Studies on Pathophysiology of Sudden Cardiac Death-Type Strongyloidosis Using an Ovine Model

January 2021

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A Dissertation Submitted to
the Graduate School of Life and Environmental Sciences,
the University of Tsukuba
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Biological Science
(Doctoral Program in Biological Sciences)

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Abstract

Strongyloides papillosus is a nematode parasite of ruminant animals and rabbits that is distributed worldwide. Infective larvae of the parasite invade a host by skin penetration to migrate up to the larynx, and mature into parasitic females in the small intestine to lay eggs in parthenogenesis. Beginning in 1978 in Japan, outbreaks of the fatal disease, which would later be identified as sudden cardiac death-type strongyloidosis, were reported with no premonitory signs on farms that raised calves in small pens using sawdust as litter. Field surveys and experimental infections disclosed that heavy infection with *S. papillosus* causes the disease, in which calves develop continuous sinus tachycardia and final sudden cardiac arrest by ventricular fibrillation (VF).

I considered that two questions are of primary importance for further studies to elucidate the disease mechanism: which developmental stage of the parasite is responsible for the disease; and whether the cardiac disorders are based on reversible and curable change. The research target can be decided by answering the first question. Solving the second question may clarify certain characteristics of the disease, such as whether the disturbance of cardiac regulation is functional or organic lesion-based. The present studies were aimed at solving these questions.

Rabbits never show cardiac disorders following heavy infection with *S. papillosus*, and therefore are not useful as an animal model. However, if lambs could be used as a model animal, experimental trials would become easier compared to those using calves due to the body size of animals. In Chapter 1, as a preliminary study, the course of heavy strongyloidosis in lambs was analyzed to determine whether they could serve as an animal

model of this disease. Lambs were percutaneously infected with 1,000-32,000 infective larvae of *S. papillosus* per kg body weight. The animals given 3,200 or more larvae/kg developed sudden death on days 11-20 after infection, having high fecal egg output and intestinal worm burdens. Electrocardiogram (ECG) monitoring on several animals revealed a series of arrhythmias consisting of continuous sinus tachycardia and final VF in the intestinal phase of infection. The animals appeared normal with no diarrhea or other clinical signs until the time of death, with the exception that some animals exhibited a loss of body weight. Throughout the course of infection, there was no discernible pattern of elevated endotoxin levels or inflammatory cytokine induction common to the animals that ultimately died. These results indicated that lambs follow almost the same pathophysiological disease course including the development of cardiac disorders as calves after infection, and can thus serve as a model for the study of sudden cardiac death-type strongyloidosis. The results also suggested that the disease is not associated with endotoxic shock or a shock-like state that is usually induced by inflammatory cytokines.

Parasitic females would appear to be the cause of the disease, since animals developed the cardiac disorders in the intestinal phase of infection. However, the possibility of a few larvae migrating into the heart tissue cannot be excluded as a cause of cardiac dysfunction, since a portion of larvae pass through the lungs during their migration. The study in Chapter 2 was thus aimed at elucidating the responsibility of parasitic females for the disease. For this purpose, I focused on two specific characteristics of parasitic females, namely, they never migrate back from the intestines and their eggs never hatch into larvae inside the intestines. Parasitic females were collected from infected donor rabbits. Recipient lambs were intraduodenally inoculated with sufficient numbers of live

and homogenized worms. These animals never had larvae in their bodies. The lambs inoculated with live worms developed continuous sinus tachycardia shortly after inoculation and died of sudden cardiac arrest by VF on days 2-9 after inoculation, having high fecal egg output and intestinal worm burdens. The animals appeared normal until the onset of VF. The course of disease was identical to that observed in the intestinal phase of percutaneous larval infection. Inoculation with homogenized worms did not produce fatal arrhythmias. These results indicated that active parasitic females in the small intestine are responsible for sudden cardiac death-type strongyloidosis regardless of the presence or absence of migratory larvae.

Most reversible and irreversible arrhythmias are likely to result from disorders without and with pathological lesions of the heart, respectively. The study in Chapter 3 was aimed at elucidating the reversibility of arrhythmias in heavy strongyloidosis. For these experiments, I focused on an anthelmintic treatment that is effective against parasitic females during the middle stage of infection. Lambs were percutaneously infected with a lethal dose of infective larvae, and received ivermectin or remained untreated upon developing continuous sinus tachycardia accompanied by prolonged PQ interval (the portion of ECG between the P wave and the QRS complex). In the treated animals, the combined arrhythmia disappeared within 39 h after treatment and thereafter no arrhythmias were detected. Fecal egg output became negative within 61 h after treatment. Untreated control animals developed sudden cardiac death. These results indicated that the supraventricular arrhythmias generated in heavy strongyloidosis are reversible and curable following worm elimination. The functional disturbance of the heart with few or no lesions is more likely to cause the arrhythmias in the disease rather than the organic

disorders with lesions.

To date, it has never been demonstrated that any gastrointestinal parasite other than *S. papillosus* induces fatal cardiac dysfunction in animals or humans having no accompanying diseases. The present studies contribute new and unexpected knowledge to the field of parasitology. Parasitic females of *S. papillosus* may excrete or secrete a cardioactive substance capable of modulating cardiac rhythms in the disease. The generation of sinus tachycardia accompanied by prolonged PQ interval can hardly be accounted for by a simple unbalance of the autonomic nervous system, since the two arrhythmias are physiologically derived from the opposite phase of the innervation. One possible hypothesis is that parasitic females elicit enhanced automaticity in the atria, which is known to produce both increased heart rate and prolonged PQ interval, via a route independent of the autonomic nervous system. Such abnormal automaticity would lead to an alteration in the pacing rate of the sinoatrial node and disturbance of conduction. The derangement would induce disorganized excitations in the ventricles, ultimately resulting in VF.

The present studies provided clear scientific evidence that worm elimination is effective for recovery from the cardiac dysfunction in sudden cardiac death-type strongyloidosis. The occurrence of sudden cardiac death can be prevented by anthelmintic treatment of animals having high fecal egg output. The results of the present studies make a major contribution to solving an important problem in the field of livestock husbandry in Japan. Further studies are required to clarify the factors associated with the mechanism underlying the cardiac disorders induced by *S. papillosus*. The ovine model is expected to facilitate such studies.

Abbreviations

ATCC	American Type Culture Collection
AV	atrioventricular
(AV block	atrioventricular block)
(AV node	atrioventricular node)
ECG	electrocardiogram(s)
EPG	eggs per gram of feces
ES	excretory and secretory
(ES products	excretory and secretory products)
IgG	immunoglobulin G
IL-1	interleukin-1
IL-6	interleukin-6
PMSF	phenylmethylsulfonylfluoride
SD	standard deviation
TNFα	tumor necrosis factor alpha
U	unit(s)
(U/ml	unit(s)/ml)
VF	ventricular fibrillation

General Introduction

The nematode genus *Strongyloides* is a member of the family Strongyloididae belonging to the order Rhabditida in the class Chromadorea of the phylum Nematoda (Mehlhorn, 2016; ITIS, 2020). The genus is made up of 52 species found in amphibians, reptiles, birds and mammalians. These include *S. papillosus* in ruminants, *S. ransomi* in pigs, *S. westeri* in horses, *S. ratti*/*S. venezuelensis* in rats, *S. fuelleborni* in monkeys, and *S. stercoralis* in humans (Speare, 1989). *Strongyloides papillosus* (Wedl, 1856) is a small intestinal parasite of cattle, sheep, goats, and rabbits. It is commonly distributed in Japan and worldwide, is almost universally prevalent in ruminants, and was previously considered to be slightly or not at all pathogenic to its hosts (Itagaki and Ooishi, 1984; Bowman, 1995).

As illustrated in Figure 1, *S. papillosus* has a unique and complex life cycle which consists of parasitic and free-living generations (Basir, 1950; Triantaphyllou and Moncol, 1977; Schad, 1989; Speare, 1989; Mehlhorn, 2016). In the parasitic homogonic generation, all the adult worms in the small intestine of the host are females; these individuals, known as *parasitic females*, lay embryonated eggs in parthenogenesis. The eggs are excreted outside the body of the host in its feces; they never hatch within the host intestines. The selection of mode of life is genetically predetermined depending on their set of chromosomes. The majority of the eggs have three chromosomal sets, and the newly hatched first-stage rhabditiform larvae develop into third-stage filariform larvae that are called *infective larvae* after two molts. It takes 2-3 days between the discharge of eggs outside a host and the development of hatched larvae into infective larvae under

suitable environmental conditions. Infective larvae of *S. papillosus* invade a host by skin penetration, gradually spread to the subcutaneous connective tissues, and then migrate up the body of the host. The migratory larvae molt into fourth-stage larvae in the larynx, lungs, trachea, or esophagus during migration, and reach the small intestine, where the last molt into fifth-stage larvae and then maturation into parasitic females take place. Approximately 6-9 days are required for initiation of laying eggs by parasitic females following infection. Parasitic females produce eggs for approximately one month, and never migrate back again from the small intestine.

In the other generation, the free-living heterogonic generation, the first-stage larvae hatched from eggs having one or two chromosomal sets, develop into male or female adult worms, respectively, after four molts on the ground with no infection to any host. After copulation, free-living female adult worms lay eggs which are destined to hatch and develop into infective larvae, turning back to the parasitic generation.

The fatal disease, which would be identified as sudden cardiac death-type strongyloidosis later, was first recognized in August 1978 on a farm raising dairy calves for beef production in Kagoshima Prefecture on Kyushu Island, Japan. In that year and each of the subsequent 9 years, the farm experienced the death of several calves due to unknown cause (Taira and Ura, 1991a; Yokomine et al., 1991). From 1979, outbreaks of similar cases were also reported on other farms in Kagoshima Prefecture, and farms in other Prefectures of Kyushu as well as from Prefectures of Shikoku and Honshu (Maeshima et al., 1983; Kubo, 1986; Amahashi et al., 1991; Taira and Ura, 1991a; Tomishita et al., 1991; Ideguchi et al., 1992; Taira and Nakanishi, 1995). The following findings were commonly noted among the dead calves. 1) The disease occurred in

summer and/or autumn. 2) The disease occurred in calves that were several months of age, in most cases 2-6 months old. 3) The calves that died were raised in small pens using sawdust as litter. 4) The calves appeared healthy with no preceding clinical signs, then suddenly collapsed followed by agonizing vocal noises and clonic spasms, and died within a few minutes. 5) Scabs were often observed at the coronary area of the ankles at necropsy. 6) The occurrence was limited to certain farms or certain pens in a farm (Maeshima et al., 1983; Nishitateno 1986; Taira, 1991; Taira and Ura, 1991a; Taira and Ura, 1991b; Taira et al., 1992b). In several cases veterinarians happened to be present during these sudden deaths, and they reported that no heart sounds were auscultated in the animals even immediately after their collapse (Amahashi et al., 1991; Nishitateno 1991). Various kinds of examinations on bacteriology, virology, biochemistry and pathology were applied to the field cases, but none of these efforts revealed or suggested any causes for the sudden death (Hase et al., 1983; Kubo, 1984; Kubo, 1986; Nishitateno, 1986). The mysterious disease was tentatively labeled *Pokkuri disease of calves*, and engendered much anxiety, in addition to the actual loss of animals, at the beef production farms in Japan.

