species as intermediate hosts (Giraudoux et al. 2006, Wang et al. 2008). Our results revealed that the Tibetan fox consumed a higher proportion of small mammals than did the red fox. This finding suggests that the Tibetan fox is likely to be a more important definitive host of *Echinococcus* in the Tibetan plateau because of its high level of predation on infected intermediate hosts. Previous work has shown that the infection rate of *E. multilocularis* among red foxes is a function of the rate of fox predation on voles, which are key intermediate hosts for *E. multilocularis* (Saitoh and Takahashi 1998, Yokohata and Kamiya 2004, Tsukada 2005, Tanner et al. 2006, Hegglin et al. 2007, Raoul et al. 2010). Interestingly, prevalence of *E. multilocularis* infection is broadly similar in Tibetan foxes (33.3–59.1%) and red foxes (15 – 59.3%; Wang et al. 2008). Additionally, a survey of helminth fauna in the Tibetan and red fox also showed no difference in the prevalence of taeniid cestodes, including *E. multilocularis*, in the two fox species (Li et al. 2013). The seasonal and regional variations in the diets of each fox species might have mitigated any differences between the two fox species in their rates of infection with *E. multilocularis*. Difference in susceptibility to *E. multilocularis* infection between the Tibetan and red fox are poorly understood but are also likely to influence infection rates. To more robustly understand the epidemiological risk to the Tibetan and red foxes of echinococcal infections in Tibetan plateau, further ecological and parasitological studies on these species will be needed.

In this study, we have revealed slight partitioning in the diets of the Tibetan and red fox, with the former species being a specialist small mammal predator. In addition, we show that there is significant overlap in the spatial distributions of these two species. Our data support the suggestion that the Tibetan fox is the key definitive host for *Echinococcus* spp. in this region. Hence, furture epidemiological surveys should focus on infection dynamics in the Tibetan fox population to elucidate the sylvatic cycle of *Echinococcus* spp. infection in the Tibetan plateau.

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Parasites & Vectors

RESEARCH Open Access

Where to deliver baits for deworming urban red foxes for *Echinococcus multilocularis* control: new protocol for micro-habitat modeling of fox denning requirements

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Abstract

Background: Deworming wild foxes by baiting with the anthelmintic praziquantel is being established as a preventive technique against environmental contamination with *Echinococcus multilocularis* eggs. Improvement of the cost-benefit performance of baiting treatment is required urgently to raise and maintain the efficacy of deworming. We established a spatial model of den site selection by urban red foxes, the definitive host, to specify the optimal micro-habitats for delivering baits in a new modeling approach modified for urban fox populations.

Methods: The model was established for two cities (Obihiro and Sapporo) in Hokkaido, Japan, in which a sylvatic cycle of *E. multilocularis* is maintained. The two cities have different degrees of urbanization. The modeling process was designed to detect the best combination of key environmental factors and spatial scale that foxes pay attention to most (here named 'heeding range') when they select den sites. All possible models were generated using logistic regression analysis, with "presence" or "absence" of fox den as the objective variable, and nine landscape categories customized for urban environments as predictor variables to detect the best subset of predictors. This procedure was conducted for each of ten sizes of concentric circles from dens and control points to detect the best circle size. Out of all models generated, the most parsimonious model was selected using Akaike's Information Criterion (AIC) inspection.

Results: Our models suggest that fox dens in Obihiro are located at the center of a circle with 500 m radius including low percentages of wide roads, narrow roads, and occupied buildings, but high percentages of green covered areas; the dens in Sapporo within 300 m radius with low percentages of wide roads, occupied buildings, but high percentages of riverbeds and green covered areas. The variation of the models suggests the necessity of accumulating models for various types of cities in order to reveal the patterns of the model.

Conclusions: Our denning models indicating suitable sites for delivering baits will improve the cost-benefit performance of the campaign. Our modeling protocol is suitable for the urban landscapes, and for extracting the heeding range when they select the den sites.

