TENACITY OF ALARIA ALATA MESOCERCARIAE IN HOMEMADE GERMAN MEAT PRODUCTS AND EFFECTS OF DIFFERENT IN VITRO CONDITIONS AND TEMPERATURES ON ITS SURVIVAL

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ABBREVIATIONS

ABBREVIATION	DEFINITION
A. alata	*Alaria alata*
AMT	*Alaria alata* mesocercariae migration technique
a_w	Water activity
cm	Centimetre
DUSN	Diffuse unilateral subacute neuroretinitis
g	Gram
h	Hour
HCl	Hydrochloric acid
IgE	Immunoglobulin E
GHP	Good Hygiene Practice
kg	Kilogram
min	Minutes
NaCl	Sodium chloride
NCS	Nitrite curing salt
USA	United States of America

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SUMMARY

The recent findings of *Alaria alata* mesocercariae in wild boars (*Sus scrofa*) and other animals in Europe reinforced the concern about the public health risk posed by this parasite. Cases of food associated human alariosis in North America suggest that a risk associated with the consumption of traditional raw cured products from infected wild boar meat cannot be neglected because the commonly applied preservation techniques may not necessarily kill the mesocercariae. A risk may also exist if the game meat is insufficiently heated or refrigerated. Cooking and freezing are effective methods for the inactivation of parasites in meat. Nevertheless, alternative methods of treatment, such as microwave heating may represent an equally effective tool for the inactivation. In addition, changes in consumer behaviour (e. g. low salt) and new preparation methods for game meat may increase the risk for food-associated parasitic infections. Thus, there is a strong need for the evaluation of the tenacity of *A. alata* mesocercariae against common preservation methods such as different physical and chemical influences. Additionally, it is relevant to understand the survival of *A. alata* mesocercariae during digestion in the human stomach. Finally, the effect of different concentrations of ethanol solutions is important for the evaluation on the viability of *A. alata* mesocercariae during the disinfection of surfaces in households and/or laboratories.

CHAPTER 1 gives a general introduction into the topic of emerging food-borne zoonoses. This chapter presents a brief history and taxonomy of *A. alata* as well as its life cycle. Further, this chapter provides an overview about the prevalence of the parasite around the world. Likewise, previous studies on the tenacity of *Alaria* spp. and the human alariosis cases caused by food are provided here. Finally, CHAPTER I describes briefly why the *A. alata* mesocercariae migration technique (AMT) was used in all the experiments reported in the next chapters.

CHAPTER 2 consists of a sound analysis of the survivability of *A. alata* mesocercariae during curing, fermentation, cold smoking and drying in meat products. The research is published in the International Journal of Food Microbiology. Traditional German meat products were prepared from naturally infected game meat and partly spiked with additional vital *A. alata* mesocercariae. The resultant products were examined for dead and viable *A. alata* mesocercariae. After 24 h of production, vital *A. alata* mesocercariae

were still found in raw type sausages but no vital parasites were detected in the final products.

CHAPTER 3 discusses the effect of different concentrations of natrium chloride (NaCl), ethanol solutions and simulated human gastric digestion on the viability of *A. alata* mesocercariae. The complete report is published in the Journal Parasitology Research. The objective was to measure the resistance of the parasite to different *in vitro* conditions which were never analyzed before. A total of 120 min were required to reliably inactivate *A. alata* mesocercariae within HCl-pepsin digestion solution with a pH of 1.5-2.0 at 37 °C. Furthermore, 3.0 % is the minimum recommended concentration of NaCl in meat products. Finally, the concentration of ethanol (70.0 %) is sufficient for the inactivation of *A. alata* mesocercariae.

CHAPTER 4 analyzes the effect of different temperatures on the survival of *A. alata* mesocercariae. Isolated vital mesocercariae were examined with respect to their resilience against heating, refrigeration, freezing and microwave heating. The present study is published in the Journal Parasitology Research. *A. alata* mesocercariae stored in Ringer's solution do not survive heating temperatures that exceed 60.0 °C. Microwave heating ensured an inactivation of all parasites after 90 sec. The parasite survived refrigeration temperatures (4.0±2 °C) in Ringer's solution for up to 13 days. An effective inactivation is only guaranteed if infested game meat is frozen to a core temperature of -13.7 °C for a minimum of 2 h.

CHAPTER 5 provides information on the combination of food treatments to inactivate the parasite in game meat products. Also, this chapter discusses the survival of *A. alata* mesocercariae to the disinfectant ethanol and how the parasite reacts to the gastric juice from human stomach. Additionally, the effects of different temperatures (freezing, refrigeration, heating and microwave heating treatment) on the resistance of *A. alata* mesocercariae were commented. Finally, recommendations to guarantee safety in the production of game meat and/or products for human consumption and further research objectives are provided.

Keywords: *Alaria alata* mesocercariae; zoonotic parasite; meat technology; food safety

ZUSAMMENFASSUNG

Die aktuellen Funde von *Alaria-alata*-Mesozerkarien in Wildschweinen und anderen Tieren in Europa haben die Sorge um ein mögliches Gesundheitsrisiko, das von diesem Parasiten ausgeht, verstärkt. Verschiedene Fälle Lebensmittel-assoziierter humaner Alariose in Nordamerika haben gezeigt, dass ein Infektionsrisiko, welches mit dem Verzehr traditioneller, roher geräucherter und/oder gepökelter Produkte aus infiziertem Wildschweinfleisch verbunden ist, nicht vernachlässigt werden kann, da diese häufig verwendeten Konservierungstechniken nicht in allen Fällen ausreichen, um die Mesozerkarien sicher abzutöten. Ein Risiko für den Verbraucher besteht vor allem dann, wenn infiziertes Wildfleisch ungenügend erhitzt oder gekühlt wird. Durcherhitzen und Einfrieren sind effektive Methoden, um den Parasiten in Fleisch zu inaktivieren. Darüber hinaus können alternative Methoden, wie z. B. die Erhitzung mittels Mikrowellen, eine gleichwertiges Verfahren der Inaktivierung darstellen.

Ein erhöhtes einer Infektionen des Verbrauchers besteht außerdem, wenn durch ein sich veränderndes Verbraucherverhalten (Salzreduktion) und neue Zubereitungsmethoden des Wildfleisches, eine sichere Inaktivierung von im Fleisch enhaltenen Parasiten nicht mehr gewährleistet ist.

Aus den beschriebenen Gründen ergibt sich die Notwendigkeit, die Tenazität der *A.-alata*-Mesozerkarien gegenüber üblichen Konservierungsmethoden wie verschiedenen physikalischen und chemischen Einflüssen zu evaluieren.

Zudem ist es für eine anschließende Bewertung des humanen Expositionsrisikos notwendig, die Überlebensfähigkeit der *A.-alata*-Mesozerkarien bei der Magen-Darm-Passage zu untersuchen. Parallel dazu kann der Effekt verschiedener Ethanol-Konzentrationen auf die Vitalität von *A.-alata*-Mesozerkarien wichtige Anhaltspunkte für die Durchführung eines geeignten Reinigung- und Desinfektionsschemas in Haushalt und Gastronomie geben.

KAPITEL 1 gibt eine allgemeine Einführung in das Thema der neuen lebensmittelbedingten Zoonosen. Dieses Kapitel präsentiert eine kurze Geschichte und Taxonomie von *A.-alata* sowie zum Lebenszyklus des Parasiten. Weiterhin zeigt dieses Kapitel eine Übersicht über die weltweite Verbreitung von *Alaria* spp. Ebenfalls werden frühere Studien zur Tenazität und den lebensmittelbedingten humanen Alariose-Fällen

dargestellt. Schließlich beschreibt KAPITEL 1 kurz, warum die neu entwickelte „*Alaria alata* mesocercariae migration technique“ (AMT) als Nachweisverfahren in allen durchgeführten Experimenten der nachfolgenden Kapitel verwendet wurde.

