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Multicomponent analyses of a hydatid cyst from an Early Neolithic hunter–fisher–gatherer from Lake Baikal, Siberia

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ABSTRACT

Calcified biological objects are occasionally found at archaeological sites and can be challenging to identify. This paper undertakes the differential diagnosis of what we suggest is an *Echinococcus granulosus* hydatid cyst from an 8000-year-old mortuary site called Shamanka II in the Lake Baikal region of Siberia. *Echinococcus* is a parasitic tapeworm that needs two hosts to complete its life cycle: herbivores and humans are intermediate hosts, and carnivores such as dogs, wolves, and foxes are definitive hosts. In the intermediate host the *Echinococcus* egg hatches in the digestive system, penetrates the intestine, and is carried via the bloodstream to an organ, where it settles and turns into an ovoid calcified structure called a hydatid cyst. For this object, identification was based on macroscopic, radiographic, and stable isotope analysis. High-resolution computed tomography scanning was used to visualize the interior structure of the object, which is morphologically consistent with the *E. granulosus* species (called cystic *Echinococcus*). Stable isotope analysis of the extracted mineral and protein components of the object narrowed down the range of species from which it could come. The stable carbon and nitrogen isotope ratios of the object's protein, and stable carbon isotope ratio of the mineral, closely match those of the likely human host. Additionally, the $\delta^{13}C$ protein-to-mineral spacing is very low, which fits expectations for a parasitic organism. To our knowledge this is the first isotopic characterization of a hydatid cyst and this method may be useful for future studies. The hydatid cyst most likely came from a probable female adult. Two additional hydatid cysts were found in a young adult female from a contemporaneous mortuary site in the same region, Lokomotiv. This manuscript ends with a brief discussion the importance of domesticated dogs in the disease's occurrence and the health implication of echinococcal infection for these Early Neolithic hunter–fisher–gatherers.

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1. Introduction

During excavation of a 7000 to 8000-year-old Early Neolithic cemetery called Shamanka II (Weber et al., 2006), located on the southern tip of Lake Baikal, Siberia, Russian Federation (Fig. 1), an ellipsoidal calcified biological object was found in the abdominal area of a human skeleton. The term calcification refers to the deposition of calcium phosphate salts in tissues (whereas, the term ossification indicates calcium deposition in a collagen matrix). About the size of a chicken egg, this ovoid and hollow object (Fig. 2; described in more detail below) was found in grave 23 with individual 2 (Fig. 3), amidst many grave goods including animal bones, implements, and ornaments. Initial examination of the object resulted in suggestions that it may be an organ cyst (e.g. ovarian or renal), a teratoma, a calcification of any number of normal or pathological structures (e.g. gallbladder, ovary, appendix, lymph node, fibroma, neoplasm, lipoleiomyoma, etc.), or a parasitic hydatid cyst that forms from infection by the tapeworm *Echinococcus granulosus*. Therefore, various methods were employed to identify the object and its possible significance in the lives of Early Neolithic foragers.

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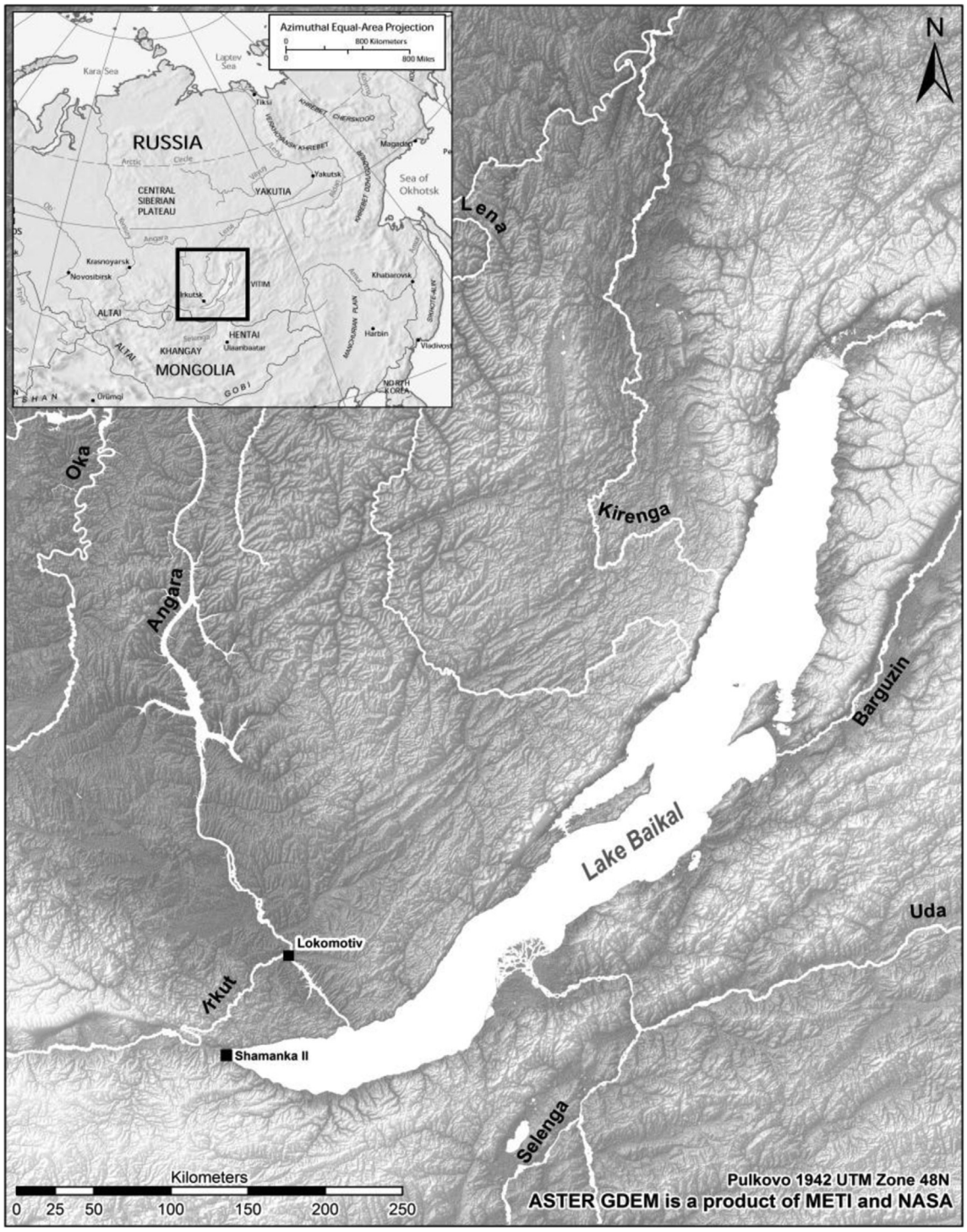

Fig. 1. Map of Lake Baikal showing the location of the mortuary sites Shamanka II and Lokomotiv, Siberia.