Keywords: *Echinococcus multilocularis*, Baiting strategy, Cost-benefit performance, *Vulpes vulpes*, Urban red fox, Den site selection, Key environmental factors, Key spatial scale, Requisite spatial scale, Heeding range

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Background

The establishment of effective strategies for zoonoses control is needed urgently in order to minimize infection risks to humans, because wildlife and human habitats are becoming rapidly overlapped [1].

Echinococcus multilocularis Leuckart, 1863 is a parasite perpetuated in a sylvatic cycle mainly between wild carnivores (definitive hosts) and rodents (intermediate hosts). Infection of humans occurs by the accidental ingestion of the parasite eggs, which are provided from the feces of the definitive hosts. This ingestion will cause human alveolar echinococcosis (HAE), which constitutes a serious zoonosis. The number of cases of HAE has been increasing in recent years in central Europe, parts of North America, and parts of Asia including Japan [2,3].

In Japan, HAE is endemic in Hokkaido, the northernmost prefecture. Here, the red fox, *Vulpes vulpes* Linnaeus, 1758, is the main definitive host [4] and acts as a vector of *E. multilocularis* toward humans.

The red fox is common wildlife in Hokkaido, and it is known to have a high capacity for adaptation to artificial environments. In fact, their habitat has expanded into urban areas of many cities worldwide in recent decades [5-8]. This urbanization of red foxes has been reported in Hokkaido as well [9-12]. Moreover, *E. multilocularis* is prevalent among the urban fox population there [9,12]. The urbanization of infected red foxes leads to contamination of these areas with eggs of *E. multilocularis* and raises the exposure risk of residents to the pathogenic eggs.

Deworming of foxes by baiting with anthelmintic praziquantel could prevent the contamination of areas with the eggs of *E. multilocularis*. Previous studies have demonstrated that this approach successfully reduced *E. multilocularis* prevalence in the red fox population in several countries [7,13-22].

Although effective, anthelmintic baiting requires continuous effort to keep the fox population in the target area free from parasites. Even if family members are effectively treated, the risk of re-infection increases again during the annual immigration. Achieving the maximum effect at the minimum cost is fundamental for sustainable baiting, hence identifying the most suitable locations for delivering baits is necessary [23-26], especially areas having low densities of foxes such as cities in Hokkaido (e.g. 0.080 families/km^2 in Sapporo [11]).

Clarifying the pattern of habitat use to standardize the target locations for delivering baits could improve the cost-benefit performance of anthelmintic baiting. The target location should be related to the habitat use of red foxes [8,27,28], especially around dens, which are the pivot of their habitation. A red fox family usually has several dens in different places and they depend on the sites throughout the breeding season. They are likely to intake bait around the dens constantly because they invariably come back at least once a day to any one of their dens during the breeding season. Indeed, a camera trap study revealed that foxes accepted baits frequently at their dens during the season [29]. Hence, the requirements for fox denning are the key to determining the target location where bait should be delivered. Standardized denning requirements could be clarified by establishing a model that extracts key environmental factors.

A general modeling method exists for standardizing the habitat selection of arthropods [25,30-35]; however, this method is not applicable to modeling the habitat use of urban foxes. This general modeling method is appropriate for risk prediction of vector-borne diseases mechanically transmitted by arthropods, which targets the macro-scale area. On the other hand, the fox model is intended for the risk prediction of echinococcosis, which is a parasitic zoonosis indirectly transmitted by a mid-sized, generalist species inhabiting urban landscapes. Three major problems must be solved to apply the existing method to habitat use modeling for red foxes: 1) the general modeling approach uses the "abundance" of vector individuals as its modeling target; however, "presence or absence" is suitable for fox modeling especially in the areas in which they inhabit in low densities; 2) the general modeling approach uses variables on existing thematic maps, but these variables for foxes should be based on their individual habitat use, not general land use nor general vegetation; 3) the size of unit in the general modeling approach is based on the grid size (resolution) of existing thematic maps; however, neither fox territories nor their habitat use can be represented by the resolution of these maps.