KAPITEL 2 besteht aus einer Analyse der Überlebensfähigkeit von *A.-alata*-Mesozerkarien während des Pökelns, der Fermentierung, des Kalträucherns und der Trocknung in Wurstwaren. Die Studie wurde im International Journal of Food Microbiology veröffentlicht. Traditionelle deutsche Wurstwaren, die aus natürlich infiziertem Wildfleisch hergestellt wurden, sind teilweise mit zusätzlichen lebenden *A.-alata*-Mesozerkarien gespickt worden. Die hergestellten Produkte wurden nach toten und lebensfähigen *A.-alata*-Mesozerkarien untersucht. 24 Stunden nach der Herstellung wurden noch lebensfähige *A.-alata*-Mesozerkarien in den Produkten gefunden, in den Endprodukten konnten dann jedoch keine lebenden Parasiten mehr festgestellt werden.

KAPITEL 3 erörtert die Wirkung verschiedener NaCl-Konzentrationen und unterschiedlich konzentrierter Ethanollösungen sowie einer simulierten menschlichen Magenverdauung auf die Lebensfähigkeit von *A.-alata*-Mesozerkarien. Diese Studie wurde in der Fachzeitschrift Parasitology Research publiziert. Das Ziel war, die Resistenz des Parasiten in Bezug auf verschiedene In-vitro-Bedingungen zu messen, die bisher noch nie analysiert wurden. In einer HCl-Pepsin-Verdauungslösung mit einem pH-Wert von 1,5 - 2,0 dauerte es 120 Minuten bei 37 °C, um die *A.-alata*-Mesozerkarien zuverlässig zu inaktivieren. Außerdem war festzustellen, dass in Wurstwaren, die für den menschlichen Verzehr hergestellt werden, mindestens 3.0 % NaCl eingesetzt werden sollten. Die allgemein im Rahmen der Desinfektion angewendeten Ethanol-Konzentrationen (70.0 %) sind für die Inaktivierung von *A.-alata*-Mesozerkarien ausreichend.

KAPITEL 4 analysiert die Wirkung der Temperatur auf das Überleben von *A.-alata*-Mesozerkarien. Die gewonnenen, vitalen Mesozerkarien wurden hinsichtlich ihrer Widerstandsfähigkeit gegen Erhitzung, Kühlung, Einfrieren und Mikrowellenbehandlung untersucht. Die Ergebnisse der Studie sind bereits in der Fachzeitschrift Parasitology Research veröffentlicht. *A.-alata*-Mesozerkarien, überleben Temperaturen von über 60,0 °C beim Erhitzen in Ringerlösung nicht. Mikrowellenerhitzung führt zu sicheren Inaktivierung aller Parasitenstadien nach 90 Sekunden. Die Parasiten überlebten aber eine Lagerung bei 4,0 ± 2 °C bis zur 13 Tage. Einfrieren führt nur dann zu einer wirksamen

Inaktivierung, wenn infiziertes Wildfleisch mindestens 2 Stunden eingefroren wird, wobei eine Kerntemperatur von mindestens -13,7 °C erreicht wird.

KAPITEL 5 informiert über die Kombination von verschiedenen Prozessfaktoren zur Inaktivierung des Parasiten in Wildfleischerzeugnissen. Außerdem wird in diesem Kapitel die Überlebensfähigkeit von *A.-alata*-Mesozerkarien in Anwesenheit des Desinfektionsmittels Ethanol und die Reaktion des Parasiten auf den Verdauungssaft des menschlichen Magens diskutiert. Parallel dazu wird der Einfluss verschiedener Temperaturen (bei Einfrieren, Kühlen, Erhitzen bzw. Mikrowellenbehandlung) auf die Widerstandsfähigkeit von *A.-alata*-Mesozerkarien kommentiert. Abschließend werden Empfehlungen zur Etablierung eines hohen Schutzninveaus des Verbrauchers bei der Produktion von Wildfleischerzeugnissen und Wurstwaren und für weiteführende Forschungsschwerpunkte gegeben.

Schlagwörter: *Alaria-alata*-Mesozerkarien; zoonotische Parasiten; Fleischtechnologie; Lebensmittelsicherheit

CHAPTER 1 GENERAL INTRODUCTION

1.1 EMERGING FOOD-BORNE ZOONOSES

Food-borne zoonoses are defined as diseases and/or infections which are naturally transmissible indirectly between animals and humans through food (Hugh-Jones et al., 1995; Fosse et al., 2008). Annually, 10.0 % of the human population in industrialised countries may suffer from food-borne zoonoses (Schlundt et al., 2004; Fosse et al., 2008). In this sense, emerging zoonoses have been defined as zoonoses that are newly recognised or newly evolved, or that have occurred previously but show an increase in incidence or expansion in geographical, host or vector range (Report of the WHO/FAO/OIE Joint Consultation on Emerging Zoonotic Diseases). It is estimated that 75.0 % of emerging human pathogens are zoonotic (Woolhouse, 2002; Chomel, 2008; Dorny et al., 2009).

Food and waterborne infections have received recently considerable attention. In the past, the risk of human infection with parasites was considered to be limited to specific geographic regions because of the parasites' adaptations to definitive hosts, selected intermediate hosts and particular environmental conditions (Orlandi et al., 2002). Other factors that may explain the emergence of some zoonotic parasitic diseases are the increase of the population of highly susceptible persons because of malnutrition, ageing and other medical conditions. Also the changes in lifestyle, such as the increase in the number of people eating meals prepared in fast food restaurants, canteens and as well as from street food vendors who do not always respect food safety (WHO, 2002; Dorny et al., 2009).

Taylor et al. (2001) identified 1415 species of infectious organisms known to be pathogenic to humans, including 217 viruses and prions, 538 bacteria, 307 fungi, 66 protozoa and 287 helminths. Of these, 868 (61 %) were identified as zoonotic and 175 pathogenic species were associated with diseases considered to be "new" or "emerging", 132 of those (75 %) being zoonotic (Chomel, 2008). In a past study, Jones et al. (2008) showed that emerging infectious disease events are dominated by zoonoses (60.3 %) and the majority of those (71.8 %) originate in wildlife.

However, food-borne parasites can be controlled with some basic strategies such as sanitation and proper cooking of foods. Frequent hand washing, use of clean utensils, and temperature measurement are basic strategies to prevent cross-contamination during food processing. In developed countries, husbandry practices that limit exposure of pigs to contaminated feed and feces have been successful in reducing human infections with *Trichinella* spp. (Doyle, 2003).

Additionally, meat processing techniques such as fermentation, curing, drying, cooking, among others are effective ways to reduce the risk of infection when consuming meat (Dorny et al., 2009). Some traditional recipes for long or short-ripened dry sausages do not incorporate bacterial starter cultures (Incze, 2010) and rely on sodium nitrate or combinations of both as curing agent to eliminate bacteria or parasites (Paulsen et al., 2011).

Likewise, cooking is effective in killing parasites if the appropriate temperature is reached in the core of the meat product (Dorny et al., 2009). Therefore, consumers should be advised to properly cook meat. For example, meat that may be infected with *Trichinella* spp. should reach an internal temperature of at least 71.0 °C to eliminate the risk of infection (Doyle, 2003). Application of microwave radiation is another technological procedure to cook food. However, microwave heating does not reliably kill all parasites in meat because the heating temperature is uneven and cooler spots may permit survival. Supplementary, freezing for several days can inactivate or kill parasites in meat. It is recommended to freeze pork meat at -17.0 °C for 20 days, -23.3 °C for 10 days or -29.0 °C for 6 days destroys *Trichinella* spp. (CDC, 2003).