Two methods were applied to identify the object[1] and, in the case of it being parasitic, the host organism. First, high-resolution computed tomography (CT) scanning was undertaken to discern the structure of the object's interior. Second, stable isotope analyses of the protein and mineral component was undertaken to narrow the range of species from which it could derive. To our knowledge this is the first isotopic characterization of this type of calcified object. Two similarly shaped calcified biological objects were found at a nearby contemporaneous site called Lokomotiv, making it possible these Early Neolithic hunter–fisher–gatherers were similarly affected.

[1] The extensive handling of the object, and its antiquity, made the success of aDNA analysis very unlikely. Additionally, a significant portion of the object would need to be destroyed. Thus, aDNA extraction and analysis was not attempted.

Fig. 2. Calcified biological object from grave 23, individual 2, Shamanka II, Siberia (post-mortem damage visible as small holes and a crack on the left lower side and the large opening on the upper end).

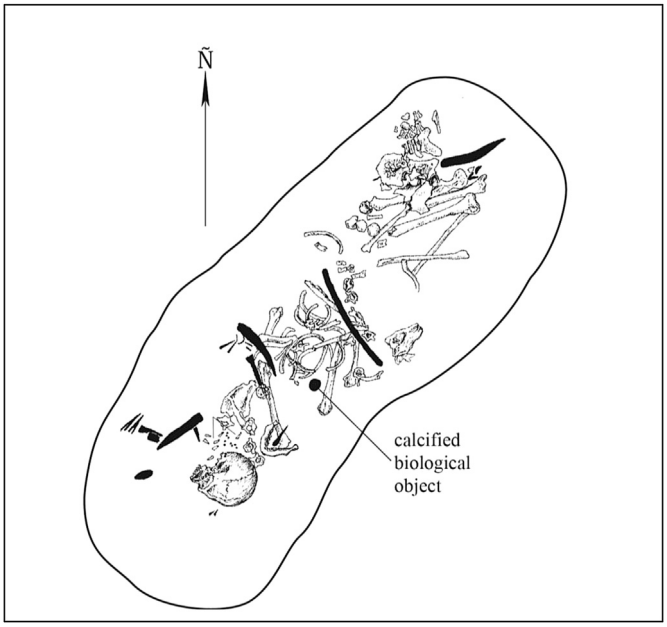

Fig. 3. Plan view of grave 23, individual 2, Shamanka II, Siberia, with location of the calcified biological object indicated. Other objects shown in black are grave inclusions (artifacts, animal remains).

After the materials section below, this manuscript is divided into three sections. Section I contains the methods, results, and discussion concerning the differential diagnosis of the object, including the CT data. Section II contains the methods, results, and discussion of the stable isotope analysis of the object. Finally, Section III concludes with a brief discussion of possible morbidity and mortality implications of the affliction and the role that domesticated dogs may have played. The significance of this case study lies in the analytical techniques that are applied, and the identification of a previously undiagnosed disease in these northern hunter–fisher–gatherers. More broadly, this research demonstrates the close interaction of people and dogs in the Early Neolithic period. Dogs are an important vector for infections that affect humans, sharing at least 65 diseases (McNeill, 1976), including for example tuberculosis (Bathurst and Barta, 2004), Chagas' disease (Aufderheide et al., 2004), and hydatid disease (Ortner, 2003).

2. Material

2.1. Grave composition and human skeletal osteobiography

The depositional environment at Shamanka II resulted in generally very good organic preservation. However, various taphonomic factors caused comingling and disturbance in some graves, including, to a certain extent, in grave 23. Five individuals are present in the grave pit: individual 23-1 is a relatively complete male of 35–45 years; while the remains of individuals 23-3, 23-4 and 23-5 were incomplete and fragmentary, resulting in age determinations of only 'adult', and no morphological sex estimations (Lieverse et al., n.d.); however, subsequent aDNA results identified 23-4 and 23-5 as male (Thomson, 2006). Individual 23-2, with the calcified object, is estimated as a probable female based on postcranial skeletal morphology, while an age of 18 + years ('adult') was estimated based on the complete fusion of all skeletal elements (no skull or teeth were identifiable to this individual) (Lieverse et al., n.d.; Lieverse, 2005). Because of the comingling within grave 23, and the presence of a number of graves goods of animal bone, teeth, and antler, it is not certain that the calcified object came from one of the humans in the grave. At Shamanka II burial inclusions of deer (red deer (*Cervus elaphus*), roe deer (*Capreolus pygargus*)) are most common, and moose (*Alces alces*) and musk deer (*Moschus moschiferus*) are also present (Losey et al., 2013a,b).

2.2. Unidentified calcified object

Weighing 10.35 g, the object's maximum dimensions are 3.48 cm length, 3.12 cm width, and 9.62 cm circumference (Figs. 1 and 4). A post-mortem break on one end shows an internal structure of thin dispersed calcified layers that could be described as lattice-like. The outer surface is coarse with disorganized fiber-like

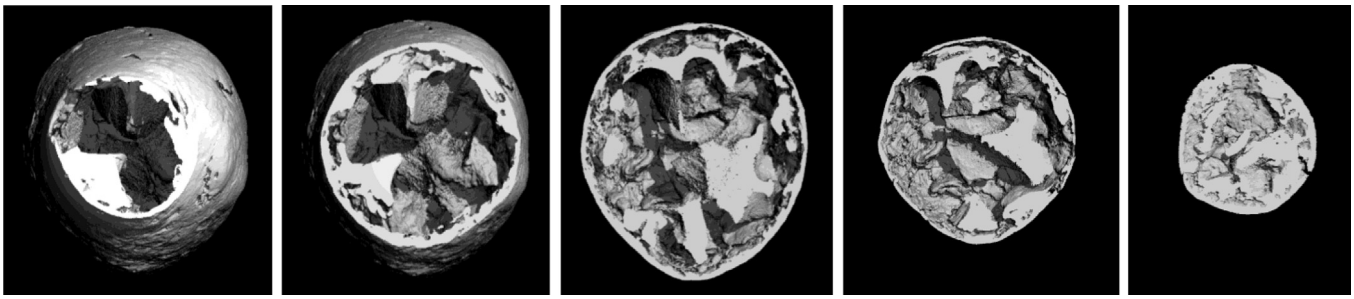

Fig. 4. Five serial CT scan sections through the calcified object demonstrating the internal septa. http://dx.doi.org/10.1016/j.jas.2014.06.015.

patches distributed around the entire object (Fig. 1). Note, there are a few other small post-mortem cracks and breaks.