In the present study, we specified the potential habitat of urban fox dens by establishing an innovative fox denning model, which identifies the suitable locations for delivering anthelmintic baits. The fox denning model was designed as a den site selection model that can extract the best combination of key environmental factors and key spatial scale for denning simultaneously. The presence or absence of fox dens was set as the modeling target, which is applicable for analyzing fox denning instead of abundance of individuals. The new modeling method simultaneously extracts the best combination of critical factors and an optimal size of modeling unit from all combinations. This is the first approach to establish a comprehensive micro-habitat model for mid-sized and generalist mammals in consideration of specifying the requisite spatial scales for the target populations. The protocol for the modeling process is presented visually. In addition to spatial modeling, a comparison is made between the results of denning factors extracted by our new model and by two traditional univariate analyses. The extracted factors by the traditional analyses are also compared with the results from other places reported in

previous studies to discuss the differences in fox denning requirements depending on habitat types. Control strategies for *E. multilocularis* are also discussed.

Methods

We established a new spatial model to specify the potential habitat of urban red fox dens to identify the suitable location to deliver anthelmintic baits. The model clarifies the critical environmental requirements for den site selection by urban red foxes. Models were constructed for urban areas of Obihiro and Sapporo cities in Hokkaido, Japan, in which red fox populations have been established. The modeling protocol is given below (see Analysis: "Den site selection modeling").

In addition to establishment of the new model above, we extracted denning factors using two other traditional univariate analyses to compare the results between the methods. The factors extracted by the traditional approaches are also compared with the results from previous studies conducted in non-urban areas [36-43] to discuss the differences in fox denning requirements depending on habitat type. The protocols of the two traditional analyses are also given below (see Analysis: "Supplemental analyses by traditional methods").

Study areas

The study areas were urban regions of Obihiro and Sapporo cities in Hokkaido, the northernmost prefecture of Japan (N 41°21′-45°33′, E 139°20′-148°53′). Hokkaido belongs to the subarctic zone and shows a continental climate, and it usually snows from November to March although the annual amount of snowfall varies depending on the province. Obihiro City is a small city located in the eastern part of the island. Sapporo City is the prefectural capital and located in the western part of Hokkaido Island, and in which *E. multilocularis* has been fixed in red foxes. The densities of red foxes in urban areas of Hokkaido is relatively lower (e.g. 0.080 families/km^2 in Sapporo [11]) than in other cities in Europe [20,44-46]. Both of the study areas are composed almost entirely of artificial environments, including urban parks and farmland; however, these two study areas are different in the scale of each component, i.e. surface area, human population size, and human population density.

A map of the Obihiro study area is given in Figure 1-A. This study area (about 59.8 km^2) consists of the whole of the Urbanization Promoting Area (UPA; about 41.9 km^2) and its surrounding suburban area (about 17.9 km^2). The UPA is composed of a mosaic of dwellings, commercial areas, urban parks, urban green spaces, and riverbeds. The surrounding suburban area is composed of urban parks, an area of continuous farmlands, and riverbeds of two large rivers, plus some small rivers and streams. The human population of the study area is approximately 167,000, which amounts to 96% of the total population of whole city. The population density is about 4400 people/km^2.

A map of the Sapporo study area is given in Figure 1-B. This study area (about 367.9 km^2) consists of the whole of the UPA (about 249.3 km^2) and its surrounding suburban area (about 73.6 km^2). The UPA is composed of a mosaic of dwellings, commercial areas, urban parks, urban green spaces, and riverbeds. The surrounding suburban area is composed of large urban parks, urban farmland, and riverbeds of two big rivers, plus some small rivers and streams. The human population of the study area is approximately 1,855,000, which is around 99% of the total population of the whole city. The population density is about 7400 people/km^2.