Nowadays, game meat is becoming more and more popular and the consumption of game meat is increasing economic relevance. In Germany, game meat is considered to be a high-quality product and the per capita consumption is rising steadily (BfR, 2014). A total of 36,126 tons of wild animals were hunted from 2005 to 2006. These were divided into 19,000 tons of wild boar (461,881 animals), 11,300 tons of roe deer (905,387), and about 4,000 tons of red deer (60,664) (DJV, 2006). According to FAOstat (FAO, 2013), from the year of 2010 to 2011, Germany produced 137,000 tonnes game meat and 585,244 wild boars were hunted in the year 2010 and there has been a considerable increase compared to year 2009 with 440,354 hunted animals (DJV, 2012). In 2011,

approx. 15,000 tons of wild boar meat was consumed (Stahl, 2012). The average amount of annual wild meat consumption is about 0.45 kg/person and accounts for 0.8 % of the total meat consumption (Gurrath, 2008). About 62.0 % of retailed game meat originates from animals hunted. Only 3.0 % of the meat is from animals that are grown in captivity, with fallow deer (*Dama dama*) the most frequently grown captive animal (Hurlin and Schulze, 2007). In compliance with the legal regulations, hunters are educated in meat inspection and hygiene rules (Miko et al., 2009). Inspected and acceptable carcasses are allowed to proceed to immediate sale to individuals, restaurants, and food handlers. For safety reasons, processing of game meat must occur separately from processing of other meat; when processing of game meat is conducted on a larger scale, it must be performed in special meat-processing facilities (Hurlin and Schulze, 2007).

As mentioned above, wild boars are commonly hunted in Germany. Free-living swine populations pose not only ecological concerns but infectious disease concerns as well (Meng et al., 2009). On this subject, wild boars may harbour infectious agents that are transmissible to domestic pigs and other animal species which can include humans. In some regions of the world, recreational hunting and consumption of wild boar meat is the ideal environment for the transmission of pathogens between wild boars and humans (Gibbs, 1997). The rise of wild boar populations contribute to the development of a commercial hunting (Acevedo et al., 2006), and this could further complicate the transmission of pathogens. An increasing number of findings of *A. alata* mesocercariae in meat of wild boars during the official meat inspection and the reports on human cases of alariosis initiated a reassessment of the potential food borne human health risk posed by this parasite (Große and Wüste, 2004; BfR, 2007; Möhl et al., 2009; Riehn et al., 2010). The digenean trematode *Alaria alata* is widely distributed in Europe. The Federal Office for the Environment (FOEN) in Switzerland categorized *A. alata* as a stage 2 risk (Z) for parasites with zoonotic potential (Anonymus, 2003). In addition, *Alaria* spp. was identified by Dorny et al. (2009) as one of the most important emerging food-borne parasites and Fosse et al. (2008) as biological hazard for the assessment of human exposure. Because of its occupational health risks, the Federal Institute of Risk Assessment (BfR, 2007) concluded in its risk assessment that game meat which contains *A. alata* mesocercariae should be considered as unfit for human consumption.

1.2 Brief History of A. alata

Goeze (1782) gave the original taxonomic description of the adult stage of *A. alata*. Subsequently, Gestaldi (1854) first described the larval stage of the trematode (*Distoma tetracystis*) in frogs (Möhl et al., 2009). Later on, Duncker studied and described the sexually immature trematodes which were found in swine muscles during trichinellosis control in Saxony, Germany (1881a; 1881b; 1884; 1896; 1897). Finally, Stefañski and Tarczyñski (1953) demonstrated that a direct relation between *A. alata* (Goeze, 1782) and *Distomum musculorum suis (*Duncker, 1896) exists. Therefore, the parasite can be found in the literature as: *Alaria alata* (Goeze, 1782), *Distoma tetracystis* (Gestaldi, 1854), *Distomum musculorum suis* (Duncker, 1896) or *Duncker'scher Muskelegel* (in German).

Alaria alata (*Diplosomatidea, Strigeata*) is a parasitic trematode of carnivores in Europe and the former Soviet republics (Schnieder, 2006; Möhl et al., 2009). Further *Alaria* species can be found in North and South America: *A. mustelae* (Bosma, 1931; Möhl et al., 2009), *A. intermedia* (Olivier and Odlaug, 1938; Möhl et al., 2009), *A. marcianae* (LaRue, 1917; Möhl et al., 2009), *A. arisaemoides* (Augustine and Uribe, 1927; Möhl et al., 2009), *A. canis* (LaRue and Fallis, 1936; Möhl et al., 2009).

1.3 Life cycle of A. alata

The definite hosts of Alaria spp. are felids, canids, and other carnivores, and the adults parasitize the guts of these hosts. Eggs, shed with the host's feces, embryonate and hatch in the water. For the completion of the parasite's life cycle, two intermediate hosts are necessary: a planorbid snail (cercarial stage) (Ruszkowski 1921; Portier et al. 2012) and an amphibian host (mesocercarial stage) (Ruszkowski 1921; Skrjabin 1965). The second intermediate hosts are ingested either by a definitive host or by a transport or paratenic host. Many mammalian, avian, and reptile species, among them wild boars (Dollfus and Chabaud 1953), can serve as paratenic hosts. The liberated mesocercariae remain within these hosts in a dormant state, i.e. they do not complete development to adult flukes, but can wait for months until a definite host ingests the paratenic host and they can resume development to adults (Möhl et al., 2009). The mesocercariae can survive many paratenic

host transitions unharmed and still remain infective for the definitive host. Moreover, it is important to point out that the paratenic host can also infect each other, which leads to a notable expansion of the infection cycle (Möhl et al., 2009; Murphy et al., 2012).

1.4 Prevalence of A. alata around the world

Alaria species are distributed worldwide (Schnieder, 2006; Mehlhorn, 2008). Moreover, the spectrum of definitive hosts is very broad and includes, depending upon the *Alaria* species, a wide range of carnivores in the respective geographic range (Table 1 and 2). Mehlhorn (2008) estimated that about 30.0 % of the wild canides in Europe are carriers of *A. alata*. However, the list increased since the recent findings during the last years.

Table 1 List of definitive hosts of different species of *Alaria* in North and South America.

Country	Definitive host	Alaria species
NORTH AMERICA		
Canada [2]	Red fox (*Vulpes fulva*) [2,3]	*A. americana*
USA [3, 5]	Domestic dog (*Canis familiaris*) [5]	
	Coyote *(Canis latrans)*[3]	
Canada	Eastern wolf (*Canis lyacon*) [7]	*A. canis*
USA	Red fox (*Vulpes fulva*) [1,2,3,8]	*A. arisaemoides*
	Domestic dog (*Canis familiaris*) [4, 9]	
USA	Red fox *(Vulpes fulva)* [2]	*A. mustelae* (Synonym:
	Short-tailed weasel *(Mustela erminea)*[10]	*Alaria. taxideae* and
	Short-tailed weasel (*Mustela erminea)* [11,12]	*Alaria canadensis*)
	American badger (*Taxidea taxus)*[11,12]	
	Common raccoon (*Procyon lotor)* [11,12]	
	American mink (*Mustela vison)*[11,12]	
	Eastern spotted skunk (*Spilogale putorius)*[11,12]	
	Domestic dog (*Canis familiaris)*[13]	
	Domestic cat (*Felis familiaris)*[13]	
SOUTH AMERICA		
Uruguay	Domestic cat (*Felis familiaris)*[14]	*A. alata*
Argentina	Geoffroy's cat (*Oncifelis geoffroy*) [14, 15, 17]	*A. alata*
	Domestic dog (*Canis familiaris)* [14, 15, 17]	
Brazil	Pampas fox (*Pseudalopex gymnocercus*) [18]	*A. alata*
	Crab-eating fox (*Cerdocyon thous*) [18]	

Adapted and modified from Buller, 2012

Table 2 List of definitive hosts of different species of *Alaria* in Eurasia.