In 1984, a parasitological survey was conducted on a farm where coccidiosis was a problem for the calves raised in sawdust litter pens, but where no sudden deaths of the animals had occurred (Taira and Shimura, 1988). The survey revealed that some of the affected calves had a high fecal egg output of *S. papillosus* on the order of 10^4 eggs per gram of feces (EPG). Similar cases were reported in 1985 and 1986 on two farms where calves died of diarrhea, not so-called Pokkuri disease, with high EPG values of *S. papillosus* on the order of 10^4-10^5 (Yachi et al., 1987; Ideguchi et al., 1992). These

reports suggested that sawdust litter provided a suitable environment for the development of *S. papillosus* from hatching of eggs to survival of larvae, functioning as an effective medium to produce heavy infection by the parasite that was never established in calves raised in a pasture. Based on this information, attention was next focused on the parasitological examination of calves that died of the still-unexplained Pokkuri disease on several farms in 1987 (Amahashi et al., 1991; Taira, 1991; Taira and Ura, 1991a; Yokomine et al., 1991; Taira et al., 1992b). The investigations revealed that all the dead calves were heavily infected with *S. papillosus*, showing both EPG values of rectal feces and burdens of parasitic females in the small intestine on the order of 10^4-10^5. A large number of infective larvae were recovered by humidified incubation of the rectal feces collected from the dead animals and mixed with sawdust. In addition, anthelmintic treatment of a group of calves prevented further occurrence of sudden death after the recognition of *S. papillosus* infection at the farms (Ito, 1991; Matsutani et al., 1991; Taira and Ura, 1991a; Taira and Ura, 1991b; Tomishita et al., 1991; Almeida et al., 2005). Thus, it was indicated that the unknown sudden death of calves was associated with heavy infection with *S. papillosus* resulting from the contamination of sawdust litter in pens with a large number of infective larvae. By 1994, outbreaks of the disease had been reported from a total of 29 Prefectures, including Hokkaido and Okinawa (Taira and Nakanishi, 1995). At this point, experimental infections with *S. papillosus* were required to determine whether the parasite was the cause of the sudden death.

Experimental infection trials to confirm the field occurrence in calves started in 1990 (Taira et al., 1992a; Taira et al., 1992b). Calves were percutaneously infected with six different doses of *S. papillosus* infective larvae between 1,000 and 320,000 larvae per kg

body weight. Three out of five calves given 3,200 larvae/kg and all eight calves given 10,000 or more larvae/kg died between days 11 and 28 after infection (see Figure 2 in Chapter 1). Among the 11 calves that died, one calf suffered from diarrhea before death, but the other 10 calves developed sudden death with no premonitory signs. These animals fell down suddenly, exhibited agonizing vocalizations and clonic spasms, and then their corneal reflexes were completely disappeared within a few minutes. Heavy infection with *S. papillosus* was observed in the animals which died suddenly, with maximum fecal egg counts of more than 49,000 EPG throughout the course of infection and intestinal worm burdens of more than 21,000 parasitic females at necropsy. No consistent and significant changes suggesting a cause of death were confirmed in hematological, biochemical and histopathological examinations of the animals (Nakanishi et al., 1993). The course of death and the results of examinations in the experimental animals were the same as those found in the field cases of sudden death. The remaining calves given 3,200 larvae/kg and two calves given 1,000 larvae/kg survived the entire course of infection with no abnormal findings, having maximum EPG values between 7,000 and 70,000.

Throughout the course of experimental infection, electrocardiograms (ECG) and pneumograms had been continuously recorded in eight animals (Tsuji et al., 1992; Ura et al., 1993b). Six of the animals monitored in this manner developed sudden death. In all six of these cases, continuous sinus tachycardia had been recorded from 5-16 days after infection until the time of death. A few days before death, these animals showed patterns of sinus tachycardia including sporadic appearance of second degree atrioventricular block, ventricular premature beat and paroxysmal ventricular tachycardia, then finally

developed sudden cardiac arrest by ventricular fibrillation (VF). Neither abnormal behaviors nor abnormal respiration was observed until the animals developed VF. The onset of VF immediately triggered accelerated abdominal breathing for a few minutes followed by complete cessation of respiration. On the other hand, in the two surviving animals that were subjected to ECG and pneumograms, no abnormal recordings were detected during the experiment. The results of the experimental trials thus confirmed that heavy infection with *S. papillosus* causes sudden cardiac death in calves. Following these trials, the sudden death previously called Pokkuri disease, outbreaks of which had been spread throughout Japan, was named sudden cardiac death-type strongyloidosis (Taira and Ura, 1991b; Tsuji et al., 1992; Ura et al., 1993b; Taira and Ura, 2005).

Collectively, the above studies clearly disclosed the existence of sudden cardiac death-type strongyloidosis in calves. However, the pathophysiological mechanism underlying the fatal effect of *S. papillosus* infection on the regulation of cardiac rhythms remained entirely unknown. I considered that two questions are of prime importance when designing further studies to elucidate the disease mechanism. The first question is which of the developmental stages of *S. papillosus* that make up the complex infection course of the parasite is responsible for the disease. The research target(s) can be decided by answering this question. The answer to this question may also contribute to the establishment of a strategy for treatment and prevention of the disease. The second question is whether cardiac disorders developed in the disease are based on reversible and curable change or progressive and unrecoverable change. Solving this question may provide important clues for understanding certain characteristics of the disease, such as whether the disturbance of cardiac regulation is functional or organic lesion-based.

Before initiating studies to solve these questions, I tried to establish an experimental model for the disease using lambs. Rabbits could not be used, since they have been shown to develop a wasting condition, not sudden cardiac death, in response to heavy infection with *S. papillosus* (Nakamura et al., 1994; Nakamura and Motokawa, 2000). However, it has not been established whether lambs, a ruminant host of *S. papillosus* like calves, develop sudden cardiac death following heavy infection with the parasite. If lambs could be used as a model animal for sudden cardiac death-type strongyloidosis, then the costs, labor and required number of parasites in the experimental trials could be reduced due to the smaller body size of lambs compared to calves.

These studies presented in three Chapters were aimed at solving the two important questions mentioned above, following the establishment of an ovine experimental model. In Chapter 1, I analyze the course of heavy infection of lambs with *S. papillosus* as a preliminary study. I demonstrate that lambs are as sensitive as calves to the development of sudden cardiac death through the same course following infection, and that lambs can thus be used as a model animal in studies for sudden cardiac death-type strongyloidosis. In Chapter 2, I investigate the effect of direct inoculation of parasitic females of *S. papillosus* into the duodenum on the cardiac rhythms of lambs, focusing on the fact that such animals have no migratory larvae. I demonstrate that parasitic females in the small intestine are the developmental stage responsible for the cardiac disorders found in sudden cardiac death-type strongyloidosis. In Chapter 3, I investigate alterations of the cardiac disorders in lambs infected with a lethal dose of *S. papillosus* larvae following anthelmintic treatment that has been proven effective against many kinds of adult nematodes when administered to the animals upon the development of continuous sinus

tachycardia. I demonstrate that cardiac disorders generated in the patent infection are reversible and curable following worm elimination. Finally, in the General Discussion, I discuss the pathophysiology of heavy infection with *S. papillosus*, including the fully clarified and still uncertain aspects, and the application of current results to the prevention of sudden cardiac death-type strongyloidosis in the field of animal husbandry.

Figure

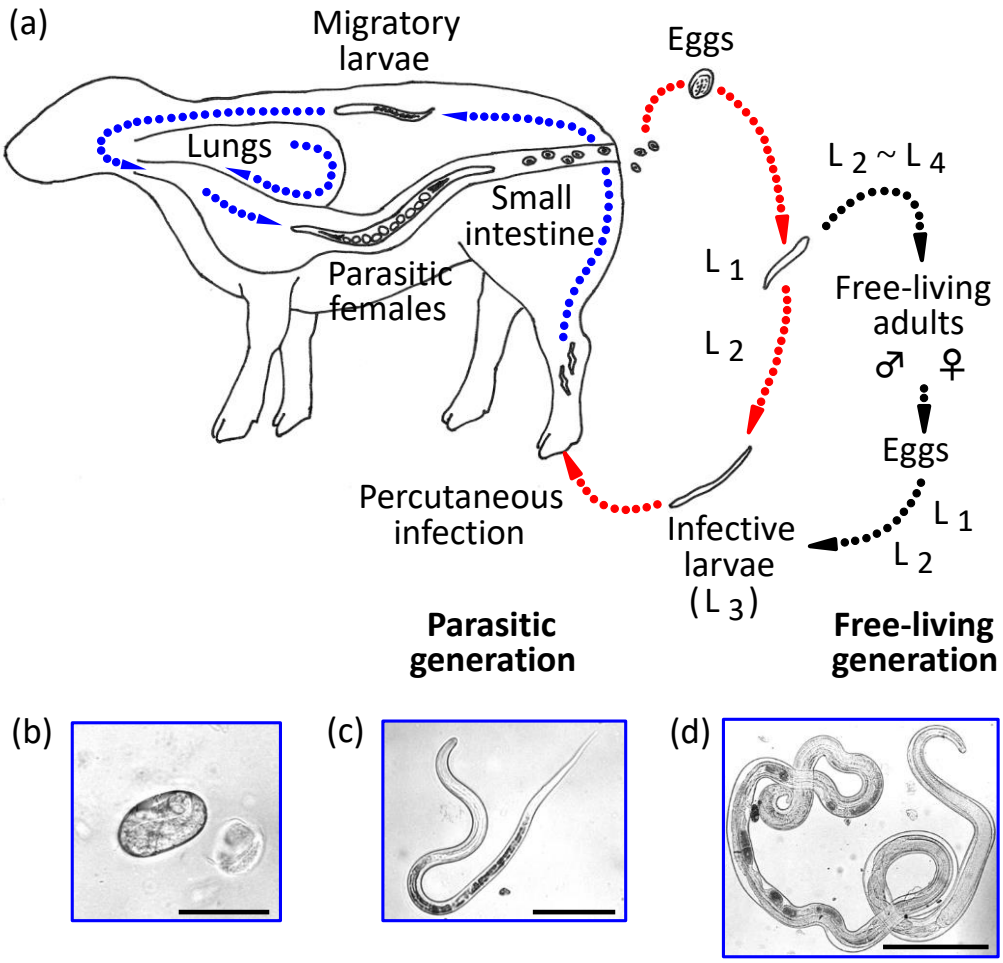

Figure 1. Life cycle and developmental stages of *Strongyloides papillosus*. (a) Life cycle. L_1: first-stage larvae, L_2: second-stage larvae, L_3: third-stage larvae, L_4: fourth-stage larvae. (b) Egg. bar = 50 μm. (c) Infective larva (third-stage larva of parasitic generation). bar = 0.1 mm. (d) Parasitic female (adult of parasitic generation). bar = 0.5 mm.