3. Section I

3.1. CT methods and results for differential diagnosis

The primary method of differential diagnosis was to first identify the main attributes of the object, and then use these to produce a list of conditions that may have resulted in formation of the object. Using the Scanco viva-CT 40, the object was scanned to characterize its internal structure, with an isotropic image resolution of 38 μm. The resulting three-dimensional image was globally thresholded to reduce noise.

CT imaging of the object reveals a well-defined hollow mass with continuous curvilinear external calcification and several intra-object calcified septa ('walls') that indicate the presence of multiple chambers (Fig. 4) [Video S1]. Because it was not always possible to determine the boundary of the outer wall and septae, it was difficult to determine average wall thickness for the entire structure. However, in regions where septae are absent, wall thickness averages 0.63 mm. The volume of the object is 153.0 mm.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.jas.2014.06.015.

Therefore, for the differential diagnosis the main attributes of the unidentified object are that: 1) it is ellipsoid in shape and hollow; 2) it is small to medium in size; 3) it has a continuous outer shell (or if there was an opening, where the post-mortem break is now, the outer shell was near-continuous); and 4) it lacks external vascularization (i.e. it was not connected to the individual's vascular system). A fifth attribute, the presence of calcified internal septa indicating a multi-chambered structure, is considered for its presence or absence in each possible condition as listed in Table 1.

3.2. Differential diagnosis results

Based on these attributes a list of the conditions that could have formed the object is presented in Table 1, with a brief description of each condition. Note, the table does not include conditions/forms that do not match the key attributes of this object. This rules out calcified urinary or renal calculus, ovarian fibroma, appendicolith, leiomyoma, calcified omental nodule, mesenteric carcinoid tumor, psammoma body, mesenteric cyst, myostitis ossificans and any non-cyst-like heterotopic ossification, and fibrodysplasia ossificans progressiva.

3.3. Differential diagnosis discussion

The fact that the calcified object has internally calcified septa helps narrow down the range of possible objects, with only those objects from Table 1 that can potentially contain internally calcified septa discussed further. The other objects are listed to demonstrate the rather high number of causes/diseases that can result in medium-sized ovoid calcified bodies. Both types of teratoma, the cystic (non-ovarian) and the ovarian dermoid type, may contain calcified septa, but also often contain other tissues that can be preserved, such as teeth and bone (i.e. Armentano et al., 2012), making this diagnosis less likely. The object in question is larger with thicker walls than typical modern documented calcified lymph nodes and is seemingly singular, whereas usually more than one lymph node becomes calcified (Gawne-Cain and Hansell, 1996; Gross et al., 1980; Jacobson et al., 1967), suggesting it is unlikely to be a calcified lymph node.

This size of the object fits well within the range of a calcified amputated ovary (Abd-el-Maeboud and Salama, 1997; Currarino and Rutledge, 1989; Fletcher et al., 1988; Ledesma-Medina et al., 1992). Calcified amputated ovary is very rare and usually reported in subadults (Fletcher et al., 1988; Lester and McAlister, 1970; Nixon and Condon, 1977; Uygun et al., 2012). The calcifications can be amorphous and irregular, or curvilinear and regular, and are variable as to residual evidence of vascularity (Kennedy et al., 1981). While some amputated ovaries have internal septa, these are rarely calcified (Haliloglu et al., 2004; Ledesma-Medina et al., 1992), and certainly not so completely, so it is unlikely this is the source of the object.

Calcification of renal cysts can occur in the wall or the septa of benign as well as malignant lesions (Kim et al., 1981; Weyman et al., 1982). Renal cysts are usually larger than the object in question, but there are exceptions (Israel and Bosniak, 2003). The strongest reason that the object in question is unlikely to be a renal cyst is that its calcification is continuous (there are no non-calcified areas) and homogenous (the thickness and morphology of the calcification is similar). In contrast, calcified renal cysts are very variable: they may only have one small patch of calcification, in the wall or septum, and these calcifications can vary noticeably in thickness and appearance (i.e. as nodular calcifications) (i.e. see Israel and Bosniak, 2003). As well, some calcified renal cysts have a lobulated appearance, thus dissimilar to the object in question. Finally, in renal cysts that have been calcifying over many years, the layers may become very thick such that the cyst is almost filled (Israel and Bosniak, 2003); this is also not in keeping with the object's morphology. In sum, in our opinion it is very unlikely that a renal cyst would have calcified into something that looks like the object. Therefore, we propose that the object is most likely to be a calcified hydatid cyst.

Aufderheide and Rodriguez-Martin (1998) note how difficult it can be to definitively identify a hydatid cyst based only on the calcified remains because of the large number of other objects that are similar in appearance (clearly seen in Table 1). The hydatid cyst forms from *Echinococcus* infection, a parasite tapeworm that needs two hosts to complete its lifecycle: herbivores and humans are intermediate hosts, while carnivores are the definite host. Hydatid cysts can form in non-human animals as well as humans. In the intermediate host the *Echinococcus* egg hatches in the digestive system, penetrates the intestine, and is carried via the bloodstream to an organ, where it settles and turns into a bladder-like calcified structure – the hydatid cyst. The object corresponds to the morphology of a hydatid cyst in terms of being ellipsoid in shape, hollow, small to medium in size, formed of a continuous calcified shell, without evidence of vascularization, and its inner morphology contains calcified septa. *E. granulosus* hydatid cysts usually grow slowly, on average one cm per year, reaching typical sizes of one to ten cm (Canda et al., 2003; Lucas, 1994; Mandell and Mandell, 2010). Once a cyst reaches a diameter of approximately one cm, its wall differentiates into a thick outer, non-cellular membrane which eventually becomes completely calcified, covering a thin germinal epithelium (Canda et al., 2003). Hydatid cysts are not connected to the host's vascular system. In about two-thirds of cases, hydatid cysts are singular and with time may develop internal daughter cysts that result in inner septations (walls) which can eventually calcify (Canda et al., 2003; Moro and Schantz, 2009). Cysts are most common in the liver (75%), lungs (5–15%), and may also develop in the kidneys, spleen, brain, muscles, and bone (Canda et al., 2003).