Country or region	Definitive host	Alaria species
EURASIA		
Germany (east) [21] Belarus [30] Romania (Danube delta) [20] Volga Delta [22]	Raccoon dog (*Nyctereutes procyonoides*)	*A. alata*
Belarus	American mink (*Mustela vison*) [29] Weasel (*Mustela nivalis L.*) [28] European otter (*Lutra lutra*) [27] Stoat (*Mustela erminea L.*) [28]	*A. alata*
Poland [6]	Eurasian lynx (*Lynx lynx*)	*A. alata*
Russia	Domestic cat (*Felis familiaris)* [35, 52] Domestic dog (*Canis familiaris)* [35, 52]	*A. alata* mesocercariae
Greece [34] Turkey [32] Sweden [33]	Domesticated dog (*Canis lupus familiaris*)	*A. alata*
Poland [23] Estonia [37] Belarus [31]	European wolf *(Canis lupus L.*)	*A. alata*
Denmark [41, 50] Finland [39] Sweden [24] Wales [42] Austria [43] Poland [25,38] Yugoslavia [40] Bulgaria [44] Portugal [51] Netherlands [46] Belarus [49] Ireland [48] Germany [26,45,47]	Red fox (*Vulpes vulpes*)	*A. alata*

Adapted and modified from Möhl et al., 2009

Alaria spp. infects multiple hosts, including snails, amphibians, and mammals, to complete its life cycle. Additionally, *Alaria* spp. is a genus with various definitive hosts differently affected by the environment. Generally, a significant prevalence of *A. alata* mesocercariae in wild animal populations can be expected in water-rich areas in which the suitable host species (snails, amphibians, and definitive hosts) are present (Möhl 2009). However, the spectrum of snails, frogs, and amphibians and their preference vary depending upon *Alaria* species (Möhl et al., 2009). The incidence of early developmental

stages of *A. alata* in amphibians has been studied mainly in Eurasia and North America (Table 3).

Table 3 List of second intermediate hosts of different species of *Alaria* in North America and Eurasia.

Country	Second intermediate host	*Alaria species*
NORTH AMERICA		
USA	Northern leopard frog (*Rana pipens)* [54]	A. marcianae *mesocercariae*
USA	Northern leopard frog (*Rana pipiens*) [53] Lowland Leopard Frog *(Rana yavapaiensis)* [55]	*Alaria* spp. *mesocercariae*
USA	American tod (*Bufo americanus)* [55] Wood frog (*Rana sylvatica)* [55] Northern leopard frog *(Rana pipiens)* [11,55]	*A. arisaemoides*
Canada	American tod *(Bufo americanus)* [55] wood frog *(Rana sylvatica)* [55] Northern leopard frog *(Rana pipiens)* [55] Southern chorus frog *(Pseudacis nigrita)* [55]	*A. canis mesocercariae*
USA	Pacific tree frog *(Pseudacris regilla)* [56] Northern leopard frog *(Rana pipiens)* [11,13] Green frog *(Rana clamitans)* [13] American bullfrog *(Rana catesbeiana)* [13]	*A. mustelae mesocercariae* (Synonym: *Alaria. taxideae* and *Alaria canadensis*)
EURASIA		
Germany [61] Russia [59] Ukraine [57] Belarus [27] France [16]	Common water frog *(Rana esculenta)* [36,61] European common brown frog *(Rana temporaria)* [36, 59, 61] Spadefoot toad (*Pelobates fusca)* [57, 59] Moor frog *(Rana arvalis)* [61] European green toad *(Bufo viridis)* [27] First intermediate host: Freshwater snail *(Planorbis planorbis* and *Anisus vortex)* [16]	A. alata *mesocercariae*

Adapted and modified from Buller, 2012 and Möhl et al., 2009

A wide range of paratenic hosts (e.g., wild boars, raccoons) can acquire infection by ingesting tadpoles and frogs, or infected paratenic hosts (Table 4). Since 2002 up to date a steadily rise in the number of *A. alata* mesocercariae is found in wild animals (Große and Wüste, 2004, 2006). Besides, Wolfe et al. (2001) reported significantly lower infection of foxes with the parasite during the breeding season (January to June) in contrast to the non-breeding (July to December) season, because snails and frogs would be most active during the summer when the parasite would develop optimally, so that foxes would be infected by eating frogs in late summer when it had sufficient time to develop.

Table 4 List of paratenic hosts of different species of *Alaria* in North America and Eurasia.

Country	Paratenic Host	*Alaria species*
NORTH AMERICA		
Canada	Chicken *(Gallus gallus)* [55]	*A. arisaemoides*
	House mouse *(Mus musculus)* [55]	
	Deer mouse *(Peromyscus maniculatus)* [55]	
Canada [55, 63]	Chicken (*Gallus gallus)* [55]	*A. canis*
USA [62]	Domestic duck *(Anas boschas)* [55]	
	House mouse *(Mus musculus)* [55]	
	Deer mouse (*Peromyscus Maniculatus)* [55]	
	European polecat (*Mustela putorius)* [55]	
	North American river Otter (*Lutra Canadensis)* [55]	
	Domestic cat (*Felis domestica)* [62]	
	Domestic dog (*Canis familiaris)* [63]	
Canada [10, 65]	Fisher (*Martes pennanti*) [10]	*A. mustelae*
USA [13, 64]	American mink *(Neovison vison)* [64]	(Synonym: *Alaria. taxideae* and *Alaria canadensis*)
	American marten *(Martes americana)* [65]	
	Northern white-footed mouse (*Peromyscus leucopus noveboracensis)* [13]	
	American mink (*Mustela vison)* [13]	
	Common raccoon (*Procyon lotor)* [13]	
EUROPE		
Germany	Domestic pig *(Sus scrofa domesticus)* [66]	*A. alata mesocercariae*
Germany	Common raccoon (*Procyon lotor)* [67]	*A. alata mesocercariae*
Ireland	Red fox (*Vulpes fulva*) [68]	*A. alata mesocercariae*
Germany [69]	Wild boars (*Sus scrofa* Linnaeus, 1758)	*A. alata mesocercariae*
Austria [70]		
Bulgaria [71]		
France [58, 60]		
Czech Republic [19]		

Adapted and modified from Buller, 2012 and Möhl et al., 2009

(1) Augustine and Uribe, 1927 (2) Smith, 1978 (3) Davidson et al., 1992 (4) Dyer et al., 1997 (5) Hall and Wigdor, 1918 (6) Szczesna et al., 2008 (7) Swales, 1933 (8) Wirsing et al. 2007 (9) Allen and Mills, 1971 (10) Dick and Leonard, 1979 (11) Johnson, 1970 (12) Swanson and Erikson, 1946 (13) Bosma, 1934 (14) Castro et al., 2009 (15) Martínez, 1986 (16) Portier et al., 2012 (17) Lombardero and Santa Cruz, 1986 (18) Ruas et al., 2008 (19) Paulsen et al., 2013 (20) Barbu, 1972 (21) Schuster et al., 1993 (22) Ivanov and Semenova, 2000 (23) Popiolek et al., 2007 (24) Persson and Christensson, 1971 (25) Furmaga and Wysocki, 1951 (26) Saar, 1957 (27) Shimalov et al., 2000a (28) Shimalov et al., 2001a (29) Shimalov et al.,2001b (30) Shimalov et al., 2002 (31) Shimalov et al., 2000b (32) Umur, 1998 (33) Jogeland et al., 2002 (34) Papazahariadou et al., 2007 (35) Yastrebov et al., 2005 (36) Bugge 1942 (37) Moks et al., 2006 (38) Kozlowska, 1957 (39) Freeman, 1966 (40) Lozanic, 1966 (41) Guildal and Clausen, 1972 (42) Williams, 1976 (43) Hinaidy, 1976 (44) Jancev and Ridjakov, 1978 (45) Loos-Frank and Zeyhle, 1982 (46) Borgsteede, 1984 (47) Lucius et al., 1988 (48) Wolfe et al., 2001 (49) Shimalov et al., 2003 (50) Saeed et al., 2006 (51) Eira et al., 2006 (52) Möhl et al. 2009 (53) Goldberg et al., 2001 (54) Cort, 1918 (55) Pearson, 1956 (56) Johnson et al. 1999 (57) Timofeev, 1900 (58) Portier et al., 2014 (59) Potekhina, 1951 (60) Portier et al., 2011 (61) Andreas, 2006 (62) Burrows and Lillis, 1965 (63) LaRue and Fallis, 1936 (64) Foster et al. 2007 (65) Poole et al. 1983 (66) Duncker, 1896 (67) Rentería-Solís et al., 2013 (68) Murphy et al., 2012 (69) Riehn et al., 2012 (70) Paulsen et al., 2012 (71) Riehn et al., 2013