Chapter 1

Establishment of an Ovine Model for Sudden Cardiac Death-Type Strongyloidosis

Summary

Calves develop sudden cardiac death following heavy infection with *Strongyloides papillosus*. The disease is characterized by cardiac disorders showing continuous sinus tachycardia and final ventricular fibrillation (VF). Affected animals show almost no clinical signs until the onset of VF when they collapse suddenly. In the present study, the pathophysiological course of heavy *S. papillosus* infection was analyzed in lambs in order to clarify whether lambs could be used as a model animal for the disease. Lambs were percutaneously infected with 1,000, 3,200, 10,000, and 32,000 infective larvae per kg body weight. Eggs per gram of feces (EPG) were determined. Electrocardiogram (ECG), pneumogram and video recordings were continuously carried out on one animal each from the dose groups given 3,200 or more larvae/kg. Plasma endotoxin levels and serum profiles of tumor necrosis factor alpha (TNFα), interleukin-1 (IL-1), and interleukin-6 (IL-6) were also determined to ascertain the relation between the disease and a state of shock usually induced by inflammatory cytokines. At necropsy, gross lesions were examined. Intestinal worm burdens and migratory larvae in the tissues were counted. Two animals given the lowest dose showed no abnormalities during the experimental period, having maximum EPG values of less than 20,000. Eleven animals given higher larval doses died on days 11-20 after infection, having maximum EPG values of more than 40,000. They showed no significant abnormalities including diarrhea until the time of death, except that five of them lost 1.0 kg or more body weight. In the three animals with ECG monitoring, continuous sinus tachycardia started between days 6 and 9 after infection. In the late stage of infection, several kinds of ventricular arrhythmias

appeared among the patterns of sinus tachycardia and finally degenerated into VF. The onset of VF caused sudden cardiac arrest followed by accelerated breathing and then clonic spasms until complete death within 3 min. At necropsy, no gross lesions suggesting a cause of death were observed. Parasitic females ranging in number approximately from 3,000 to 159,000 were recovered from the small intestine of the animals that died. A few migratory larvae were recovered mainly from the forelegs and lungs but none were detected in the brain or heart. Plasma endotoxin levels remained normal. Inflammatory cytokine activities were transiently detected in some animals, but there were no induction profiles common to all the animals that died. These results indicated that lambs develop sudden cardiac death following heavy infection with *S. papillosus* through the same course as calves. Lambs can thus be used as a model to study sudden cardiac death-type strongyloidosis. The present study also suggested that the pathophysiology of the disease is not associated with inflammatory cytokine responses.

Introduction

Strongyloides papillosus is a small intestinal nematode commonly distributed worldwide. Natural low dose infections with the parasite are universally found in grazing domestic ruminants, and rabbits can serve as an experimental host (Itagaki and Ooishi, 1984; Bowman, 1995; Mehlhorn, 2016). The parasite had long been considered almost apathogenic until the discovery of sudden cardiac death-type strongyloidosis in calves (Chinone, 2007, Bowman, 2009). From 1978 in Japan, outbreaks of sudden death due to unknown cause in calves were frequently reported on beef production farms (Taira and Ura, 1991a; Taira and Ura, 1991b; Taira and Nakanishi, 1995). Heavy percutaneous infection with *S. papillosus* was experimentally demonstrated to be the cause of sudden cardiac death in calves by 1992 (Taira et al., 1992a; Taira et al., 1992b; Tsuji et al., 1992; Ura et al., 1992; Ura et al., 1993b; Taira and Ura, 2005). These studies indicated that calves develop continuous sinus tachycardia in the intestinal phase of infection and finally die of sudden cardiac arrest by ventricular fibrillation (VF). The possible minimum dose for lethal infection, which was estimated as approximately the 50% lethal dose, is 3,200 infective larvae per kg body weight, and the earlier times of death are associated with the higher larval doses. Affected calves appear healthy with almost no clinical signs until the onset of VF when they collapse suddenly, and then start to show accelerated abdominal breathing and clonic spasms.

The pathophysiological mechanism by which *S. papillosus* provokes fatal interference with the cardiac rhythms of calves remains unsolved. The dramatic change in the clinical appearance of animals in the most terminal stage of the disease is remindful of a state of

shock, such as endotoxic shock, a condition which also frequently results in acute death preceded by increased heart rate in cattle and humans (Isogai and Isogai, 1986; Andersen, 2003; Bullock and Benham, 2020). Endotoxic shock and a variety of other acute shocks are mediated by inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin-1 (IL-1), and interleukin-6 (IL-6) (Cannon et al., 1990; Titus et al., 1991; Mitsuyama, 1992; Martin et al., 1997; Andersen, 2003; Cavaillon, 2018). These cytokines are also associated with some heart diseases such as acute myocardial infarction, cardiomyopathy, myocarditis, heart failure, and cardiogenic shock (Ikeda et al., 1992; Matsumori et al, 1994; Yamada et al., 1994; Hedayat, 2010; Müller-Werdan et al., 2016). However, the relation between sudden cardiac death-type strongyloidosis and inflammatory cytokines remains unknown.

Experimental investigation into sudden cardiac death-type strongyloidosis has been impeded by several factors related to the available animal models. Calves themselves are comparatively large animals, and thus their handling is costly and labor intensive, and the parasites used to infect them must be prepared on a large scale. Rabbits, the smallest natural host of *S. papillosus*, develop a wasting condition but never show cardiac disorders following heavy infection with the parasite (Nakamura et al., 1994; Nakamura and Motokawa, 2000). Rabbits therefore cannot be used as a model animal for sudden cardiac death-type strongyloidosis, although they are useful for the maintenance and propagation of *S. papillosus* in serial passages. Lambs are smaller and more docile in nature than calves. If it could be established that *S. papillosus* induces cardiac disorders in lambs, much progress could be expected in studies on sudden cardiac death-type strongyloidosis by substituting lambs as a model animal.

In general, little attention has been paid to the pathogenic effects of *S. papillosu*s infection on sheep (Itagaki and Ooishi, 1984; Onyali et al., 1989; Pienaar et al., 1999), but there have been occasional case reports noting a clinical disease in lambs ascribed to *S. papillosus* infection under natural conditions (Turner and Wilson, 1958; Round, 1963; Taira and Kato, 1986; Onyali et al., 1989; Pienaar et al., 1999). According to these reports, lambs suffering from *S. papillosus* infection developed emaciation, anemia, and diarrhea with enteritis, and some of the animals died. On the other hand, a few studies on experimental single infection with *S. papillosus* described that lambs given 5,000 to 10,000 or more larvae/kg died in the intestinal phase of infection, showing no clear clinical signs except for slight to moderate anemia (Turner, 1955; Turner, 1959; Bezubik and Turner, 1964). Neither the reports on natural infections nor the studies on experimental infections included recordings and analyses of cardiac rhythms of the animals. Thus, the pathogenic effects of heavy infection with *S. papillosus* on the cardiac rhythms of lambs remain unknown.

The purpose of this Chapter was to establish an ovine experimental model for sudden cardiac death-type strongyloidosis. This Chapter describes a preliminary study designed to clarify whether lambs develop fatal arrhythmias following heavy infection with *S. papillosus* in a manner similar to calves. I investigated the course of percutaneous infection with four different doses of *S. papillosus* larvae in lambs, and recorded electrocardiograms (ECG) and pneumograms in some of the animals following infection. In addition, I analyzed endotoxin, TNFα, IL-1, and IL-6 activities in the blood of animals to ascertain whether the sudden death was associated with a state of shock.

Materials and Methods

Animals

Thirteen helminth-free Suffolk lambs were employed in this study. Their sex, ages, and body weights are listed in Table 1. They were fed a diet of hay and concentrate twice a day. Water was provided *ad libitum*. Three of them were loosely restrained by an individual neck stanchion on a drainboard from 7 days before infection for the recording of ECG and pneumogram in a quiet air-conditioned room. The recording room was sufficiently lighted to allow continuous observation of their behavior by a camcorder. The animals were accustomed to the stanchion before the experiment. The remaining 10 animals were maintained two each in concrete-floored pens. The pens were cleaned twice a day. Specific pathogen-free male Japanese-White rabbits weighing 2-3 kg were used for the passage of *S. papillosus*. They were kept separately in stainless steel wire cages with a drainboard and a feces tray. Standard pelleted food and water were provided *ad libitum*. All experiments using animals were approved by the Ethics Committee of the National Institute of Animal Health in Japan. Based on the results described in the previous studies using calves (Tsuji et al., 1992; Ura et al., 1993b), eventual occurrence of sudden cardiac death would be predicted if lambs developed continuous sinus tachycardia. In these experiments, however, such animals were neither treated nor euthanized in order to confirm the entire course of the disease. Animals showed almost no significant symptoms and abnormal behavior until the critical moments of sudden death, suggesting they experienced little or no pain and suffering throughout the experiment.

Parasites

The Himeji strain of *S. papillosus* was used in this study. The strain was originally isolated from a calf that developed sudden death in association with *S. papillosus* infection at a farm in Himeji City, Hyogo Prefecture in 1988 (Taira et al., 1991; Taira et al., 1992a). The parasite was maintained through monthly serial passages in rabbits by percutaneous infection with 100,000 to 200,000 infective larvae per animal as previously described (Nakamura et al., 1994).

Percutaneous infection of lambs with infective larvae

Feces were collected from infected rabbits, crushed and suspended in water. The fecal suspension was filtered with a 100-mesh sieve. The filtrate was filtered through cotton wool pads placed in a funnel set on an aspiration bottle. Each of the wet cotton wool pads containing *S. papillosus* eggs was heat-sealed in a polyethylene bag and incubated at 25 ºC for 7 days. Infective larvae that accumulated in the bag were suspended in water. The numbers of active infective larvae in 0.1 ml of suspension were counted eight times to confirm the larval density of the suspension. The third-stage rhabditiform larvae of heterogonic generation were never seen at counting. The volume of suspension containing a planned dose of larvae was filtered through cotton wool pads of 10 x 10 cm each.

Lambs were percutaneously exposed to 1,000 (n = 2), 3,200 (n = 3), 10,000 (n = 4), and 32,000 (n = 4) infective larvae/kg by attaching cotton wool pads that collectively contained the appropriate dose to the skin of the forelegs for 3 h (Table 1). The cotton wool pads were covered with polyethylene film and fixed with tape to the forelegs of

lambs. After exposure, the cotton wool pads were cut into pieces and the remaining larvae were examined by a modified Baermann technique (Tsuji et al., 1991). The numbers of larvae that remained in the cotton wool pads were 0.4% -12.3% (mean 2.8%) of those initially present (Table 1).