Calcified hydatid cysts can be classified into two types based on appearance (adapted after von Sinner et al., 1991; Polat et al., 2003). Type I is a simple cyst with no internal architecture and Type II is a cyst with internal architecture because of the existence of one or more daughter cysts that result in an internal matrix. As well, two categories of cysts with daughter cysts exist: type A have round

Table 1
List of conditions considered in the differential diagnosis of the calcified object.

Condition	Description	Appearance of calcified object	Presence of multi-chambered internal structure
1. Calcified hydatid cyst from *Echinococcosis granulosus* (cystic echinococcus)	A parasitic organism, *Echinococcosis granulosus*, that grows into a hydatid cyst (usually located in the liver and lungs) and can become calcified.	*E. granulosus* cyst is typically unilocular, spherical or sub-spherical, filled with clear fluid, and composed of a single main chamber. It can be multi-chambered if daughter cysts formed within the main cyst (Brunetti et al., 2010). Small to medium in size (1.0–10.0 cm in diameter).	Possible – common
2. Calcified renal cyst	Calcification of relatively common cystic renal masses (collections of fluid in kidney). 1–3% of cysts calcify.	Usually unilocular, hollow, with an oval or circular shape. Calcification can be thin and smooth or thick and nodular, and can occur in the wall and/or septa if present. Small to medium in size (2.5–6.0 cm in diameter) (Israel and Bosniak, 2003).	Possible – common
3. Calcified amputated ovary	Torsion of the adnexa with subsequent amputation and possible calcification of the ovary. This can move around the pelvic cavity or become attached to a structure.	Solid or partially calcified, oval in shape. Small in size (1.5–4.0 cm diameter) (Kennedy et al., 1981).	Possible – rare
4. Calcified lymph node of the egg-shell type	Calcification around the periphery of the lymph node, of unknown cause but common with prolonged or repeated infection.	Wall very thin (<2 mm). May or may not have internal calcifications (Gross et al., 1980; Jacobson et al., 1967). Usually two or more involved. Small in size with short axis diameter <2.0–3.0 cm (Gawne-Cain and Hansell, 1996).	Possible
5. Calcified ovarian dermoid cyst (also known as an ovarian teratoma)	Calcification of fluid-filled cyst sacs that can be common in the ovary. Calcified ovarian cysts are almost always of the dermoid type (also known as a teratoma) meaning they contain tissue from more than one germ layer, frequently all three. May contain hair, teeth or bone.	Usually thin outer calcification with variable interior morphology, including a hollow fluid (fat and water) filled form and more solid masses containing hair, teeth, or bone. Of variable size, usually medium to large (>6.0 cm diameter) (Outwater et al., 2001).	Possible (see Armentano et al., 2012)
6. Cystic teratoma (non-ovarian)	A teratoma is an encapsulated tumor with tissue or organ components resembling normal derivatives of two or all three germ layers. There are solid and cystic (fluid or semi-fluid filled) forms. The outer shall often calcifies.	Sometimes the teratoma has within its capsule one or more fluid-filled cysts. May occur anywhere depending upon its derivative cell type. The most common types (besides ovary listed above) are saccrococcygeal, testicular, and mediastinal (Friedman et al., 1982). Of variable size, from medium (~5 cm) to large (>15 cm) (Moeller et al., 1997).	Possible
7. Calcified hydatid cyst from *Echinococcus multilocularis* (alveolar echinococcus)	A parasitic organism, *Echinococcus multilocularis*, with metacestodes that develop primarily in the liver and cause necrosis with scattered or pseudo-cystic calcification.	Comparatively more complex structure than *E. granulosus* cysts. The *E. multilocularis* cyst is always multivesicular as alveolar (blister-like) tissue forms, with infiltrative (tumor-like) rather than expansive growth. Of variable size depending upon extent of growth (can destroy entire organ) (Brunetti et al., 2010).	No
8. Calcified uterine lipoleiomyoma	A rare benign tumor of the uterus composed of masses of fat cells and fibrous tissue that can calcify. It results from degeneration of the smooth muscle cells (considered a	Usually round and without internal calcification (fat-filled mass). Small to medium sized (a few mm to a few cm) (Avritscher et al., 2001; Sudhamani et al., 2010).	No

(*continued on next page*)

Table 1 (*continued*)

Condition	Description	Appearance of calcified object	Presence of multi-chambered internal structure
	distinct variety of uterine leiomyoma).		
9. Calcified fat necrosis (usually pancreas, sometimes breast)	Metabolically or traumatically induced areas of fat necrosis that can calcify. Most often in or near the pancreas and possibly the breast.	Highly variable in size and form. In breast eggshell or rim calcifications, the wall is usually less than 1 mm in thickness (Smithuis and Pijnappel, 2008). Extra-pancreatic cysts are usually 3–5 cm in diameter (Klöppel and Maillet, 1991).	No
10. Calcified gallbladder	Calcification of the gallbladder. Also known as a porcelain gallbladder. The incidence at autopsy is 0.6–0.8%, with a male-to-female ratio of 1:5 (Polk, 1966).	Calcium encrustation of the gallbladder wall usually resulting in a hollow, semilunar or pear-like object with hard, bluish-white texture resembling porcelain ceramic. Avascular with a 'shaggy' fibrous surface. Small to medium in size (Ashur et al., 1978).	No
11. Calcified granuloma	Soft tissue granulomatous lesions may calcify. Granulomas can form in tuberculosis, schistosomiasis, sarcoidosis, various fungal infections, and even around foreign bodies.	Single or multiple, partial or complete calcification. Can be very small (<1 cm) to, rarely, medium (6 cm) in size (Marchiori et al., 2005; also see Salo et al., 1994 for TB example).	No
12. Generalized calcified neoplasm	A general category because any abnormal mass of tissue that results in a lump or tumor can become calcified (because of tumor necrosis).	Variable in every regard: may be singular or multiple, may be circular/oval or more amorphous, outer wall may be thin or thick, may be hollow or solid, many to very small (<1 cm) to very large (>50 cm) (Stewart et al., 1983). Often show vascularization.	No
13. Intra-testicular tumors/ cysts (not including teratoma listed above)	After Vikram et al. (2001): a) Intratesticular tumors derived from the Sertoli and Leydig cells of the seminiferous tubules sometimes calcify (especially the large-cell calcifying Stertoli cell tumour). b) Epidermoid tumours may have calcification of the wall c) Simple testicular cysts are usually thin walled and may contain calcification within the rim	After Vikram et al. (2001): a) Usually solitary but sometimes bilateral/multifocal (~20%), average 2 cm diameter, range from 1 to 6 cm b) Usually solitary, range in diameter from 1 to 3 cm c) Usually solitary, range from 2 mm to 2 cm in diameter	No
14. Calcified faecalith (also called fecaloma)	Calcification of a hard mass of dried feces that can occur in the appendix and large or small intestine.	Usually solitary, rarely two or more, oval or circular in shape with a smooth outer wall. Small in size at 1–2 cm in diameter (Antonopoulos et al., 2006).	No