1.5 PREVIOUS STUDIES ON TENACITY OF ALARIA SPP.

Several authors have previously studied the tenacity of *A. alata* mesocercariae against chemical and physical factors. Moreover, the mesocercarial stage of *Alaria* spp. seems to be resistant to different temperatures. In this regard, Duncker (1896) and Bugge (1942) published that the parasite survived for 3-4 days and 16-18 days in refrigeration. Walters et al. (1975) demonstrated that at refrigeration (8.0 °C), the mesocercariae survived in non-sterile Ringer's solution for approx. 3 months. Living mesocercariae subjected to repeated cycles of refrigeration and then room temperature, survived for over a month (Freeman et al., 1976). Additionally, the freezing tolerance of *A. alata* mesocercariae has been documented, where the parasite in pork meat survived up to 8 weeks at -20.0 °C (Hiepe, 1985) and just one parasite survived after 5 days of refrigeration at -18±2 °C (Portier et al., 2011).

Shoop and Corkum (1983b; 1984a; 1984b; 1987) reported about the possibility of a vertical transmission of *Alaria* mesocercarial stages. Ingested mesocercariae of *A. marcianae*, migrate from the stomach to the subcutaneous fat of mammalian, paratenic hosts and then to the mammary glands during lactation (Shoop and Corkum, 1983a; 1983b; 1984b). *A. marcianae* mesocercariae was detected in five amphibian, seven reptilian, and two mammalian species in Louisiana, USA (Shoop and Corkum, 1981). Natural infections were also found in the subcutaneous fat of raccoons and opossums. Examination of three gravid opossums showed a marked preference of the mesocercariae for the mammary glands. In this sense, an artificial experiment with lactating mice from Shoop and Corkum (1984a) showed that mesocercariae of *A. marcianae* penetrated the dense connective tissue surrounding the lobules. Within the stroma the larvae migrated along tracks of fat cells and were consistently found in pools of milk created from the destruction of alveoli. These pools of milk led directly into the large lactiferous ducts. Moreover, Shoop and Corkum (1984b) studied nursed newborn cats and mice that were infected with *A. marcianae* from females that had been experimentally infected with the parasite. Lactating females showed infection with the mesocercarial stage of the parasite in their mammary glands. Of the two mammalian species, the mouse was the closest model of the larval dynamics in a human. If the results in mice are extrapolated to

humans, then one reasonably assumption will be that a human female, once infected, will transmit mesocercariae to all the children she nurses. These migrating larvae might invade vital organs of a newborn, leading to physical or mental impairment, or even death.

Walters et al. (1975) showed in experiments with rabbits that the mesocercariae of *Alaria* spp. can direct penetrate through the cornea, the mesocercariae were capable of prolonged survival within the eye. The inflammatory reactions in the eye were relatively mild. Prolonged survival of the parasite led to chronic inflammatory reaction with eosinophils.

Foster et al. (2008) identified *A. marcianae* mesocercariae in the lungs of three freshly dead panther neonates in Florida, USA. The 11-, 12-, and 17-day-old neonates were presumably fed only with milk from the mother since birth. Milk was the only item found in the gastrointestinal tract of the whelps. Mesocercariae of *A. marcianae* were collected from the lungs of the three neonates, indicating a transmammary route of infection. The probable paratenic host for the *A. marcianae* infection in the adult panther is the raccoon (Möhl et al., 2009).

Regarding drugs that act against infections caused by parasitic worms, it was Freeman et al. (1976) who studied the activity of antihelmintic against *A. alata* mesocercariae. In these experiments, Bithional and Niridazole (antiparasitic) were used. Results showed that *A. alata* mesocercariae exposed to a minimal concentration, which is the normal administration of pharmacological doses, immobilize and kill the flukes within 20 min.

Furthermore, Johnson et al. (1985) investigated the development and survival of *A. arisaemoides, A. marcianae, and A. mustelae* mesocercariae in different culture media: NCTC 135 and Triple Eagle Medium. Each were used alone or supplemented with chicken, dog, horse, calf, fetal calf and human serum. It was found that the culture systems used in this study failed to produce advanced development of the mesocercariae of *A. arisaemoides, A. marcianae, and A. mustelae*; however, prolonged periods of survival (up to 27 days) were observed. The low percentage of survival of *A. arisaemoides* and *A. marcianae* in dog serum was surprising, since the dog can serve as final host for both species.

To the author's knowledge, the only case of former studies involving food processing technologies is the one from Hiepe (1985) where it was observed that *A. alata* mesocercariae can survive in pork meat up to 10 days during curing.

1.6 HUMAN ALARIOSIS CASES CAUSED BY FOOD

Few reports on human larval alariosis exist, all of them exclusively linked to American *Alaria* species, and none have been reported in Germany (Möhl et al., 2009). The pathogenicity of *A. alata* mesocercariae is correlated to high infestation densities, in particular, after repetitive intake of it (Möhl et al., 2009). Löscher and Sonnenburg (2005) reported that most infections with trematodes are found to be associated with the increase in the number of eosinophils in the blood or tissues (eosinophilia) and the increase of serum immunoglobulin E (IgE), which means that a general anaphylactic reaction may arise from a repetitive oral intake of infected material. The symptoms of an anaphylactic shock range from tachycardia and drop in blood pressure to respiratory collapse, confusion, agitation, anxiety and loss of consciousness (Bork, 1985; Egger, 2005).

Human alariosis manifests in various clinical signs which range from low-grade respiratory and cutaneous symptoms to a diffuse unilateral subacute neuroretinitis (DUSN) which includes symptoms of visual loss, vitreous cells, optic disc inflammation and leakage, and transient recurrent crops of grey-white outer retinal lesions, and to an anaphylactic shock with lethal consequence (Hedges, 2000).

The first report of human larval alariosis was described by Byers and Kimura (1974). A 17-year-old boy from California, USA presented a living larva which could not be clearly identified. Following administration of anthelmintic, the larvae died within a few days. Also, Shea et al. (1973) reported the infection with *A. alata* mesocercariae on the left eye of a 29-year-old woman from Ontario, Canada. The larva was only identified visually as mesocercariae, further diagnosis was not made. The patient complained about intermittent low vision in the left eye over 6 years. She perceived a shadow with parasite-like movements while looking to the light. After examination, a small, white, moving parasite was found next to the retina. The larva was killed by laser. It is suspected

that the mesocercariae was living in the eye of the woman for several years before she was diagnosed. The authors believed that the woman may have been infected by rubbing her eyes while cleaning frog legs for cooking at home.

For the first time, a generalized infection of a person was described by Freeman et al. (1976) and Fernandez et al. (1976). A 24-year-old Canadian male complained of tightness in the chest and abdominal symptoms. After initial symptoms, the patient died in the hospital. At autopsy, practically all viscera showed extensive local or diffuse haemorrhage. Several thousand mesocercariae were estimated to have been present within the viscera and nearly all organs. The cause of death was asphyxiation from extensive pulmonary haemorrhage, probably due to immune-mediated mechanisms, after repetitive oral intake of *A. americana* mesocercariae. The authors concluded that the victim ate uncooked or more likely inadequately cooked frog legs heavily infected with mesocercariae.