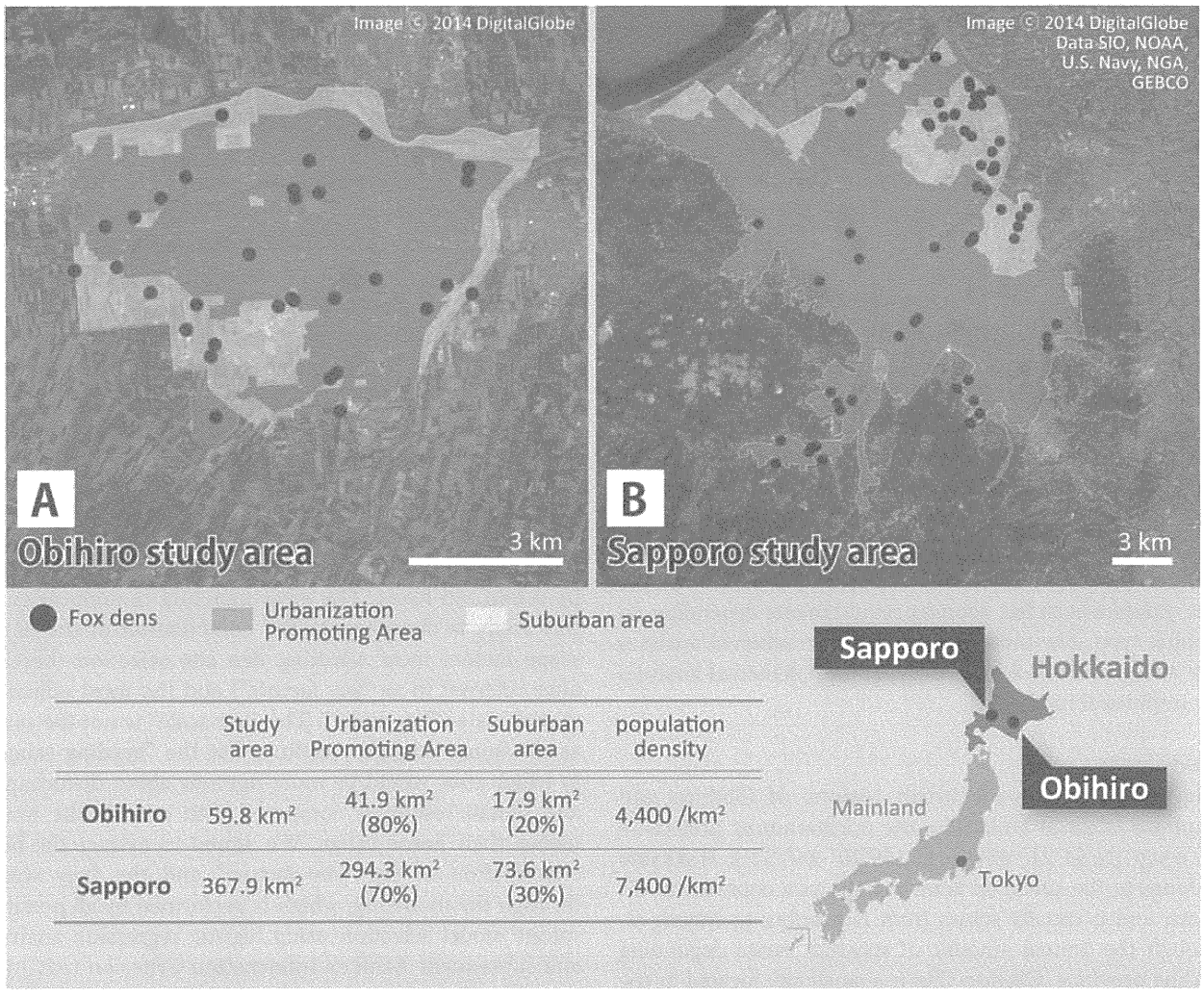
Analyses

Den site selection modeling

The modeling process was designed to extract the critical environmental requirements for den site selection by urban red foxes. The environmental requirements in this study is described as the combination of the landscape factors most affecting den site selection (hereinafter referred to as "key factors") and the most affecting spatial scale ("key scale"). The "key scale" is not the same as the home range or territory but the "heeding range", in which they would be more nervous about disturbance and secure resources compared with outside the range within their home range. We aimed to extract the best combination of the "key factors" and the "key scale" through the modeling, which is performed by all possible subset model selection using logistic regression analysis and subsequent Akaike's Information Criterion (AIC) inspection. The protocol for the modeling process is given below and in Figure 2.

Assumptions of the modeling The regression analysis consisted of the presence or absence of a fox den as the objective variable, and nine categories of landscape features as the predictor variables. The nine predictor variables were presented by percentages of area occupied by nine categories of landscape feature: "wide road" (WROAD), "narrow road" (NROAD), "occupied building" (OCPBL), "vacant building" (VCTBL), "water place" (WATER), "riverbed" (RIVER), "farmland" (FARM), "green covered area" (GREEN), and "blank space" (BLANK). These were equipped for analyzing urban habitat use by red foxes based on previous studies on fox habitat selection [36-43]. These variables were carefully chosen to reflect the sensitivity of foxes against artificial structures when they select the den sites.

Detailed definitions of landscape feature categories and those of corresponding variables and abbreviations are shown in Table 1.



	Study area	Urbanization Promoting Area	Suburban area	population density