daughter cysts arranged at the periphery of the mother cyst; type B lesions contain larger, irregularly shaped daughter cysts that occupy almost the entire volume of the mother cyst. Clearly, this hydatid cyst is a Type II-B.

4. Section II

4.1. Hydatid cyst structure

Now that we have established that the object is most likely a hydatid cyst, a brief overview of its structure and biochemical composition is provided in order to understand the subsequent stable isotope analyses. A hydatid cyst has three layers (Fig. 5): an outer host derived reaction (pericyst) and two internal parasite derived layers (endocyst). Polat et al. (2003) describes the outer adventitial layer, which is the layer that can calcify, as consisting of modified host cells, fibroblasts, giant cells, and eosinophils that form a rigid protective layer a few millimeters thick. The middle laminated membrane is acellular, easily ruptured, and has the purpose of the passage of nutrients (Polat et al., 2003). The inner germinal layer is thin and responsible for the formation of the other layers, as well as the hydatid fluid and brood capsules within the cyst. The infectious embryonic tapeworms (protoscoleces) develop from outpouchings of the germinal layer and are known as the brood capsules (Polat et al., 2003). Fig. 5 also shows a daughter cyst with brood capsules within it. The cyst fluid is clear and is a transudate of serum containing proteins (Lewall and McCorkell, 1985). The thickness of these layers depends on the tissue in which the cyst is located. The layers tend to be thick in the liver and less developed in muscle (Polat et al., 2003). During the natural course

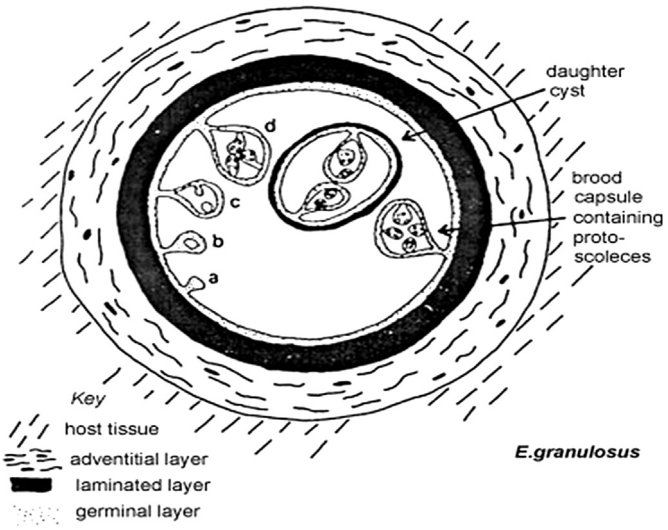

Fig. 5. Diagrammatic representation of *Echinococcus granulosus* cyst structure (from Brunetti et al., 2010).

of a hydatid cyst, complete calcification occurs, which basically indicates the death of the cyst (Polat et al., 2003). Calcification is more common in hydatid cysts of the liver, spleen, and kidney (Polat et al., 2003).

4.2. Hydatid cyst biochemical composition

It is necessary to know the biomolecule composition and polypeptide profile of hydatid cysts, especially the calcified pericyst, in order to understand what we are analyzing isotopically. The pericyst consists of two sub-layers with different formative mechanisms: the outer adventitial layer, directing toward the host organ, is the result of fibrosis and compression of paraenchyma cells and blood vessels of the host organ. The inner layer of the pericyst, directing toward the parasite, is a granuloma-like layer (Chen et al., 2004; Derbel et al., 2012; Peng et al., 2005). Osteopontin, a highly acidic glycoprotein, has been found to be a prominent constituent of calcified and calcifying pericysts (Peng et al., 2006). Osteopontin has an arinine-glycine-aspartic-acid calcium binding site which is responsible for the regulated mineralization progression (O'Regan and Berman, 2000). The fibrous tissue of the adventitial also contains collagen (Peng et al., 2006). Several studies have detected host-derived proteins including immunoglobulin in the cyst wall (Coltorti and Varela-Diaz, 1974; Taherkhani and Rogan, 2000; Varela-Diaz and Coltorti, 1973).

In the parasite derived endocyst layers, protein by far is the most abundant biomolecular component by weight (Irshadullah and Nizami, 1997; Irshadullah and Rani, 2011). The laminated (middle) layer is a polysaccharide protein complex, mostly glycoprotein (Kilejian et al., 1962; Kilejian and Schwabe, 1971; Richards, 1984; Taherkhani, 2001). In the inner germinal layer the proteins identified belong to the disulphide group, and the most common amino acids are arginine and tryptophan (Richards, 1984) with any protoscolex containing the proteins albumin and globulin (Frayha and Haddad, 1980). The cyst fluid also contains the proteins albumin and especially globulin (Frayha and Haddad, 1980).

Therefore, if there is preserved protein in the hydatid cyst it will be of the glycoprotein type, most likely osteopontin and collagen, and maybe immunoglobulin. The amino acid composition of osteopontin is predominated by the non-essential amino acids aspartate, serine, and glutamate (Kiefer et al., 1989), which is quite different from type I collagen which is predominated by glycine and proline, with a relatively high hydroxyproline content (Eastoe, 1955). Parasite derived globulin or albumin may be present if any of the endocyst tissue became incorporated into the calcification.