Beaver et al. (1977) reported a 43-year-old man from Louisiana, USA who complained of a small (1x1 cm) intra dermal mass, which was evident for about 3 weeks on the upper thigh. After some days, the patient returned because a similar lesion had appeared in the lumbar area. The "mass" was removed, which later on, was identified as mesocercariae belonging to the subfamily *Alariine.* On questioning him later, it was learned that he ate part of a cooked raccoon and different wild game meat including deer, rabbit, and squirrel. McDonald et al. (1994) reported two cases of human intraocular infection with mesocercariae of *Alaria* spp. as causative factor, involving unrelated Asian men who had unilateral decreased vision. The first case was a 35-year-old Asian man from San Francisco, USA who complained of bad vision in his right eye. It turned out that he had a restriction of vision for the past 2-3 years. The parasite in the first case was diagnosed as an *A.* mesocercariae on the basis of its shape, size (500 x 150 microns) and movement. After all, it was successfully killed with laser. The second patient was a 38-year-old, Asian man, again from San Francisco, USA, who presented blurred vision on his left eye. However, no larva was found. After repeated treatment, a moveable, grey-white cyst was found inside the anterior chamber of the eye. The parasite was removed surgically and identified as mesocercariae, most likely an *A. americana*. In both patients, the author

referred that the probable source of infection was the ingestion of undercooked frogs' legs containing the parasite in local restaurants.

Finally, Kramer et al. (1996) reported the infection with mesocercariae in a 38-year-old man with no history of pulmonary disease developed intermittent hives and bronchospasms shortly after returning from a hunting and fishing trip in the Hudson Bay, Canada. Approximately one year later, examination of an excised subcutaneous nodule demonstrated infection with a mesocercariae. Eating undercooked wild goose meat during the hunting trip was the most likely source of infection. Treatment with oral antihistamines and inhaled corticosteroids led 2 months later to an improvement of symptoms. However, one year later, the patient presented symptoms again. Beside the undercooked wild goose, it was recorded that the patient consumed frog legs approx. 4 months in advance. At the end, it was not clear, whether the infection was caused by an earlier consumed wild goose meat or frog legs.

A. alata mesocercariae are distributed in meat of wild boars, although it was assumed that the parasite would not imply any risk to consumers (Ostertag and Schönberg, 1955; Lerche et al., 1957). Odening (1963) reported a massive *A. alata* mesocercariae infection during an *in vivo* experiment with Rhesus monkeys (*Macaca mulatta*). He identified the presence of *A. alata mesocercariae* in several vital organs including the heart (Portier et al., 2014). These findings represented clear evidence that a host closely related to humans can become infected with the parasite (Odening, 1963; Rentería-Solís et al., 2013). It is well known that humans get infected by eating raw or undercooked meat infected with cyst stages of *T. gondii*, *Sarcocystis* spp., *Taenia* spp. and *Trichinella* spp. In most countries measures are taken to prevent humans from becoming infected with meat-borne helminths by inspecting the meat in the slaughterhouse or laboratory (Dorny et al., 2009).

1.7 A. ALATA MESOCERCARIAE MIGRATION TECHNIQUE (AMT)

Although the detection of adult *A. alata* is well standardized (Szczesna et al., 2008; Bagrade et al., 2009; Riehn et al., 2010), specific detection methods for *A. alata* mesocercariae were not available until the *A. alata* mesocercariae migration technique (AMT) was introduced. At first, these encysted parasites, especially on the serous membranes, were identified by the naked eye or with a magnifying glass (Odening, 1960; 1963; Hiepe, 1985; Riehn et al., 2010). Trichinoscopy was used in the past as the only available method for *Trichinella* spp. detection until the pooled sample artificial digestion (HCl/Pepsin) method was introduced in the 1970's and 1980's (Jakšic et al. 2002). The actual official method for the detection of *Trichinella* spp. in muscle tissue included in the Annex I, Chapter I of the regulation (EC) No. 2075/2005 (European Commission, 2005) is unsuitable for the detection of *A. alata* mesocercariae since *A. alata* does not present the encapsulation as in the case of *Trichinella* spp. Digestion liquid from the official method harms and destroys the mesocercariae.

The AMT refers to so-called Baermann technique (“Baermanization”) for nematodes. The first step is chopping the sample material into pieces. The sample (30 g) contains muscle, adipose, connective, and glandular tissue. Later, the prepared sample is transferred to a plastic sieve placed in the funnel and immersed in 150 ml of lukewarm tap water. The sample has to be totally emerged in the water. Next, the sample is allowed to stand for 90 min at room temperature. After 90 min, a 20 ml sample of fluid is quickly run off into the measuring cylinder and transferred to larval counting Petri dish. At that time, the sample is examined by stereomicroscope at a magnification of 15 to 20 times (Riehn et al. 2010).

AMT method suits better for the identification of *A. alata* mesocercariae because of the parasite’s specific and pronounced mobility, its ability to actively move from the sample, and its inability to swim against gravity. The warm liquid, used in the AMT method, stimulates the migration of the parasite as they will move actively into the free liquid. Furthermore, AMT works without chemicals and guarantees a distinctly higher survival rate and motility of the parasite and facilitates diagnosis. Additionally, the chemical-free implementation minimizes the environmental risks and reduces costs (Riehn et al. 2010).

1.8 OBJECTIVES

At present, little is known about the characteristics of survival of *A. alata* in different meat products under various food processing technologies.

The first objective of this thesis was to analyse through different German homemade raw cured products (raw ham, salami and raw sausage) and their meat processing technology (fermentation, curing, cold smoking, drying) the tenacity of *A. alata* mesocercariae. It is relevant to understand if the parasite can be eliminated by food processing and if the traditional meat products made from *A. alata* mesocercariae positive game meat represent a possible source of infection for the consumer.

The second objective targets the resistance of *A. alata* mesocercariae to different concentrations of NaCl and ethanol. Also, to understand the condition/time function that allows the elimination of the parasite in the human stomach gastric acid.

Finally, the third objective is to elucidate the mortality of the parasite at different temperature/time profiles and to gather information of which thermal treatment processes can be recommended for the treatment of game meat and game meat products.

CHAPTER 2 TENACITY OF ALARIA ALATA MESOCERCARIAE IN HOMEMADE GERMAN MEAT PRODUCTS

CHAPTER 2 IS PUBLISHED IN THE INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY

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CHAPTER 3 EFFECTS OF IN VITRO CONDITIONS ON THE SURVIVAL OF ALARIA ALATA MESOCERCARIAE

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CHAPTER 4 EFFECT OF TEMPERATURE ON THE SURVIVAL OF ALARIA ALATA MESOCERCARIAE

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CHAPTER 5 GENERAL DISCUSSION

5.1 General problem

The recent presence of *A. alata* in wild boars in Germany (Riehn et al., 2012), Austria (Paulsen et al., 2012), Poland (Wasiluk, 2013), Bulgaria (Riehn et al., 2013), Czech Republic (Paulsen et al., 2013) and France (Portier et al., 2011; 2014) are a clear proof that viable *A. alata* mesocercariae can be present in game meat. Besides, the cases of human alariosis in the America region linked to the consumption of insufficiently heated game meat show that the transmission of vital *A. alata* mesocercariae and subsequent infection of humans are possible.

Up to now, a scientific assessment of the human exposition risks and information on the tenacity of *A. alata* mesocercariae with respect to established meat processing technologies, combination of time-temperature treatment and the reaction of the parasite to different *in vitro* conditions is not available.

It is known that the presence of the parasite poses a potential risk for humans when uncooked or undercooked game meat is consumed. It is presumed that insufficiently cooked, frozen or refrigerated game meat can act as a source of infection of alariosis. In general, cooking and freezing are considered to be effective in killing parasites. Additionally, refrigeration of game meat is considered as an important part of the safety chain production, whereas microwave heating has not been studied on the inactivation of *A. alata* mesocercariae in game meat. Therefore, it is important to determine how long the parasite survives when exposed to cooking, refrigeration, freezing and microwave heating.

5.2 Isolation

All parasites were collected from naturally infected wild animals (wild boar; raccoon; and raccoon dog) originated from different regions in eastern Germany. Wild animal carcasses were stored at 2.0 °C until AMT technique was performed within 24 h.