Obihiro	59.8 km²	41.9 km² (80%)	17.9 km² (20%)	4,400 /km²
Sapporo	367.9 km²	294.3 km² (70%)	73.6 km² (30%)	7,400 /km²

Figure 1 Maps showing the landscape structures and fox den distributions in the two study areas. Panel **A** shows Obihiro study area and panel **B** shows Sapporo study area. Each study area consists of Urbanization Promoting Area (orange area) and surrounding suburban area (yellow area). Black dots in the maps indicate red fox dens.

Modeling process The detailed modeling process is described below. The process consists of the preparation of data sets (Step 1–5) and model selection (Step 6–7). A series of modeling processes was performed using statistical software R 3.0.3 (The R Project for Statistical Computing) [47] and the R packages of all.logistic [48] and glm2 [49].

Step 1. Customization of analytical base maps

A specialized analytical base map was prepared for each study area by customizing existing thematic maps to render the whole study area in nine categories of landscape feature: "wide road", "narrow road", "occupied building", "vacant building", "water place", "riverbed", "farmland", "green covered area", and "blank space".

The categories "occupied building" and "vacant building" were distinguished to investigate whether foxes were sensitive to the presence of humans or artificial structures. "Water place" and "riverbed" were distinguished for detailed investigation of the reason why foxes prefer den sites near a river. It was reported previously that red foxes prefer sites near a river; however, it has not yet been clarified whether they are attracted to rivers just as a source of water or whether they are attracted to other environmental factors associated with the river, such as a riverbed with a slope and dry sand that may enable them to dig easily, fewer invaders, many rodents as food, etc. [41,50]. The category "farmland" was distinguished from "green covered area" to determine if foxes are sensitive to disturbance by farmers or tractors.