4.3. Stable isotope methods

A small fragment (0.5 g) of the outer surface of the cyst was obtained from a pre-existing broken edge. The sample was cleaned ultrasonically in $ddH^2 0$ and dried. To isolate the protein fraction and dissolve the mineral fraction, a portion of the sample was soaked in four washes of 0.5% hydrochloric acid (HCl) for 24–48 h, similar to the method of Sealy (1986). The remaining organic material was soaked in a 0.125 M sodium hydroxide (NaOH) solution for 20 h in order to remove any humic acids. The resultant gelatinous residue was then rinsed to neutrality in ddH^2O and freeze-dried. The gelatinous residue was analyzed on a Finnigan Mat Delta + mass spectrometer interfaced with a Carlo Erba gas analyzer in the Isotope Science Laboratory, University of Calgary, under the direction of Stephen Taylor. Isotope ratios are reported in permil (‰) relative to V-PDB for carbon and AIR for nitrogen. Precision of analysis for both $\delta^{13}C$ and $\delta^{15}N$ is 0.2‰ as determined by repeat analyses of an internal laboratory standard. C/N ratios as well as %C and %N are provided by the Carlo Erba gas analyzer. Precision of %C and %N is 5%.

The mineral fraction was isolated by the method described by Lee-Thorp et al. (1989) using the unprocessed portion of the sample. Ground tissue was soaked in four washes of dilute sodium hypochlorite (2%) for 48 h to remove organic matter. Dilute acetic acid (0.1 M) was used to remove any recently deposited carbonate from the burial environment. The resultant carbonate was then rinsed to neutrality in ddH^2O and freeze-dried. Further treatment and analyses were carried out in the Institute of Geology and Mineralogy, University of Erlangen, under the direction of Dr. Michael Joachimski. Carbonate powders were reacted with 100% phosphoric acid at 75 °C using a Kiel III online carbonate preparation line connected to a Thermo-Finnigan 252 mass spectrometer. Values are reported in permil (‰) relative to V-PDB by assigning a $\delta^{13}C$ value of +1.95‰ and a $\delta^{18}O$ value of −2.20‰ to NBS19. Reproducibility of replicate analysis of laboratory standards is better than 0.1‰ for $\delta^{13}C$ and $\delta^{18}O$.

4.4. Stable isotope results

Table 2 depicts the stable isotope and preservation indicator results. As we do not know the exact composition of the analyzed protein(s), it is not possible to use the preservation indicators (i.e. C/

Table 2
Stable isotope ratios and preservation results for purported hydatid cyst.

	Value
Protein	
$\delta^{13}C$	−17.2‰
$\delta^{15}N$	17.0‰
Atomic C/N ratio	4.0
%C	53
%N	15
Protein yield	8.3
Mineral	
$\delta^{13}C$	−14.3‰
$\delta^{18}O$	−14.1‰
Mineral yield	54.3
$\delta^{13}C$ protein–mineral spacing	2.9

N ratio, %C, %N, protein yield, and mineral yield) in the same way as with bone collagen samples. We can however, offer a few points that suggest the extracted hydatid cyst protein and mineral are adequately preserved and not diagenetically altered. First, the bone collagen and apatite of individual 23-2, as well as the other individuals in grave 23, is very well preserved and uncontaminated as evidenced by acceptable values for all stable isotope preservation indicators. This suggests other organic and inorganic material in the grave should be similarly well preserved. In addition, the hydatid cyst protein yield and mineral yield are sufficiently high which suggests good preservation, as small values can be associated with poor preservation (Ambrose, 1990, 1993). Similarly, the %C and %N values are sufficiently high. Finally, because we know what types of protein constitute the calcified hydatid cyst layer, we can postulate about if the C/N ratio of 4.0 is within the range of what it should be. An acceptable bone collagen C/N ratio can vary from 2.9 to 3.6 (DeNiro, 1985). As noted above, the hydatid cyst is expected to contain a significant amount of protein other than collagen, especially osteopontin. The composition of osteopontin, being predominated by the non-essential amino acids aspartate, serine, and glutamate (Kiefer et al., 1989; Prince et al., 1987), should lead to a C/N ratio higher than that of collagen alone. Studies by Howland et al. (2003), Corr et al. (2005), and Styring et al. (2010) have demonstrated considerable isotopic difference between amino acids, related to the origin of their building blocks and the biosynthetic pathways of essential versus non-essential amino acids. Aspartate (common in osteopontin) usually has a more negative $\delta^{13}C$ value than glycine (common in type I collagen) (Corr et al., 2005), and glutamate (common in osteopontin) usually has a more positive $\delta^{15}N$ value than glycine (common in type I collagen) (Styring et al., 2010). We argue this should cause the C/N ratio to be higher than 3.6, and quite possibly in the range of 4.0. Therefore, we suggest that despite the current limitations in precisely assessing cyst protein and mineral preservation, the preservation indicator data are suggestive of protein and mineral isotope values that will be indicative of *in vivo* isotope values.

Fig. 6 graphs the hydatid cyst stable isotope protein results with collagen stable isotope results from humans from the site of Shamanka II, including individual 23-2, and several faunal species. The hydatid cyst has the highest $\delta^{15}N$ value (17.0‰) of all species depicted, 1.4‰ above individual 23-2 and 2.4‰ above the mean of adults from Shamanka II. The $\delta^{15}N$ value of the cyst is over 10‰ higher than the cervid species (roe deer, red deer, musk deer, moose). The $\delta^{13}C$ value of the cyst is closest to that of humans at −17.2‰, being more negative than the mean by 0.8‰, and 1.5‰ more negative than individual 23-2, but well within the range of $\delta^{13}C$ collagen values from Shamanka II adults (range −14.7 to −18.4‰, $n = 93$).

The $\delta^{13}C$ mineral value of the hydatid cyst is −14.3‰, which is higher than almost all faunal species from Lake Baikal. Humans from Shamanka II have a mean $\delta^{13}C$ carbonate value that is slightly higher at −12.1 ± 0.7‰ (range −9.7 to −13.8‰, $n = 93$). The protein–mineral spacing value of the hydatid cyst is the lowest of all Baikal faunal species at 2.9, as well as lower than adult humans from Shamanka II, with a mean $\Delta\delta^{13}C$collagen–carbonate spacing value of 4.3 ($n = 93$).