For each experiment and all samples, *A. alata* mesocercariae were examined under a stereomicroscope at room temperature for visual evaluation of larval vitality within

1 min. *In vitro* assessment of larval viability was carried out based on larval motility (active vs. non-active). Larvae that did not show any signs of motility or movement within 1 min were considered as dead.

5.3 FOOD TREATMENTS IN GAME MEAT PRODUCTS

In general, hurdle technologies aim to preserve the safety and quality of foods during extended periods of storage. The concept of hurdle is defined as a combination of treatments to increase the safety, stability and quality of food (Leistner and Gould, 2002b). Survival of *A. alata* mesocercariae was investigated in three different meat products: salami, raw sausages and dry-cured raw hams.

Salami is defined as a cured sausage made from fermented and air-dried meat with normal maturation. The salamis used for this trial were divided in 2 different batches. Both batches were made from *A. alata* infected meat, fat tissue, nitrite curing salt (NCS), started culture and extra spices. For sausage preparation all ingredients were mixed and transferred to a meat grinder. In one batch a defined number of additional vital mesocercariae was added to the sausage meat in order to compare the parasite mortality in a highly contaminated product with normal contaminated sausage. The sausage meat was stuffed into casings with a manual hand-crank sausage stuffer. The final salami sausages were held in a fermentation room for 24 h and dried in a conditioning room.

The safety of this meat product relies on a decrease of pH and water activity (a_w) during fermentation and drying (Arnau et al., 2007). In this case, the produced salamis presented a reduction in the pH after 24 h due to the production of lactic acid, which was steady and rapid for the first 48 h. However, the presence of vital *A. alata* mesocercariae was observed after 24 h of preparation.

Raw sausages with short maturation, known in Germany as “Knackwurst”, were produced following recipes from local German hunters and game meat consumers. The small raw type sausages contained infected meat, fat tissue, and NCS. The mixed ingredients were transferred into natural pig casings. For this type of sausage, no starter culture was added. The sufficient addition of NCS to the raw sausage mixture is particularly important

since the other hurdles have not yet been established and the finished products are usually stored without refrigeration and consumed without being heated (Leistner, 1995). As in the case of the salami, the parasites were still alive after 24 h preparation during the initial steps of the sausage.

The production of dry-cured raw ham is known to be a process, which does not permit growth of pathogens (Reynolds et al., 2001). According to Leistner and Gould (2002a), raw hams are preserved by a decreased water activity, which is brought about by salting and drying. The mean of NCS concentration used in the raw hams was 3.0 %: this level of concentration has been also shown in the past to be effective for the inactivation of *Trichinella* (Zimmerman, 1971). NCS must be considered as an important factor in the devitalisation of the parasite through possible toxic action and dehydration of the parasite (Ransom et al., 1920). Additionally, cold smoking seems to influence the water loss, salt diffusion in the hams and product stability. In this meat product, only dead parasites were found.

Because of the information mentioned above, it is now feasible that *A. alata* mesocercariae can be inactivated during the processing of raw ham, salami and small raw sausage if the sequences of hurdles are met during processing. Nevertheless, the early consumption of fresh raw type sausages may represent a risk for the consumer.

5.4 Effect of NaCl

Complementary results indicate that 3.0 % of NaCl concentration is most effective in reducing *A. alata* mesocercariae because the parasite survived less than 24 h *in vitro* conditions. The clear trend of the response of *A. alata* mesocercariae to NaCl treatment varies with the concentration; this means that the lower concentration of NaCl the longer time the parasite will need to die. It is suggested that the addition of 3.0 % NaCl can be used as a secure measure when game meat products are fabricated. Usually, for the typical manufacture of dry fermented sausages, lean meat and fatty tissue are mixed with 3.0 % salt, sugar, spices, and starter culture (Lücke 1994). In this sense, game meat products produced with lower than 3.0 % NaCl concentration can represent a potential

risk of alariosis for consumers depending on the kind of product and the time within it is consumed after production.

5.5 Consumer habits

The recent demand for a variety of low NaCl meat products has increased due to meat processors and consumers awareness of the association of NaCl intake and hypertension (Ruusunen and Puolanne, 2005). However, a minimum of NaCl concentration should be recommended to assure that the game meat product will not represent a risk to the consumer due to the presence of *A. alata* mesocercariae and other pathogens.

Additionally, short ripened products “Knackwurst” are usually consumed directly after production because they are often perceived as ready-to-eat products. However, *A. alata* mesocercariae might be still alive in the early stage of the production. In terms of preventive consumer protection these products should be recommended for consumption after heating only.

Nonetheless, specific population groups are at considerably higher risk of contracting alariosis than others. These include game meat consumers and hunters. Therefore, education for consumers and hunters, especially those who consume raw or undercooked game meat and traditionally cured products, can be helpful in prevention of alariosis.

5.6 Effect of ethanol

Effective disinfectants and decontamination procedures are required in laboratories working with infectious parasites such as *A. alata* mesocercariae. Hence, procedures for a reliable inactivation of the parasite are necessary for safe handling in game meat production and/or under laboratory conditions. Different percentages of ethanol, starting from 0.1 to 70 %, solutions were prepared by mixing ethanol in distilled water. Recovered *A. alata* mesocercariae previously isolated were transferred and suspended in the ethanol concentration solutions. Ethanol is effective at reducing survival of *A. alata* mesocercariae after a short period of time (<1 min) at a concentration of 70.0 %. The finding that high

ethanol concentrations efficiently destroy *A. alata* mesocercariae suggests that it can be used as a measure to decontaminate areas that were accidentally contaminated with the parasite.

5.7 Effect of the Gastric juice

Gastric juice is the first barrier against various infectious diseases (Smith, 2003). To assess the survival of *A. alata* mesocercariae in conditions of human stomach digestion, vital *A. alata* mesocercariae underwent pepsin-HCl digestion for different intervals of time.

A loss of viability of *A. alata* mesocercariae was observed during the first 60 min of incubation. The parasites were able to survive for 30 – 110 min during fasting conditions (pH 1.0 – 2.0 at 37 °C), but were dissolved after 120 min.

Nevertheless, the consumption of a characteristic meal of a Western diet produces an immediate rise in the median gastric pH to about 6.0 (Waterman and Small, 1998). The interaction of gastric acid and ingested *A. alata* mesocercariae is conditioned by many variables in addition to gastric acid pH: number of *A. alata* mesocercariae ingested, digestion time, physical protection of the parasite by food, buffering of gastric content, use of certain drugs (e. g. antacids and proton pump inhibitors), as well as the infectivity of the parasite and the resistance of the host.

5.8 Effect of heating

Most parasites are rapidly inactivated by heat treatment. It was proved that A. *alata* mesocercariae are reliably inactivated, if temperatures of 60.0 °C and above are reached in water bath. The experiments were carried out with isolated larvae, stored in a liquid medium. Recommended core temperature for a safe preparation of game meat with respect to other parasites is already documented. For example, Gamble et al. (2000) state, that meat, which may contain *Trichinella* spp. larvae should be cooked until the game meat reaches a core temperature of at least 71.0 °C and the German Federal Institute for Risk assessment recommends at least 67.0 °C for the inactivation of

Toxoplasma gondii cysts (BfR, 2011). The impact of insufficiently cooked game meat as a potential source of human infection became already clear in the past when wild boar and goose meat were identified as the transmitting vehicle for *A. alata* mesocercariae (Portier et al., 2014).