Fecal egg counts

Rectal feces were daily taken from all the animals for fecal examination. At necropsy, cecum contents were also collected. The fecal egg count was determined in a diluted suspension containing 5 mg of feces by a modified McMaster technique (Tsuji et al., 1991). The number of eggs counted was multiplied by 200 to calculate the eggs per gram of feces (EPG) value.

Collection of blood samples

Jugular blood samples were collected into heparinized tubes weekly prior to feeding in the morning from all the animals to prepare plasma samples for the determination of plasma endotoxin levels. Jugular blood samples were taken into tubes without anticoagulant for serum collection twice a week from one animal given the lowest larval dose (Lamb No. 102) and daily from seven animals given 3,200 or more larvae/kg (Lambs Nos. 201, 301, 304, and 401 to 404). Serum was harvested 6 h later for the determination of serum cytokine levels described below. Blood was also taken within a few minutes of death when it was possible to see the moment of the animal's death. Plasma and serum samples were stored at –80 ºC until used.

Electrocardiogram, pneumogram, and video recordings

Continuous ECG and pneumogram recordings were carried out on one animal each from the larval dose groups given 3,200 (Lamb No. 201), 10,000 (Lamb No. 301), and 32,000 (Lamb No. 401) larvae/kg. The apex-base lead, which is the bipolar lead along the longitudinal heart axis, was applied to the ECG recording with wire electrodes anchored into the subcutaneous tissue of the animals under local anesthesia with procaine (Omnicain 2% Injection, 0.2g/animal; Sankyo Seiyaku (presently Daiichi-Sankyo), Tokyo, Japan) 7 days before infection (Nakamura, 1967; Sesaki et al., 1971). The anode (+) electrode was placed in the lower left part of the thorax at the cardiac apex approximately 5 cm posterior to the head of the left elbow. The cathode (-) electrode was placed in the upper right part of the thorax at the one-fourth point between the peak of the withers and the right shoulder joint. The earth electrode was placed in the position of the last right rib at the same height as the cathode electrode. Respiratory movements were monitored with a breast-belt transducer (TR-701T; Nihon Kohden, Tokyo, Japan) that was placed around the thorax of the animals 3 days before infection.

The recordings were started from 3 days before infection on a polygraph (RM-80; Nihon Kohden) placed outside the recording room. Heart and respiration rates were determined on a recording chart at least once per hour when an animal was at rest and the recording baseline was stable, excluding the period of 1 h after the start of feeding. The normal heart and respiratory rates of the animals were 50-70/min and 12-23/min, respectively. A heart rate of more than 80/min was considered tachycardia (Detweiler, 1993). Tachycardia that lasted over 12 h was arbitrarily considered continuous.

The behavior of the animals was monitored with a camcorder (SVHS-EIS; Matsushita

Denki Sangyo (presently Panasonic), Osaka, Japan) and recorded with a video recorder (NV-HX11; Matsushita Denki Sangyo). The videotapes were changed every 8 h. In addition, the rectal temperature of the animals was monitored daily during their feeding. Their normal temperature was 38.5-39.5 ºC.

Necropsy

Lambs that died were necropsied within 3 h of death or within 3 h after the discovery of their death. Surviving animals were killed on day 42 after infection by bleeding under anesthesia with xylazine (Serakutal 2% Injection, 10 mg/animal; Bayer Yakuhin, Osaka, Japan). The animals were weighed and their organs were examined for gross lesions. The small intestine was slit longitudinally, cut into several sections, and incubated on a 16-mesh sieve in water at 37 ºC for 3 h. After the intestinal sections were rinsed and removed, the solution was mixed with a 1/19 volume of formaldehyde. The numbers of recovered worms in 10ml or 20 ml of suspension were counted eight times to calculate the total number of worm recovery. Migratory larvae were recovered by a modified Baermann technique from 10 g of each of the following tissues: the connective tissue of the coronary area of the forelegs and triceps brachii muscles; abdominal and mandibular muscles; the tissue around eye orbit; brain; lungs; heart (myocardium); liver; kidneys as previously described (Tsuji et al., 1991).

Endotoxin levels

Plasma endotoxin levels were determined using a colorimetric assay kit (Endotoxin Test D; Seikagaku Corporation, Tokyo, Japan) according to the manufacturer's

instructions. The measurement range of the kit was 2.0 to 300 pg/ml.

Cytokine bioassays

Serum TNFα activity was determined by a cytotoxicity assay using WEHI164 cells derived from murine fibrosarcoma (American Type Culture Collection (ATCC) CRL-1751) and recombinant murine TNFα (Genzyme (presently Sanofi Genzyme), Cambridge, USA) as previously described (Asai et al., 1993). Ovine TNFα shows cytotoxic activity against WEHI164 cells and amino acid homology of 73% to murine TNFα (Nash et al., 1991). The amount of TNFα that induced 50% growth inhibition was defined as 1 unit (U). The lower detection limit was 4.0 U/ml. The specificity of TNFα in positive samples was confirmed by a neutralization test with hamster monoclonal anti-murine TNFα antibody (immunoglobulin G (IgG); Genzyme) and control hamster IgG (Organon Teknika, Durham, USA) as previously described (Asai et al., 1993; Nakamura, 1998). Test samples were pre-incubated with an equal volume of 50-fold diluted antibodies at room temperature for 30 min. A sample showing 50% or more reduction in cytotoxic activity in the neutralization test was considered to contain TNFα activity.

Serum IL-1 activity was determined by a cytotoxicity assay using A375.S2 cells derived from human malignant melanoma (ATCC CRL-1872) and recombinant human IL-1α (Genzyme) as previously described (Nakai et al, 1988). Molecules of IL-1α and IL-1β exert identical biological activity (Dower et al., 1986). The A375.S2 cell assay has been successfully used to detect bovine IL-1 (Yoshioka et al., 1998). Since ovine IL-1 has amino acid homologies of more than 90% to bovine homologues (Andrews et al., 1991), the assay is expected to be available for detecting ovine IL-1. Serum samples

were pre-treated at 56 ºC for 30 min before determination. The amount of IL-1 that induced 50% growth inhibition was defined as 1 U. The lower detection limit was 8.8 U/ml. The specificity of IL-1 in positive samples was confirmed by a neutralization test with goat polyclonal anti-human IL-1α antibody (IgG; R&D Systems, Minneapolis, USA) and control goat IgG (Organon Teknika) as in the TNFα assay.

Serum IL-6 activity was determined by a proliferation assay using 7TD1 cells derived from murine plasmacytoma (ATCC CRL-1851) and recombinant human IL-6 (Genzyme) as previously described (Ziegler-Heitbrock et al., 1992; Asai et al, 1994). Ovine IL-6 shows proliferation activity to 7TD1 cells and amino acid homology of 53% to human IL-6 (Andrews et al., 1993). Serum samples were pre-treated at 56 ºC for 30 min before determination. The amount of IL-6 that induced 50% proliferation was defined as 1 U. The lower detection limit was 3.4 U/ml. Because a neutralization test was not performed for the IL-6 assay, results were expressed as IL-6-like activities.

Results

Fate and clinical findings of the lambs

The two lambs given the lowest larval dose showed no clinical abnormalities for 42 days after infection. Eleven of the lambs infected with 3,200 or more larvae/kg died on days 11-20 after infection (Table 2, Figure 2). The ECG and video recordings confirmed that the three monitored animals developed sudden cardiac death as described below. Among the remaining eight animals that died, neither premonitory signs nor abnormalities predictive of death were noticed until the final observation done 1-5 h before death in seven animals and 11 h before death in one animal. The observations indicated that the eight animals developed sudden death.

Diarrhea was not observed in any of the animals during the experiment. The final body weights of the animals that died were 84% to 103% of their initial weights, with the mean weight ±standard deviation (SD) being 96±7% (Table 2). By the day of death, five of 11 animals had lost 1.0 kg or more body weight. Rectal temperature was 0.5 to 1.0 ºC higher than usual from days 8, 10 and 6 after infection until the day of death in Lambs Nos. 201, 301 and 401, respectively. Anorexia and a decrease in fecal amount were observed the day before death and the day of death in these three animals.

Fecal egg output

The first eggs were detected in rectal feces between 9 and 11 days after infection (Table 3). The 11 animals that died had maximum EPG values ranging from 43,000 to 127,000 (mean±SD = 75,636±24,772), with no remarkable variation among the different dose

groups. Almost all the values were detected in their rectal or cecum feces collected on the day of death. The two surviving animals, Lambs No. 101 and No. 102, had maximum EPG values of less than 20,000, which were clearly lower than those in the other dose groups. They had EPG values of 2,800 and 0, respectively, at necropsy on day 42 after infection.

Electrocardiogram and pneumogram findings

The ECG findings observed in Lambs Nos. 201, 301 and 401 are summarized in Table 4. The alterations of heart rate in these animals are shown in Figure 3. The ECG and pneumogram recordings of Lamb No. 301 are shown in Figure 4. In these three animals, continuous sinus tachycardia with a maximum rate of 100-140/min started between days 6 and 9 after infection, and finally terminated in loss of cardiac function by VF. Second degree atrioventricular block, ventricular premature beat (R on T) and/or paroxysmal ventricular tachycardia appeared sporadically among patterns of sinus tachycardia from several hours before the onset of VF. No significant changes were seen in respiration rate until the onset of VF at which time accelerated respiration of 45-74/min appeared for a short time. Respiration completely ceased within 3 min after the onset of VF in the three animals.

Video monitoring

Except for the fact that the animals did not eat one third to one half of their food from the day before death, no abnormal behavior was observed on the videotape recordings of Lambs Nos. 201, 301 and 401, even after they developed continuous sinus tachycardia,

until the critical moments just before death. At the fatal moments, the two standing animals (Lambs No. 301 and No. 401) collapsed suddenly and the one sitting animal (Lamb No. 201) stiffened its body. Accelerated abdominal breathing began 10-20 sec after the sudden attack and continued for 20-30 sec. Thereafter, the animals exhibited repeated clonic spasms and made several agonizing vocalizations. All movements of the animals disappeared completely within 3-5 min after the sudden attack.

Necropsy findings

Gross lesions observed at necropsy are shown in Table 5. The mucous membranes of the duodenum and jejunum were congested in five of the animals that died. Scars of petechial hemorrhages were sporadically observed in the lungs of 10 animals, including one of the surviving animals. A few scars of petechial hemorrhages were found on the adipose tissue beneath the epicardium of four animals that died. An enlarged gall bladder full of bile was found in four of the dead animals. None of these lesions were considered to be associated with the death of the animals. No gross lesions were observed in the other organs.