The landscape data was referenced from several numerical information maps from the National Land Numerical Information download service (Geographical Survey Institute, Japan [51]) and the Fundamental Geospatial Data

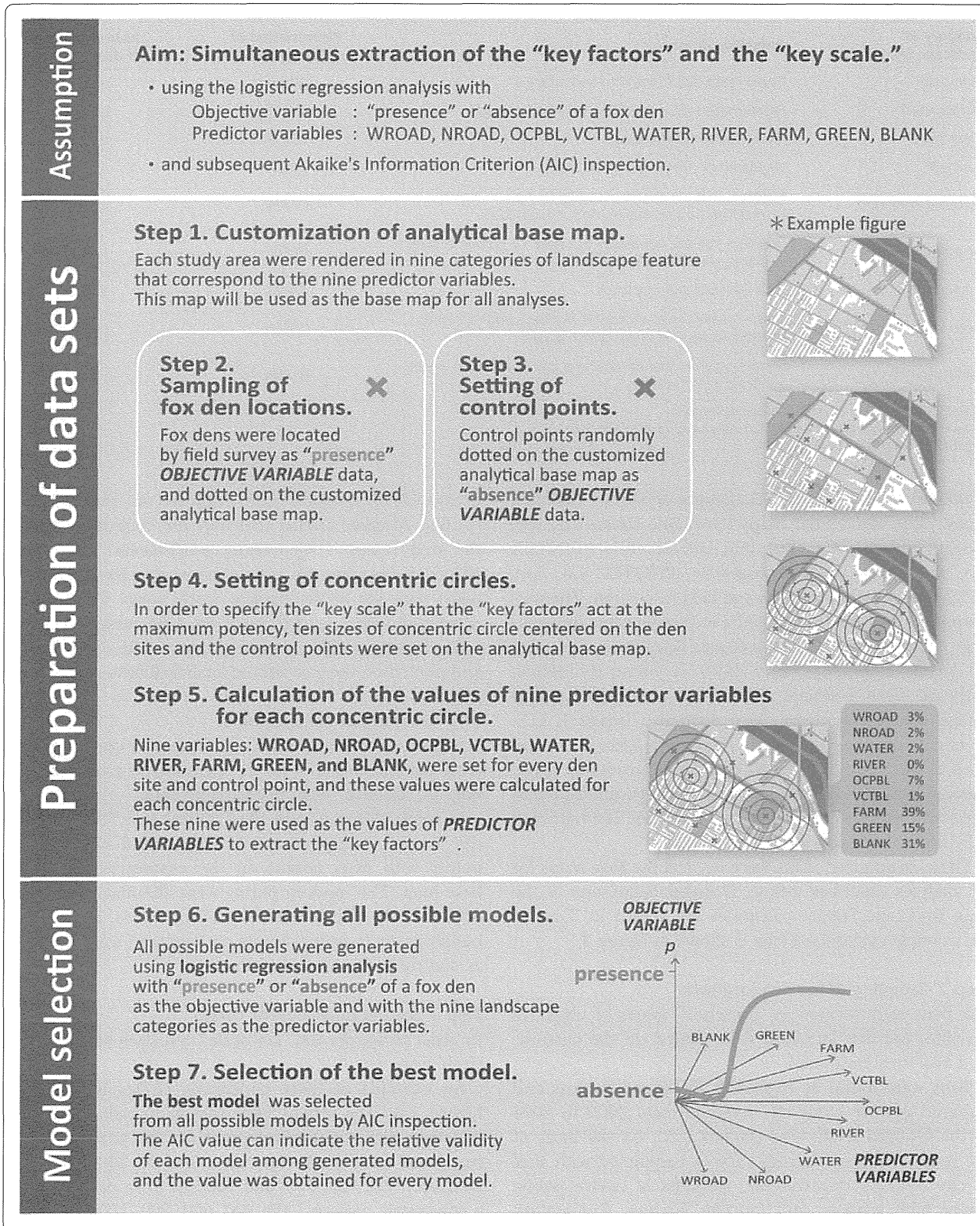


Figure 2 The protocol for the modeling process. *See also the legend of Figure 3-A.