5.9 Effect of refrigeration

European legislation states that bagged and dressed game has to be refrigerated as soon as possible. Large wild animals have to be cooled to an internal temperature of not more than 7.0 °C and small wild animals to an internal temperature of not more than 4.0 °C in order to prevent the growth of pathogenic and spoilage microorganisms on the carcass surface (European Commission, 2004). According to the results presented, it is assumed that the effect of refrigeration decreases the vitality of *A. alata* mesocercariae in game meat, but the parasites are not killed during cold storage. It was demonstrated that *A. alata* mesocercariae survive in Ringer's solution at refrigeration temperatures for up to 13 days. The results correspond with the ones from Bugge (1942) and Freeman et al. (1976), where *Alaria* spp. mesocercariae had a survival time of 16 - 18 days and *A. americana* mesocercariae survived for more than one month after being subjected to repeated refrigeration cycles. Overall, these *in vitro* studies presumed that chilling of game meat is not a suitable treatment for the inactivation of *A. alata* mesocercariae in fresh game meat, despite its great significance in terms of the Good Hygiene Practice (GHP).

5.10 Effect of microwave heating

Microwave heating is used for several processes in the food industry e.g. pasteurization and sterilization of foods (Tang et al., 2008). The major drawback associated with microwave heating is the non-uniform temperature distribution, resulting in hot and cold spots within the heated product. Several studies have been carried out to determine the effect of microwave treatment on the inactivation of some other parasites but to date no information about the effectiveness of microwave treatment with regard to the

inactivation of *A. alata* mesocercariae is available. Short time exposure to microwave treatment at a frequency of 2450 MHz decreases the survival of *A. alata* mesocercariae in game meat samples. However, it was observed that the meat samples partly remained raw in the core, although the meat's surface was partially denatured. It can be reasonably assumed in this context, that *A. alata* mesocercariae that are located close to the core may be still alive after microwave treatment of the meat because of the unevenly heat distribution within the sample. In the light of these findings, microwave heating seems not suitable for a reliable inactivation of *A. alata* mesocerariae in game meat.

5.11 Effect of freezing

Freezing can be used for the inactivation of parasites in meat. *A. alata* mesocercariae stored at -18.0 °C remained viable for 0.5, 1.0 and 1.5 h reaching an internal core temperature of the meat pieces of -4.0 °C, -7.8 °C, and -10.6 °C. Longer freezing time associated with lower temperature led to an inactivation of the larvae. Previously, Portier et al. (2011) demonstrated that one *A. alata* mesocercariae remained alive after five days of refrigeration at -18±2 °C. Additionally, Hiepe (1985) described that *A. alata* mesocercariae in pork meat survived up to 8 weeks at -20.0 °C. The freezing time needed to reach the intended target temperature in the core prolongs with the increase of the sample size. Another hypothesis aims at the tenacity of *A. alata* mesocercariae in different paratenic host species and the duration of the infection. It is possible that the resistance of *A. alata* mesocercariae is a complex function of diverse factors e.g. species, size and age of the paratenic host as well as the origin of the parasite.

5.12 Final recommendations

Based on the results it can be stated that *A. alata* mesocercariae are inactivated very effectively in the course of food technological procedures as pertained to production of traditional raw cured products such as dry cured ham and raw type sausages. However, small raw sausages are considered to be high-risk products, presumably because consumers are not aware of the safe-handling requirements for raw meat products and

due to their preference to consume these sausages fresh and unheated. Ideally, the effective and complementary combination of the usage of NCS and/or common salt, starter culture and smoking are sufficient to inactivate the parasite in game meat products but reduction or elimination of a specific hurdle may allow the survival of the parasite.

A. alata mesocercariae are particularly sensitive to salt. Although there is a new trend for meat processors to produce meat products with reduced NaCl, it is not recommended using less than 3.0 % NaCl for the preparation of game meat products. In addition, it seems difficult to change the habit of the tradition among German hunters of using wild boar or other game meat immediately after bagging and removing the viscera. Beside, changes in the consumption habits and new preparation methods for game meat (e.g. pink roasting and grilling) may increase the risk for food-associated parasitic infections.

The information presented in this thesis reveals that the effect of ethanol concentration produced an inactivation in *A. alata* mesocercariae after a short period of time. The minimum ethanol concentration of 8.0 % and the maximum ethanol concentration of 70.0 % are suitable for inactivating *A. alata* mesocercariae. Ethanol is inexpensive and can be applied to laboratories' surfaces and stainless steel surfaces.

The human stomach may act as a barrier against the parasite. *A. alata* mesocercariae survive between 30 and 110 min into an acid environment during fasting conditions (pH 1.0–2.0 at 37 °C). Digestion time depends upon individual characteristics (age, sex, health status, time of day) and food characteristics (total amount, composition, particle size). Hence, it is assumed that food intake provides a protective effect to *A. alata* mesocercariae by facilitating their survival under real digestion conditions and that infected meat products elaborated with fat tissue can contain trapped *A. alata* mesocercariae and may readily survive the acidic conditions of the stomach and pass into the intestinal tract.

Previous cases of human alariosis due to the consumption of insufficiently heated game meat and/or meat products are a clear prove that human infection is possible. Therefore, an adequate temperature treatment, especially for meat from wild and free-ranging animals, is a key measure for preventative consumer protection. The experiments with *A. alata* mesocercariae suggest that both heating and freezing temperatures are suitable for

a reliable inactivation of the parasite. A suggested proper cooking to a core temperature of at least 60.0 °C for not less than 3 min and freezing regimes that lead to core temperature of at least -13.7 °C can sustainably reduce the risk for human infection. Refrigeration and short time exposure (of up to 90 s) to microwave heating, on the other hand, cannot be recommended as reliable treatment methods for the inactivation of *A. alata* mesocercariae in game meat especially if they are used exclusively.

Thermally unprocessed game meat infected with *A. alata* mesocercariae poses a potential health risk to the consumer and should be recognized as a public health concern. This applies, in particular, to occupationally exposed persons (e.g. hunters, rangers, butchers, chefs). Persons, who handle and treat game meat, might be unaware of the contamination risk with *A. alata* mesocercariae due to improper handling and processing. Hunters and other consumers must understand that wild game should be handled and cooked with the same or even higher caution recommended for other meat since wild animals can be infected with parasites or zoonotic agents that are not or less prevalent in livestock/agriculture animals. Specific treatment guidelines with respect to cooking and freezing should be formulated for the groups of persons referenced above as well as for food control agencies and private households in order to minimize the risk of *A. alata* mesocercariae infection in humans. Game meat consumption without proper temperature treatment should be considered as a potential source of alariosis to humans.

This thesis presents for the first time the possible factors that may influence the resistance of *A. alata* mesocercariae in game meat or game meat products: different NaCl concentrations, freezing, heating, microwave treatment and cold storage, as well as ethanol as a common disinfectant for cleaning purposes and how much time the parasite survives the acidity of the human stomach. Still, in order to achieve a complete risk assessment on the viability of *A. alata* mesocercariae, it is necessary to analyze a much larger number of samples, particularly those from local hunters and homemade products, as well as other meat products considering different curing and NaCl concentrations, fermentation, smoking, drying times, freezing and refrigeration time.

The research of *A. alata* mesocercariae in game meat is still in developmental stage but has received increased attention in recent years. The experiments and results presented in this thesis will help to develop more complex test setups for future investigations. For example,

an interesting step towards more detailed studies of low temperature tolerance of *A. alata* mesocercariae and the associated influencing factors. Further experiments with *A. alata* mesocercariae infested game meat must verify to what extent these recommendations can be adopted for the inactivation of *A. alata* mesocercariae in game meat.

The studies presented here may also facilitate the competent authorities and industry to consider the problem and suggest proper set of food safety measures to be taken in game meat products processing technologies to produce wild meat products that are safe for human consumption.

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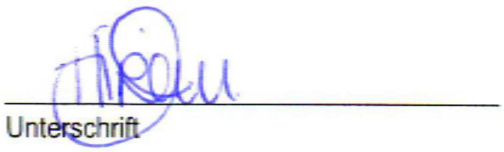

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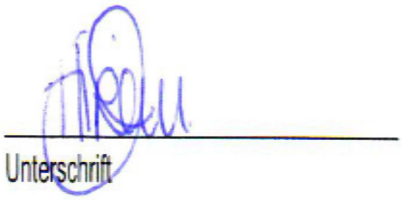

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