Parasitic females were recovered from the small intestine of the animals that died and ranged in number from 3,438 to 158,738 (Table 3). The recovery rates were 5.3% - 47.1% (mean±SD = 20.4±14.1%) against the larval dose of percutaneous infection. One of the surviving animals, Lamb No. 101, also had a small number of parasitic females in the small intestine on day 42 after infection. A small number of migratory and remaining larvae were recovered mainly from the forelegs and lungs of animals that died (Table 6). No larvae were detected in the brain, heart, liver or kidneys in any of the animals.

Blood endotoxin and cytokine levels

Plasma endotoxin levels before infection were <2.0 to 16.3 (mean±SD = 11.3±5.1) pg/ml. The levels remained below 20.0 pg/ml, a value considered normal in the present assay, in all 13 animals throughout the experiment (Figure 5a).

Serum TNFα activities were not detected before infection in any of the animals in which they were measured. Serum TNFα activity was transiently detected only in Lamb No. 401 in the middle stage of infection, with values of 8.7, 5.2, 4.0 and 4.0 U/ml on days 5, 6, 7 and 8 after infection, respectively. The value in the day-5 sample (in two-fold dilution) was reduced to <4.0 and 4.6 U/ml in the neutralization test with specific and control antibodies, respectively, which confirmed its specificity. The activities detected in the other three samples were too low to confirm its specificity in the neutralization test due to the two-fold dilution of samples with antibodies.

Serum IL-1 activity of 44.2 U/ml was detected in the last sample taken from Lamb No. 201 approximately 5 h before death on day 13 after infection. The activity in the sample (in two-fold dilution) was reduced to 11.2 and 26.5 U/ml in the neutralization test with specific and control antibodies, respectively, which confirmed its specificity. No other samples contained a detectable level of IL-1 activities.

Pre-infection levels of serum IL-6-like activity were <3.4 to 9.0 (mean±SD = 4.9±2.6) U/ml (Figure 5b). Elevated IL-6-like activities of 16.5 and 16.8 U/ml were detected in two samples taken from Lamb No. 201 approximately 5 h before death on day 13 and from Lamb No. 401 on day 9 after infection, respectively. Lamb No. 401 had serum IL-6-like activity of <3.4 U/ml at the time of death on day 11.

Discussion

The lambs percutaneously infected with high larval doses of the Himeji strain of *S. papillosus* developed sudden death in the intestinal phase of infection with high EPG values and large worm burdens in the small intestine, without significant premonitory symptoms and gross lesions which could possibly be responsible for their death. A few petechial hemorrhages beneath the epicardium found in some animals were most likely to occur at the time of sudden cardiac arrest, since such hemorrhages are common in instances of asphyxia or death in anoxia in sheep, cattle and horses (Robinson and Maxie, 1985). The petechial hemorrhages of the lungs were produced by migratory larvae. A portion of migratory larvae pass through the lungs to cause such hemorrhages in the early stage of percutaneous infection, but the injury is repaired after the disappearance of larvae from the lungs (Turner et al., 1960). This disease pattern was largely identical to that of calves experimentally infected with the same strain of the parasite in a previous study (Taira et al., 1992a). The relationship between the larval infection doses and survival times of lambs in the present study was almost the same as those in previous reports on experimentally infected lambs (Turner, 1955; Turner, 1959; Bezubik and Turner, 1964) and calves (Taira et al., 1992a). The minimum lethal dose for lambs in the present study was 3,200 larvae/kg, which was the same as that of the calves. A difference was noted in the survival rate of animals given this dose, since all three lambs developed sudden death while two of the five calves survived following infection.

The continuous ECG, pneumogram and video recordings performed on three of the lambs demonstrated that percutaneous heavy infection with *S. papillosus* also caused

sudden cardiac death in lambs. The sequence of arrhythmias and the course of behavior including accelerated respiration at the final moments in the lambs were identical to previous findings in infected calves (Tsuji et al., 1992; Ura et al., 1992; Ura et al., 1993b). The arrhythmias consisted of continuous sinus tachycardia and various ventricular arrhythmias including terminal VF. Without restoration to regular heart beats after VF, the sudden onset of attack was directly linked to death in infected lambs as in infected calves.

The present results showed that lambs offer almost the same efficiency of *S. papillosus* infection as calves. In comparison of death cases following experimental infection with up to 32,000 larvae/kg, no differences were established by Student's *t*-tests between lambs in the present study and calves in the previous study (Taira et al., 1992a) in terms of the maximum EPG values (mean±SD), which were 76±25 and 74±50 x 10^3, or the worm recovery rates (mean±SD) from the small intestine, which were 20.4±14.1% and 12.4±7.3%, respectively. Anorexia within 1 day before death and intestinal hyperemia at necropsy were also observed in some calves that developed sudden death (Taira et al., 1992a). The only major difference was concerned the change of body weight during infection. Lambs that died in the present study maintained or showed a reduction in their initial weights, resulting in a mean weight ±SD that was 96±7% of the starting weight, while calves that died in the previous study (Taira et al., 1992a) actually gained weight, with a mean weight ±SD that was 114±4% of the initial weights. These mean values were significantly different at $p = 0.00001$ by Student's *t*-test. These results, together with data on the survival time and ECG findings, showed that lambs were almost as sensitive as calves to *S. papillosus* infection, and followed the same pathophysiological

course of infection as calves, except for weight gain in the latter. The present study indicated that lambs could serve as a model to study sudden cardiac death-type strongyloidosis in calves. The appropriate dose for percutaneous infection was 3,200 to 10,000 infective larvae/kg body weight of lambs, with sudden cardiac death expected within 2 to 3 weeks after infection.

In sheep, the lungs represent the primary target organ, and respiratory failure, often unrelated to circulatory arrest, is an important feature in acute endotoxemia (Halmagyi et al., 1963). The toxic or shock-like states cause severe interstitial pneumonitis which can lead to acute respiratory distress (Dungworth, 1985). Endotoxin-exposed sheep also develop cardiovascular dysfunction due to myocardial inflammation (Seehase et al., 2011). Induction of inflammatory cytokines such as TNFα is a key event associated with the pathophysiology of affected animals (Perkowski et al., 1996; Sandor and Buc, 2005; Seehase et al., 2011). The sudden death following heavy infection with *S. papillosus* could certainly be distinguished from endotoxic shock or a shock-like state because of the normal respiration until cardiac arrest, the absence of significant lesions in the lungs, and the safe endotoxin levels in lambs that died as well as in calves (Nakanishi et al., 1993).

The present study did not find abnormal cytokine profiles in the circulation common to all the lambs that died, also suggesting that sudden death of strongyloidosis is not associated with a state of shock and endotoxemia. The serum TNFα activity transiently detected in Lamb No. 401 may have been incidentally induced by migratory larvae passing through the lungs. A portion of larvae migrate in the lungs of lambs to cause alveolar petechial hemorrhages mainly between 3 and 6 days following percutaneous infection (Turner et al., 1960). The period of TNFα induction in Lamb No. 401 could thus have

been a period of response against the large number of migratory larvae that were probably present in the lungs, since this animal received the highest larval dose. Lamb No. 201 showed detectable levels of serum IL-1 and IL-6-like activity on the day of death. It was likely that IL-1 was induced in progression of the disease rather than the fact that the disease was a result of IL-1 production, since the other animals that died had no IL-1 production. The increase in IL-6-like activity detected in Lambs No. 201 and No. 401 may have resulted from IL-1 and TNFα induction in the animals, respectively, since both cytokines stimulate IL-6 production (Titus et al., 1991). These results suggested that the pathophysiology of strongyloidosis in lambs is not associated with the circulatory induction of TNFα, IL-1, or IL-6. It has also been suggested that the wasting condition in rabbits heavily infected with *S. papillosus* has no association with cachexia induced by these inflammatory cytokines (Nakamura and Motokawa, 2000).

In this study, I revealed the course of heavy infection with *S. papillosus* in lambs and established an ovine model for sudden cardiac death-type strongyloidosis.

Tables and Figures

Table 1. Lambs used in the experiments on percutaneous infection with *Strongyloides papillosus.*

Lamb No.	Sex [1)]	Age (month)	Body weight (kg)	Infection dose per kg body weight	Larvae not infected [2)]	Larvae not infected (%) [3)]
101	F	4	22.0	1,000	275	1.3
102	F	9	18.0	1,000	NC	NC
201	M	5	21.2	3,200	NC	NC
202	M	5	27.3	3,200	413	0.5
203	M	4	23.0	3,200	9,048	12.3
301	F	5	19.5	10,000	NC	NC
302	M	4	28.0	10,000	1,100	0.4
303	M	4	23.5	10,000	1,306	0.6
304	F	9	18.0	10,000	NC	NC
401	M	4	13.0	32,000	12,350	3.0
402	F	5	22.0	32,000	NC	NC
403	M	4	24.0	32,000	32,038	4.2
404	F	4	22.4	32,000	3,713	0.5

[1)] M: male; F: female.

[2)] Larvae that remained in the cotton wool pads after exposure. NC: not counted.

[3)] Calculated as (larvae not infected) / ((infection dose per kg body weight) x (body weight)) x 100. NC: not calculated.

Table 2. Survival time and body weight at necropsy following percutaneous infection with *Strongyloides papillosus*.

Lamb No. [1]	Survival time (days)	Body weight at necropsy		
		Body weight (kg)	Weight gain (kg) [3]	Weight gain (%) [4]
101	42 [2]	21.8	-0.2	99
102	42 [2]	20.2	2.2	112
201	13	21.8	0.6	103
202	15	23.8	-3.5	87
203	20	22.0	-1.0	96
301	14	19.4	-0.1	99
302	13	27.2	-0.8	97
303	18	20.6	-2.9	88
304	15	18.0	0.0	100
401	11	13.4	0.4	103
402	11	22.5	0.5	102
403	14	22.0	-2.0	92
404	15	18.9	-3.5	84

[1] See Table 1 for infection dose.

[2] Killed.

[3] Calculated as (body weight at necropsy) - (body weight at the time of infection shown in Table 1). Negative values indicate weight loss during the experimental period.

[4] Calculated as (body weight at necropsy) / (body weight at the time of infection shown in Table 1) x 100. The mean weight gain ± standard deviation of the animals that died (Lambs Nos. 201 to 404) was 96 ± 7%.

Table 3. Fecal egg output and worm recovery from the small intestine following percutaneous infection with *Strongyloides papillosus*.