4.5. Stable isotope discussion

We argue the protein and mineral stable isotope values of the hydatid cyst demonstrate it came from a human host. It is highly unlikely the cyst came from any of the other intermediate host species, given the markedly lower $\delta^{15}N$ values of the cervids from the region. There are two possibilities to explain why the $\delta^{15}N$ of the cyst is even higher than the human collagen values. Most likely, it is due to the comparatively high $\delta^{15}N$ value of the amino acid glutamate, common in the protein osteopontin but not collagen. Similarly, the $\delta^{13}C$ values may be slightly more negative because of the differing amino acid composition of osteopontin and collagen, with osteopontin containing a high proportion of aspartate which has a more depleted $\delta^{13}C$ value than the main collagen amino acid glycine. It is also possible that cyst-derived (as opposed to host-derived) proteins became incorporated into the calcified layer and that these proteins have high $\delta^{15}N$ values because they underwent a trophic shift. As the parasitic organism is feeding off of human tissue, it is reasonable to expect the $\delta^{15}N$ values to increase as occurs in all trophic level ascents. However, while some research has found significant $\delta^{15}N$ enrichment in ectoparasites relative to the

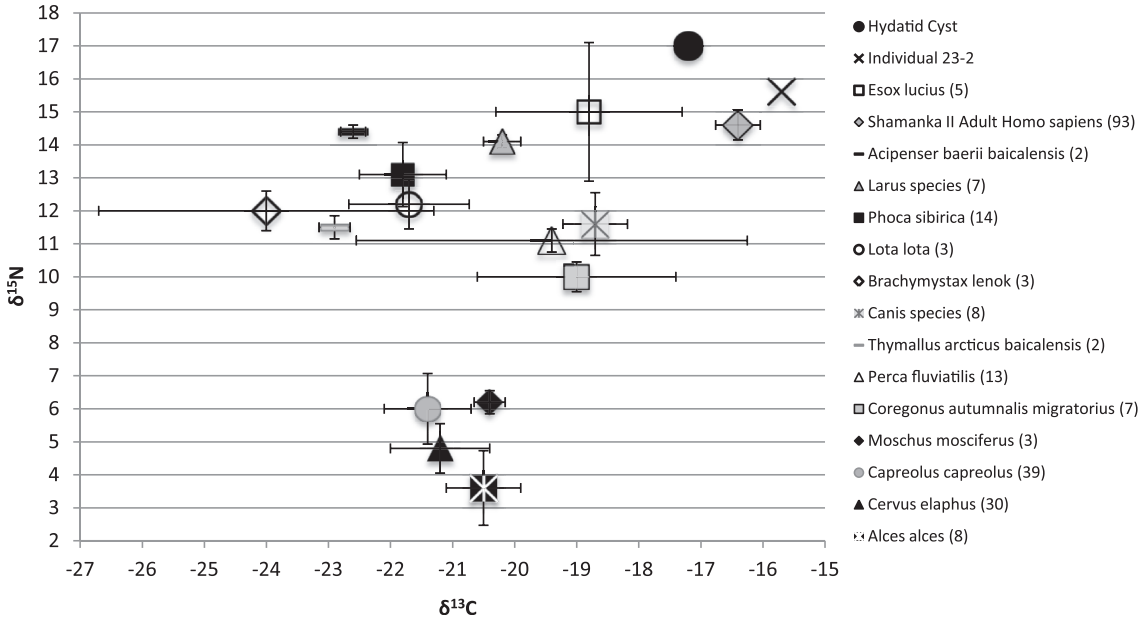

Fig. 6. Stable nitrogen and carbon ratio of hydatid cyst protein, and collagen from individual 23-2, adults from Shamanka II, and several faunal species. Error bars represent one standard deviation. Sample size is in parentheses. Human and animal data derived from Katzenberg et al. (2009, 2010, 2012) and Katzenberg and Weber (1999).

host (e.g. Gómez-Díaz and González-Solís, 2010), other studies have found that the magnitude of the ectoparasite-host fractionation is quite variable (i.e. Pinnegar et al., 2001). Each host-parasite system is complex, with the isotope system affected by a range of variables including the specific food source of the parasite (targeted amino acids or lipids) and the metabolism of both host and parasite. $\delta^{13}C$ also undergoes a slight, ~1.0‰, trophic increase (Fuller et al., 2006), which is not seen in the hydatid cyst $\delta^{13}C$ value (being more negative). Thus, in all likelihood the stable isotope values of the hydatid cyst reflect the host (human), not the parasite.

A comparison of $\delta^{13}C$ values from the protein versus mineral component of a tissue, in the form of a spacing value ($\Delta\delta^{13}C$protein–mineral, or when dealing with bone $\Delta\delta^{13}C$collagen–carbonate) can be indicative of trophic level, with herbivores showing a greater difference than carnivores (Krueger and Sullivan, 1984; Lee-Thorp et al., 1989). The physiological underpinning of this phenomenon is that different macronutrients (proteins, carbohydrates, fats) have disparate importance in different diets, with carnivores deriving energy from proteins and fats whereas herbivores derive energy from carbohydrates. Since fat is isotopically depleted in ^{13}C, relative to protein and carbohydrate (DeNiro and Epstein, 1978), an increase in its use for energy results in a less enriched bone $\delta^{13}C$ carbonate value, and hence a lower $\Delta\delta^{13}C$collagen–carbonate value. As this hydatid cyst is formed from biomolecules derived entirely from a human, its low $\Delta\delta^{13}C$protein–mineral may be indicative of its human parasitic status. In sum, stable isotope analysis of hydatid cysts is a useful method for determining the host organism, as values should be near the value of the host (keeping in mind the different amino acids compositions of the different major proteins), and the $\delta^{13}C$protein–mineral spacing may indicate the trophic level of the host from which the object was formed.

5. Section III

5.1. Discussion: echinococcosis in Early Neolithic Cis-Baikal Foragers

Echinococcosis disease has been recognized for centuries. There is mention of it in the Jewish Talmud and by several ancient Greco-Roman physicians (Hippocrates, Aretaeus, Galen) (Aufderheide and Rodriguez-Martin, 1998). Hydatid cysts are not commonly recovered from archaeological sites, but have been found in Iceland (Kristjánsdóttir and Collins, 2011), Denmark (Weiss and Møller-Christensen, 1971), the Middle East (Perry et al., 2008; Zias, 1991), Egypt (Tapp, 1986), England (Price, 1975; Wells and Dallas, 1976), and North America (Ortner, 2003; Ortner and Putschar, 1985; Williams, 1985). To our knowledge, this is the first published case of an archaeologically recovered hydatid cyst from East Asia. Molecular studies have shown that ten geographically distinct strains of *E. granulosus* exist (Moro and Schantz, 2009). The cervid strain exists in Eurasia and North America and involves cycles with dogs and wolves (definitive host) and moose and reindeer (intermediate host) (Moro and Schantz, 2009) and thus may be the strain that resulted in infection of individual 23-2. Clinical studies have found that human infection with the cervid strain involves slower and more benign growth, with less frequent side effects, than is reported for other forms (McManus and Thompson, 2003). *Echinococcus multilocularis* is also present in Russia today, with transmission to humans via hunting of wild game or ingestion of wild berries or water that has been contaminated with excrement from infected carnivores (Sergiev and Ozeretskovskaya, 2003).