Table 1 Definitions of the landscape feature categories and terms, and their corresponding variables and abbreviations

Category of landscape feature	Definition of term	Abbreviation of modeling variable	Abbreviation of linear distance variable
Wide road*	Paved roads (≥5.5 m width) and railways.	WROAD	L-wroad
Narrow road*	Paved roads (<5.5 m width) and unpaved roads.	NROAD	L-nroad
Water place*	Rivers, streams, and drains.	WATER	L-water
Riverbed*	Vegetated or dried areas along rivers.	RIVER	L-river
Occupied building**	Buildings that are always occupied by human activity, i.e. dwelling houses, outlets, and schoolhouses.	OCPBL	L-ocpbl
Vacant building**	Buildings that are not always occupied by human activity, i.e. barns, garden sheds, and garages.	VCTBL	L-vctbl
Farmland***	Meadowlands and croplands.	FARM	L-farm
Green covered area***	Green covered areas except for riverbeds and farmlands, i.e. urban parks and urban green spaces.	GREEN	L-green
Blank space***	Remaining areas that do not have any roads, rivers, water, buildings, or vegetation.	BLANK	L-blank

*Based on definition of numerical information maps.
**Based on definition of numerical information maps and house maps.
***Extracted from aerial photographs.

25000 Web Map Service (Geographical Survey Institute, Japan [52]), Residential Maps (Hokkaido-Chizu Co., Ltd. [53,54], ZENRIN Co., Ltd. [55] and ZENRIN PRINTEX Co., Ltd. [56]), aerial photographs (PHOTEC Co., Ltd. [57] and Google Earth [58]), and field inspection. The rendering process was performed using geographic information system software (free software: Quantum GIS 1.8.0, QGIS Development Team [59]), a photo-retouching software (free software: Paint.NET 3.5.10 [60]), and image analysis software (free software: Image J, U.S. National Institutes of Health [61]). The latter two were used to extract and ascertain borders of farmlands, green covered areas, and blank spaces from aerial photographs, because these landscape features were not distinguished fully in the numerical information maps.

These customized maps were used as the base maps for all analyses described below. Detailed definitions of the nine landscape feature categories are shown in Table 1. An example customized map is shown in Figure 3.

Step 2. Sampling of fox den locations

Fox dens were located as "presence" values of objective variable, and den locations were dotted on the customized analytical base map.

Dens were found by exploring all vegetated areas and unpaved ground along the riverbed from 2002 to 2004 in the Obihiro study area (Figure 1-A), on the basis of the results of questionnaire surveys conducted with staff of city cleaning departments, students of twelve public junior high schools, and farming families. Exploration was carried out from 2004 to 2007 in the Sapporo study area (Figure 1-B) with the support of hunters in addition to location data collected from farmers and previous reports [9,12]. All tunnels with a diameter of circa 20 cm excavated by animals were regarded as red fox dens [41,62]. Another animal that may use such dens around the study areas is *Nyctereutes procyonoides* Gray, 1834 (Raccoon dog), but this is a nonnative species and has not taken root yet in the present study areas. The location data of all dens found through the field surveys were recorded using a GPS receiver (Garmin Ltd., GPS 12CX), and plotted on the customized analytical base map.

Step 3. Setting of control points

As against the points with dens present, control points were dotted randomly on the customized analytical base map as "absence" objective variable data.

In total, 120 points in the Obihiro and 730 points in the Sapporo study areas were generated randomly as points with dens absent on the customized analytical base map. The random points were eliminated and generated newly if they were located on roads, in occupied buildings, or in water. Points on farmland were accepted as control points in this study.

Step 4. Setting of concentric circles

In order to specify the "key scale", ten sizes of concentric circle were set.

An example of these concentric circles is shown in Figure 3-A. The circles were 100 m in radius centered on every den and control point on the analytical base map, and each circle was expanded to 200, 300, 400, 500, 600, 700, 800, 900, and 1000 m from each point in a concentric pattern (200, 400, 600, 800, 1000 m circles are shown in Figure 3-A). The "key scale" was determined from these circles. The values of variables defined in Step 5 were calculated for each circle around all den sites and control points.