Lamb No. [1]	Prepatent period [2] (days)	Maximum egg output		Worm recovery	
		Eggs per gram of feces [3]	Day of maximum count [4]	Parasitic females recovered [5]	Worm recovery (%) [6]
101	10	19,800 [a]	16	1,094	5.0
102	11	10,600 [a]	25	0	0
201	10	95,800 [b]	13	NC	NC
202	10	86,700 [b]	15	15,844	18.2
203	10	49,400 [b]	19	3,438	5.3
301	10	127,000 [b]	14	91,813	47.1
302	10	43,000 [b]	13	52,688	18.9
303	11	65,800 [a]	17	27,281	11.7
304	11	69,400 [b]	15	NC	NC
401	10	75,400 [b]	11	158,738	39.3
402	9	46,600 [b]	11	121,188	17.2
403	10	87,700 [b]	14	143,875	19.5
404	11	85,200 [a]	12	43,875	6.2

[1] See Tables 1 and 2 for infection dose and survival time, respectively.

[2] Number of days from infection until the first eggs were detected.

[3] The mean maximum egg count ± standard deviation (SD) of the animals that died (Lambs Nos. 201 to 404) was 75,636 ± 24,772. a: Detected in rectal feces; b: Detected in cecum feces.

[4] Number of days from infection until the maximum count was obtained.

[5] NC: not counted.

[6] Calculated as (number of parasitic females recovered) / (number of larvae infected) x 100. The number of infected larvae was calculated as follows: (infection dose per kg body weight) x (body weight at the time of infection) - (number of larvae not infected), shown in Table 1. The mean worm recovery ± SD of the animals that died was 20.4 ± 14.1%. NC: not calculated.

Table 4. Electrocardiogram findings following percutaneous infection with *Strongyloides papillosus.*

Lamb No. [1)]	Occurrence of arrhythmia [2)]		
	Sinus tachycardia	Other arrhythmias	Final pattern
201	8 days pi ~ until death	VT (7 h bd ~)	VT → VF
301	9 days pi ~ until death	VPB (6 h bd ~) VT (6 h bd ~) 2nd AVB (5 h bd ~)	VPB → VF
401	6 days pi ~ until death	ST depression (9 h bd ~)	VPB → VF

[1)] See Tables 1 and 2 for infection dose and survival time, respectively.

[2)] pi: post-infection; bd: before death; 2nd AVB: second degree atrioventricular block; ST: the ST segment of electrocardiogram; VF: ventricular fibrillation; VPB: ventricular premature beat; VT: ventricular tachycardia.

Table 5. Gross lesions observed at necropsy.

Lamb No. [1)]	Petechial hemorrhages in the lungs	Petechial hemorrhages on the adipose tissue beneath the epicardium	Congestion of the mucous membranes of the duodenum and jejunum	Enlarged gall bladder
101	+	-	-	-
102	-	-	-	-
201	-	-	+	+
202	+	-	-	-
203	+	+	-	-
301	+	-	-	+
302	+	+	-	-
303	-	-	-	-
304	+	-	+	-
401	+	-	+	+
402	+	+	+	-
403	+	+	-	+
404	+	-	+	-

[1)] See Tables 1 and 2 for infection dose and survival time, respectively.

+ : Observed; - : Not observed.

Table 6. Number of migratory larvae recovered from tissues at necropsy.

Lamb No. [1]	Tissue [2]									
	a	**b**	**c**	**d**	**e**	**f**	**g**	**h**	**i**	**j**
101	0	NC	NC	NC	NC	NC	0	NC	NC	NC
102	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
201	15	0	0	1	0	0	0	0	0	0
202	0	1	0	0	0	0	0	0	0	0
203	0	0	0	0	0	0	0	0	0	0
301	11	0	0	0	1	0	1	0	0	0
302	3	2	0	0	0	0	0	0	0	0
303	0	0	1	0	0	0	0	0	0	0
304	NC	NC	NC	NC	NC	NC	NC	0	NC	NC
401	7	12	0	0	0	NC	5	NC	0	0
402	25	12	0	0	1	0	5	0	0	0
403	486	14	1	0	0	0	5	0	0	0
404	249	8	0	0	0	0	7	0	0	0

[1] See Tables 1 and 2 for infection dose and survival time, respectively.

[2] **a**: Connective tissue of the coronary area of the forelegs (10 g each from left and right); **b**: triceps brachii muscles (10 g each from left and right); **c**: abdominal muscle (10 g); **d**: mandibular muscle (10 g); **e**: tissue around eye orbit (10 g); **f**: brain (10 g); **g**: lungs (10 g); **h**: myocardium (10 g); **i**: liver (10 g); **j**: kidneys (10 g). NC: not counted.

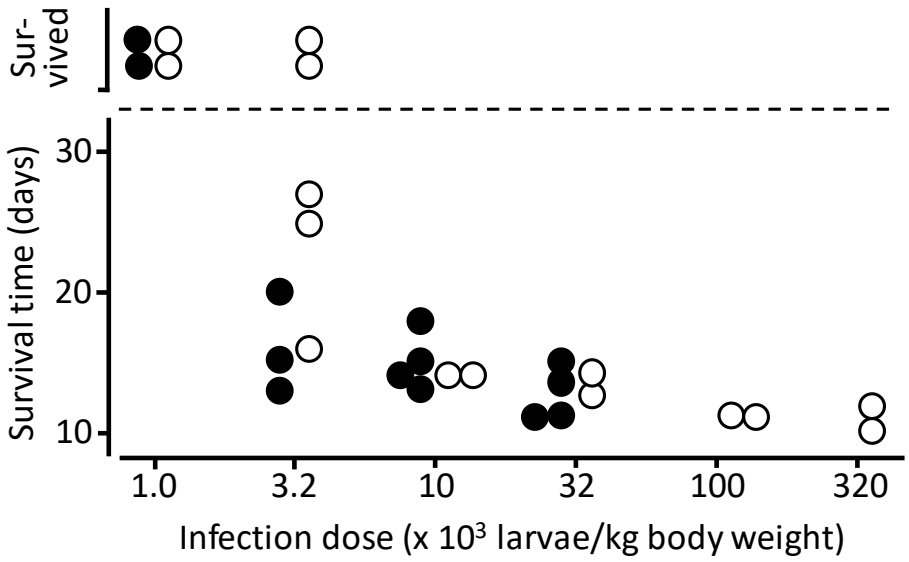

Figure 2. Survival time of animals following percutaneous infection with infective larvae of *Strongyloides papillosus*. Solid and open circles indicate lambs in the present study and calves in the previous study (Taira et al., 1992a), respectively. Lambs and calves that did not die by 42 and 34 days after infection, respectively, were considered to have survived. Note that no lambs were given the doses of 100,000 and 320,000 larvae per kg body weight.

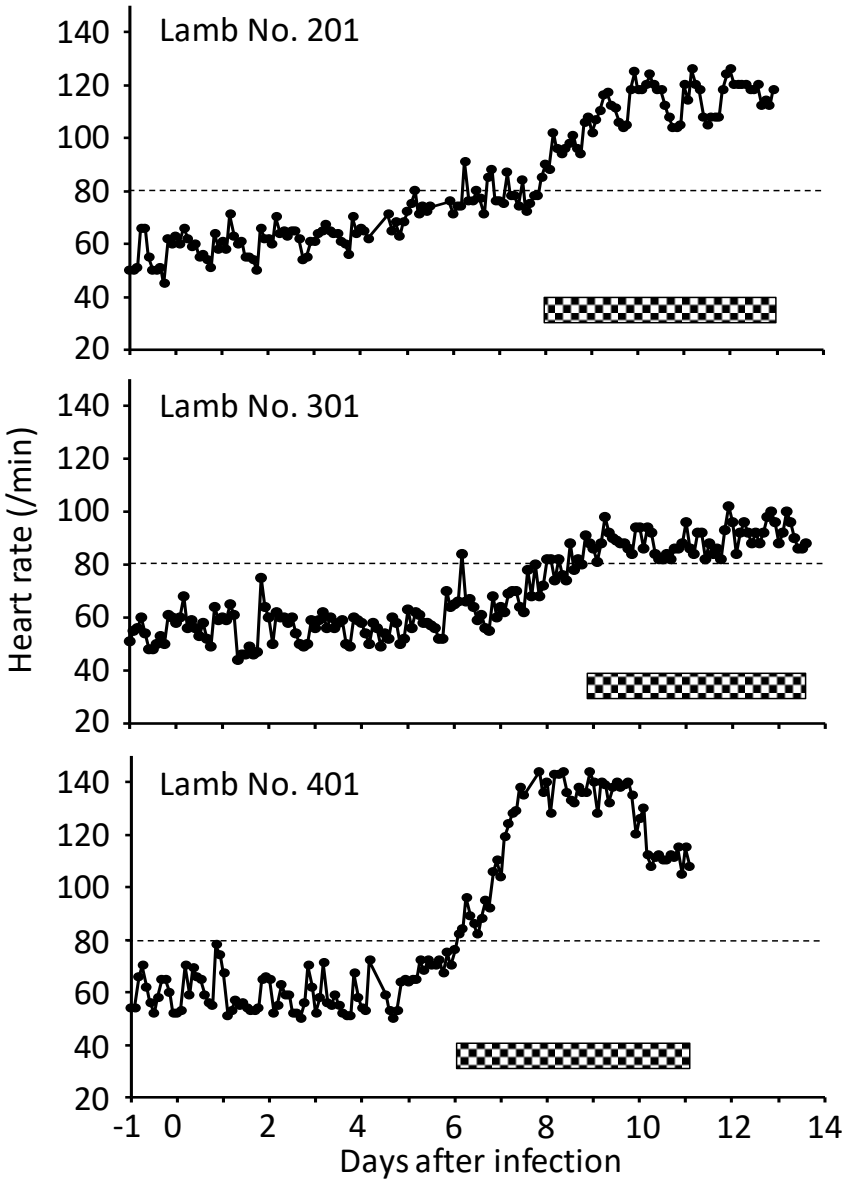

Figure 3. Alterations of heart rate in Lambs Nos. 201, 301 and 401 following percutaneous infection with 3,200, 10,000 and 32,000 infective larvae of *Strongyloides papillosus* per kg body weight, respectively. A heart rate of more than 80/min (dotted line) was considered tachycardia. Tachycardia that lasted over 12 h was considered continuous (checkered bar).