Humans can be infected by over 50 species of cestode (tapeworm) and the larval cestode echinococcus is amongst the worst for causing disease or death (Cox, 2002). Yet, hydatid cysts, especially of small size, are usually asymptomatic (Moro and Schantz, 2009; Schantz et al., 1995). Thus, it is unlikely hydatid disease in individual 23-2 affected her morbidity or mortality because of the cyst's relatively small size. As well, given that the observable calcification (>90%) was intact, it was unlikely to have ruptured. If a hydatid cyst ruptures and the fluid is released into the host's circulatory system it can cause eosinophilia or anaphylaxis, although cyst rupture may be clinically silent (Lewall and McCorkell, 1985). If a cyst's size or location puts pressure on, or causes necrosis of, an organ, it can affect function, with non-specific symptoms including weakness, fever, and pain in the affected area (Moro and Schantz, 2009). In the liver, cysts can cause bile duct obstruction leading to jaundice, nausea and vomiting, and loss of appetite, as well as causing diminishing hepatic function that may lead to ascites (Moro and Schantz, 2009; Schantz et al., 1995).

At another Early Neolithic site, Lokomotiv (see Bazaliiskiy and Savelyev, 2003), located along the Angara River (Fig. 1), a 20 to 22 year-old female (burial 25-2) was found with two probable hydatid cysts (Lieverse, 2005). The human skeletal remains were relatively complete and well-preserved, being recovered from a multiple grave with four other discretely interred individuals (three adult females and one adult male). The probable cysts, two calcified nodules, were found among the remains of burial 25-2; however, because of the close proximity to the other individuals, it is possible they came from one of the other individuals in the grave. The cysts are roughly spherical in shape, measuring two to three centimeters in diameter and, like in the present case, exhibit a coarse outer surface with disorganized fiber-like patches (Fig. 7). Several small (3–5 mm) areas of postmortem breakage revealed that the nodules were not completely hollow, but contained internal calcified structures such as septa, again much like the present case. Unfortunately, examinations of the objects were limited to macroscopic, non-destructive methods, so more detailed information for the Lokomotiv cysts is not available at this time.

The fact that the two individuals with cysts are likely female raises the question of whether there is a sex-based difference in echinococcus infection. Clinical research in areas with endemic cystic echinococcus has found that females are more affected than males because of activities, such as herding livestock and caring for dogs, that bring them in closer contact with the parasite (e.g. Abu-Hasan et al., 2002; Yang et al., 2006). It does not appear there is a biologically based difference in susceptibility or immune response to *E. granulosus* (Pawlowski et al., 2001).

In general, cystic echinococcus is more common in temperate areas (Moro and Schantz, 2006). Its occurrence in non-temperate

Fig. 7. Two probable hydatid cysts found in grave 25, with individual 2, Lokomotiv, Siberia.

zones, for example Iceland and New Zealand, is mainly due to the close association of dogs in livestock (i.e. sheep and cattle) slaughtering practices (Schantz et al., 1995). Rausch (2003) discusses four predator–prey associations that exist in the tundra and taiga that accommodate the *E. granulosus* life cycle. Two are natural cycles involving wolf (*Canis lupus*) and wild reindeer (*Rangijer tarandus*) and wolf and moose (*A. alces*). The other two involve humans. In indigenous Inupiat Alaskan populations, sled-dogs and wild reindeer were the main *E. granulosus* hosts (Ortner, 2003). In northern Siberia, the association of herding dogs and domesticated reindeer resulted in infection of 50–70 individuals per 1000 (Nemurovskaia et al., 1980). Modern indigenous Eurasian reindeer-herding groups, including many of the indigenous small-numbers peoples of Siberia and the Samii of Western Eurasia, have been shown to be heavily affected (Huldt et al., 1973; Romanenko et al., 2001). Thus, the presence of echinococcus is often indicative of a close association between humans and canids, especially domesticated dogs. In these cases, the echinococcus infection occurs by direct contact with dogs, or contamination of food (usually via the soil) or water with dog feces containing parasitic eggs. Morphological (Sablin and Khlopachev, 2002; Ovodov et al., 2011) and molecular (Druzhkova et al., 2013; Germonpré et al., 2009; Thalmann et al., 2013) studies have shown that dogs were domesticated in Eurasia long before the Neolithic period. In the Early Neolithic *Cis*-Baikal (~8000 to 7000 BP), dog burials were more common than in any other time period, often containing grave accoutrements such as beads, animal bones, and lithics (Losey et al., 2013a,b). Stable carbon and nitrogen isotopes show humans and dogs had similar diets high in aquatic protein, and Shamanka II itself contained one dog burial (Losey et al., 2011, 2013). Thus, it is possible the close interaction of humans and dogs during the Early Neolithic resulted in human parasitic infection by *E. granulosus*. Of course, it is also possible the source of infection was via undomesticated canids, similarly from contaminated food or water, or that both infection routes caused parasitic infection in these hunter–fisher–gatherers.

6. Conclusions and future research

In conclusion, this case study presents *E. granulosus* infection in an Early Neolithic *Cis*-Baikal forager, with CT and stable isotope analysis providing strong support for the object being a hydatid cyst. Stable isotope analysis of the cyst protein and mineral proved effective in identifying the host organism, and we argue a very low $\Delta\delta^{13}$Cprotein–mineral value indicates a parasitic trophic level. This is a fairly straightforward method that can be used by researchers in the future. Amino acid sequencing of the protein(s) in hydatid cysts should be undertaken to determine the exact proportion of different proteins and amino acids that are present, thereby making the comparison of cyst protein isotope results to human and animal collagen isotope results more concrete. It has been suggested that positive of identification of hydatid cysts is not possible unless histological examination reveals either the presence of characteristic hooklets, which are unlikely to be preserved, or the triple-layered cyst wall (Aufderheide and Rodriguez-Martin, 1998). We suggest that stable isotope analysis of the cyst, yielding values that would be expected of a parasitic body in a human host, is a useful step in garnering a more secure identification.

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