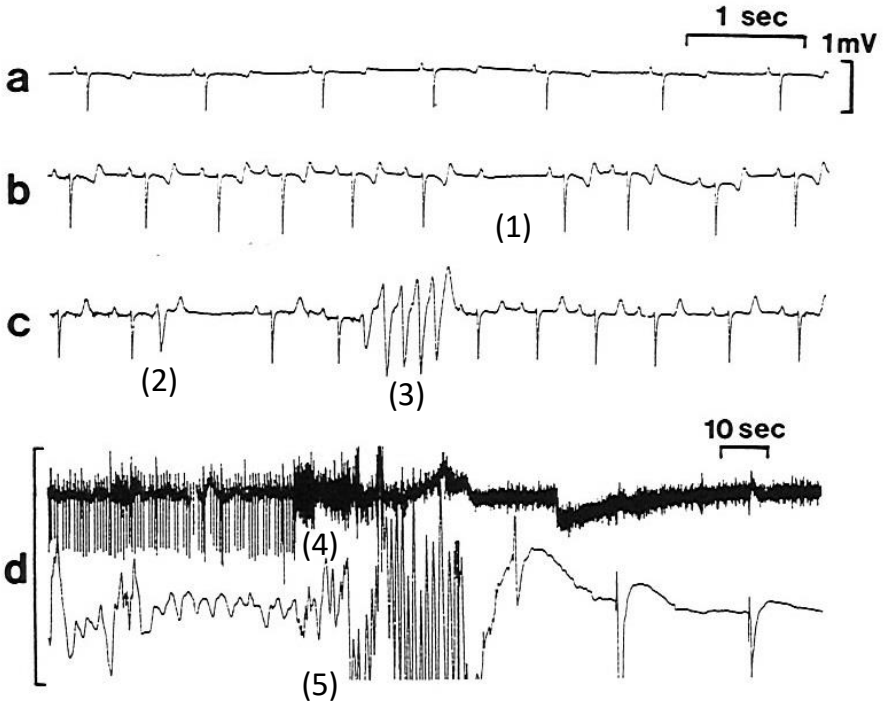

Figure 4. Electrocardiogram and pneumogram changes in Lamb No. 301 following percutaneous infection with 10,000 infective larvae of *Strongyloides papillosus* per kg body weight. **a**: Normal cardiac rhythm before infection; **b**: (1) second degree atrioventricular block among patterns of sinus tachycardia 4 h before death; **c**: (2) ventricular premature beat and (3) paroxysmal ventricular tachycardia among patterns of sinus tachycardia 4 h before death; **d**: (4) onset of ventricular fibrillation (upper strip) and (5) transient accelerated respiration (lower strip) at the time of death on day 14 after infection.

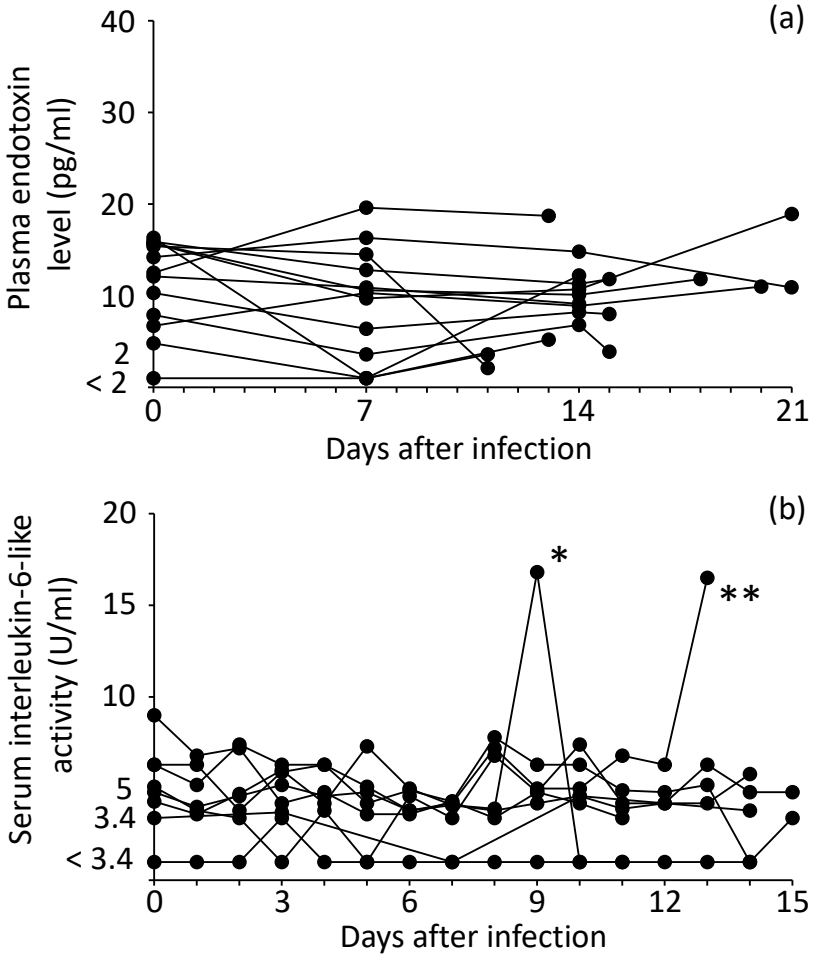

Figure 5. Plasma endotoxin level (a) and serum interleukin-6 (IL-6)-like activity (b) following percutaneous infection with *Strongyloides papillosus*. No elevated endotoxin levels were detected in any plasma samples taken from all 13 animals throughout the experiment. Serum IL-6-like activities were determined on Lambs Nos. 102, 201, 301, 304, and 401 to 404. Elevated IL-6-like activities were detected in two samples.

*: Lamb No. 401, 16.8 pg/ml, on day 9 after infection. **: Lamb No. 201, 16.5 pg/ml, 5 h before death on day 13 after infection.

Chapter 2

Parasitic Females of *Strongyloides papillosus* as a Pathogenetic Stage Responsible for Sudden Cardiac Death

Summary

Heavy percutaneous infection of calves and lambs with *Strongyloides papillosus* causes continuous sinus tachycardia and final sudden cardiac arrest by ventricular fibrillation (VF) in the intestinal phase of infection. The parasite has a unique life cycle consisting of larval migration following infection and maturation into parasitic females in the small intestine. The present study was aimed at elucidating the responsibility of parasitic females for the disease using lambs. Parasitic females were collected from the small intestine of experimentally infected donor rabbits. Three recipient lambs were intraduodenally inoculated with 2,500, 10,000 and 22,500 live worms per kg body weight (60,000, 170,000 and 270,000 worms per animal, respectively) in sterile Hanks' solution. Two animals were inoculated with the homogenate prepared with 300,000 worms in Hanks' solution. One additional control animal was injected with Hanks' solution only. Electrocardiogram, pneumogram, and video recordings were continuously carried out. The control animal and the animals inoculated with homogenized worms showed a transient sinus tachycardia over a short span immediately after inoculation. Thereafter the animals showed no abnormalities and arrhythmias during the experimental period. The animals inoculated with live worms died of sudden cardiac death between 2 and 9 days after inoculation, showing a longer survival time due to their inoculation with a lower dose of parasitic females. The animals developed continuous sinus tachycardia that began shortly after inoculation until the onset of VF. No significant clinical signs or abnormalities were observed until the time of death. The animals had high egg output with maximum values of more than 40,000 eggs per gram of feces. At necropsy, parasitic

females which had a mean recovery rate of 25% against the inoculation dose were recovered from the small intestine. The disease course was identical to that observed in the intestinal phase of percutaneous larval infection. These results demonstrated that active parasitic females of *S. papillosus* in the small intestine are responsible for the cardiac disorders in sudden cardiac death-type strongyloidosis regardless of the presence or absence of migratory larvae.

Introduction

Among gastrointestinal nematodes, *Strongyloides papillosus* has a unique life cycle in host animals. After hatching and developing into infective larvae, the parasite invades its host by skin penetration to migrate in the body, and then matures into parasitic females to produce eggs in parthenogenesis in the small intestine (Basir, 1950; Mehlhorn, 2016). The parasite takes the essential route of migration from the pharynx to the alimentary tract (Basir, 1950). It remains unclear whether *S. papillosus* larvae enter a blood vessel to migrate up to the lungs via the circulation in the early stage of infection in the manner of *S. stercoralis* in humans (Schad, 1989). Turner et al. (1960) reported that *S. papillosus* larvae were recovered from the jugular, mesenteric, and hepatic portal veins of sheep between 12 h and 72 h after percutaneous infection but failed to find any larvae in tissues of the heart, liver, kidneys, and spleen throughout the course of infection. Nwaorgu and Connan (1980) reported that almost no larvae were recovered from the circulation of rabbits between 30 min and 192 h after infection.

In calves, it has been experimentally demonstrated that a heavy infection with *S. papillosus* causes continuous sinus tachycardia and final sudden cardiac arrest by ventricular fibrillation (VF) (Taira et al., 1992a; Tsuji et al., 1992). In lambs, I previously showed that heavy infection with the parasite similarly results in sudden cardiac death as described in Chapter 1. All cases of sudden death due to *S. papillosus* infection in calves (Taira and Ura, 1991a; Taira and Ura, 1991b; Taira et al., 1992a) and in lambs (see Chapter 1), irrespective of whether the infections were natural or experimental, occurred after the prepatent period of the parasite, when nearly all larvae had reached the small intestine and

matured to lay eggs. At the time of death, all animals had high fecal egg output and high intestinal worm burdens of the parasite. Parasitic females would thus appear to be the cause of sudden death. However, the developmental stage of the parasite responsible for the fatal arrhythmias in infected animals, whether migratory larvae or parasitic females, has not yet been ascertained. When a portion of the migratory larvae pass through the lungs, the possibility of a few larvae straying into the tissue of the heart cannot be completely excluded as a cause of the cardiac dysfunction. The top priority among studies elucidating the pathophysiology of sudden cardiac death-type strongyloidosis is thus to clarify the developmental stage of the parasite responsible for the disease.

The purpose of this Chapter was to identify the developmental stage of *S. papillosus* responsible for sudden cardiac death-type strongyloidosis. I hypothesized that parasitic females in the small intestine would be responsible for the series of arrhythmias in the disease of calves and lambs. To verify this hypothesis, I performed an experiment in which the live and homogenized parasitic females of *S. papillosus* recovered from rabbit infections were directly inoculated into the duodenum of susceptible lambs. Once they reach the intestines, parasitic females never migrate back to any other locations inside a host (Basir, 1950; Turner et al., 1960). Autoinfection is impossible in *S. papillosus* because the eggs laid by parasitic females never hatch to larvae inside the intestines of a host (Speare, 1989). Therefore, there are absolutely no larvae in the bodies of lambs inoculated with parasitic females into the duodenum. I investigated the course of intraduodenal inoculation with parasitic females in lambs, by monitoring electrocardiograms (ECG) and pneumograms.

Materials and Methods

Animals

Six helminth-free male Suffolk lambs were employed in this study. Their ages and body weights are listed in Table 7. They were fed a diet of hay and concentrate twice a day. Water was provided *ad libitum*. The animals were loosely restrained by an individual neck stanchion in a recording room as described in Chapter 1. Specific pathogen-free male Japanese-White rabbits weighing 2-3 kg were used for the passage of *S. papillosus* and for the collection of parasitic females from the small intestine. They were kept separately in stainless steel wire cages with a drainboard and a feces tray. Standard pelleted food and water were provided *ad libitum*. All experiments using animals were approved by the Ethics Committee of the National Institute of Animal Health in Japan. Based on the results described in Chapter 1, eventual occurrence of sudden cardiac death would be predicted if lambs developed continuous sinus tachycardia. In these experiments, however, such animals were neither treated nor euthanized in order to confirm the entire course of the disease. Animals showed almost no significant symptoms and abnormal behavior until the final onset of VF, suggesting they experienced little or no pain and suffering throughout the experiment.

Parasitic females

The Himeji strain of *S. papillosus* (Taira et al., 1991; Taira et al., 1992a) was used in this study. Rabbits were percutaneously infected with 500,000 infective larvae each as previously described (Nakamura et al., 1994). The rabbits were fasted on day 9 and

killed on day 10 after infection by injection with sodium pentobarbital (Somnopentyl, 200 mg/kg; Kyoritsu Seiyaku, Tokyo, Japan). The small intestine was removed, slit longitudinally and cut into several sections. After being rinsed with sterile saline, the intestinal sections were incubated on a 16-mesh sieve in sterile Hanks' solution (pH 7.4; Nissui Pharmaceutical, Tokyo, Japan) containing 200 units/ml penicillin and 1 mg/ml streptomycin (Sigma-Aldrich Japan, Tokyo, Japan) at 37 ºC for 3 h in a humidified 5% CO_2 incubator. Parasitic females of *S. papillosus* which passed through the sieve were collected and washed with Hanks' solution by natural sedimentation and aspiration of the supernatant, then suspended again in fresh Hanks' solution. The numbers of motile parasitic females in 1.0 ml of suspension were counted eight times to confirm the worm density of the suspension. At the time of collection, more than 95% of the worms were alive and motile. For live worm inoculation, the worms were kept in fresh Hanks' solution at 37 ºC. For homogenized worm inoculation, the worms were disrupted in Hanks' solution containing 1 mM phenylmethylsulfonylfluoride (PMSF; Sigma-Aldrich Japan) with a glass homogenizer on ice. The resultant suspension (not filtered for sterilization) was added to 24 volumes of Hanks' solution without PMSF and kept at 37 ºC. Both live and homogenized worms were inoculated into the duodenum of lambs as described below within 1 h of preparation.

Intraduodenal inoculation of parasitic females into lambs

Lambs were fasted on the day before and the day of surgery. The animals were anesthetized with xylazine (Serakutal 2% Injection, 0.2 mg/kg; Bayer Yakuhin, Osaka, Japan) and placed in left lateral recumbency. An incision 10 cm long and 4 cm away

from the last rib was made on the right abdominal wall of the lambs under a local anesthesia with procaine (Omnicain 2% Injection, 0.2g/animal; Sankyo Seiyaku (presently Daiichi-Sankyo), Tokyo, Japan). Using a glass syringe with a rubber catheter that was approximately 10 cm long and connected to a 1.5 mm inside diameter needle, 2,500, 10,000 and 22,500 live worms per kg body weight (60,000, 170,000 and 270,000 worms per animal, respectively) in Hanks' solution were gently inoculated into the duodenum 3 cm posterior to the pylorus of three animals (Lambs Nos. 502, 503 and 504, respectively). The inoculation volumes were 60, 90 and 150 ml, respectively, and were easily injectable without clogging the needle with worms. Two animals (Lambs No. 505 and No. 506) were inoculated with the worm homogenate (300,000 worms per animal equivalent) in 60 ml of Hanks' solution, and one animal (Lamb No. 501) that served as a control was injected with 150 ml of Hanks' solution only, into the duodenum by the same method used for live worm inoculation. The surgery was completed by suturing the abdominal wall and administering intramuscularly an injection of penicillin and streptomycin (Mycillin Sol Meiji, 5 ml/animal; Meiji Seika, Tokyo, Japan). The lambs regained consciousness within 30 min of the surgery.

Electrocardiogram, pneumogram, and video recordings

Continuous ECG, pneumogram, and video recordings were carried out on all the animals from 3 days before inoculation as described in Chapter 1. The rectal temperature of the animals was monitored daily. A heart rate of more than 80/min was considered tachycardia (Detweiler, 1993). Tachycardia that lasted over 12 h was arbitrarily considered continuous.

Fecal egg counts

The fecal examination of rectal samples collected from all the animals but Lamb No. 501 was carried out daily to determine the number of eggs per gram of feces (EPG) as described in Chapter 1. At necropsy, fecal egg counts were also determined in cecum contents collected from all the animals but Lamb No. 501.

Plasma endotoxin levels

Jugular blood samples were collected into heparinized tubes weekly from all the animals and were also taken within a few minutes of death in the case of animals that died. Plasma endotoxin levels were determined as described in Chapter 1.

Necropsy

Lambs that died were necropsied within 3 h of death. Surviving animals were killed on day 14 or on day 21 after inoculation by bleeding under anesthesia with xylazine (10 mg/animal). The animals were weighed and their organs were examined for gross lesions. Worms in the small intestine were recovered and counted as described in Chapter 1. Migratory larvae were recovered from 10 g of each of the brain, lungs, and heart (myocardium) of the animals inoculated with live parasitic females, by a modified Baermann technique as previously described (Tsuji et al., 1991).

Results

Fate and clinical findings of the lambs

The control animal (Lamb No. 501) injected with Hanks's solution, and the two animals (Lambs No. 505 and No. 506) inoculated with homogenized parasitic females into the duodenum, did not show any abnormalities for 21 and 14 days, respectively, except for a decrease in daily fecal amount on the day of surgery.

The three animals (Lambs Nos. 502, 503 and 504) inoculated with live parasitic females into the duodenum died on days 9, 7 and 2 after inoculation, respectively (Table 8). The lower the inoculation dose given to an animal was, the longer was the survival time of the animal. The ECG and video recordings confirmed that the three animals developed sudden cardiac death as described below. Clinical signs observed up to the time of death were as follows: a higher rectal temperature by 0.5 ºC to 1.0 ºC than usual in Lamb No. 502 from day 2 until the day of death and in Lamb No. 503 transiently on days 1 to 3 after inoculation; anorexia in Lamb No. 504 on the day of death; and a decrease in daily fecal amount in Lambs No. 502 and No. 504 on the day of death. In addition, a decrease in daily fecal amount was observed in all three animals on the day of surgery. No diarrhea was observed in the three animals throughout the experiment. The final body weights of the three animals were 93% to 106% of their initial weights (Table 8). Lamb No. 502 had a weight loss of 1.7 kg over the longest survival time, while Lamb No. 504 had a weight gain of 0.7 kg over the shortest survival time among the experimental animals.

Fecal egg output

The first eggs were detected in rectal feces on day 2 after inoculation in Lambs No. 502 and No. 503 inoculated with live parasitic females (Table 9). Lamb No. 504 had no egg output in rectal feces until day 2 after inoculation when the animal died. The three animals had maximum EPG values of more than 40,000, with the values of Lambs No. 503 and No. 504 being detected in their cecum contents at necropsy.

Electrocardiogram, pneumogram, and video findings

The ECG findings and the alterations of heart rate observed in the six lambs are summarized in Table 10 and shown in Figures 6a and 6b, respectively. The ECG and pneumogram recordings of Lamb No. 502 are shown in Figure 7. Immediately after inoculation with control solution or homogenized worms, Lambs Nos. 501, 505 and 506 showed a transient sinus tachycardia with a maximum rate of 96-150/min over a short span of 10-24 min. Thereafter the animals showed no arrhythmias. No abnormal respiration and behavior were recorded on pneumograms and videotape recordings.

The sequence of ECG and respiratory changes seen in Lambs Nos. 502, 503 and 504 after inoculation with live parasitic females were identical to that observed in the fatal cases of percutaneous larval infection described in Chapter 1. The three animals developed continuous sinus tachycardia with a maximum rate of 120-160/min starting from 5 min to 1 h after inoculation and continuing until the onset of terminal VF. Various arrhythmias appeared sporadically among patterns of sinus tachycardia, and final ventricular tachycardia or ventricular premature beat was followed by VF.

Videotape recordings of the three animals inoculated with live parasitic females

confirmed that there were no abnormal behaviors until the critical moments of death, with the exception that Lamb No. 504 left all food on the day of death. Normal rumination in a sitting position was recorded in Lamb No. 503 until just before death.

Plasma endotoxin levels

Plasma endotoxin levels before inoculation were 8.2 to 14.8 (mean ±standard deviation = 11.7±2.3) pg/ml. The levels remained below 19.0 pg/ml, a value considered normal in the present assay, in all six animals throughout the experiment.

Necropsy findings

At necropsy, no inflammatory changes were observed on or near the surgery site in all six animals. No gross lesions were observed in the control animal (Lamb No. 501) or the animals inoculated with homogenized parasitic females (Lambs No. 505 and No. 506). Among the animals inoculated with live parasitic females, Lambs No. 502 and No. 504 had hyperemia in the mucous membranes of the duodenum and jejunum and a few petechial hemorrhages on the adipose tissue beneath the epicardium. No gross lesions were observed in the other organs of the two animals or in any organs of Lamb No. 503.

Parasitic females were recovered from the small intestine of the three animals inoculated with live parasitic females, and ranged in number from 19, 219 to 81,875 (Table 9). The recovery rates were 12.9% -32.0% (mean 25.1%) against the inoculation dose of live parasitic females. No larvae were recovered from the brain, lungs, or myocardium of the three animals. Infective larvae, which were cultured from the eggs in the cecum content of Lamb No. 503, produced a patent infection in percutaneously infected rabbits

in a normal fashion.

Discussion

Live parasitic females of *S. papillosus* caused sudden cardiac death in lambs, following direct inoculation into the duodenum. The three lambs which were thus inoculated died of the sudden onset of VF in the final moments, preceded by a period of continuous sinus tachycardia that began shortly after inoculation with live parasitic females. The course of the disease was identical to that observed in the intestinal phase of percutaneous infection with infective larvae. Parasitic females were active and maintained their ability to lay eggs after transplantation from the small intestine of donor rabbits to the duodenum of a recipient lamb. The eggs produced by the transplanted parasitic females successfully hatched to develop into infective larvae, which established an infection when percutaneously exposed to rabbits as a new host animal. It was strongly suggested that the death of the lambs was unrelated to secondary microbial infections through surgical worm inoculation, since the two animals intraduodenally inoculated with homogenized worms, which had not been sterilized, survived with no abnormalities except for transient sinus tachycardia just after inoculation. This idea was also supported by the absence of significant increase in plasma endotoxin levels in any animals. The transient sinus tachycardia just after inoculation of homogenized worms might be induced by a normal reflex against the volume of Hanks' solution injected into the duodenum, since it was observed even in the control animal injected with Hanks' solution only.

For the reasons described in the Introduction, the adult worms were present only in the intestine of the administered animals, and there were absolutely no larvae at any other

locations in the body. Therefore, the animals were never affected by any unfavorable effects, if any, associated with the invasion or migration of larvae, such as secondary infections, physical damage or chemical stimulation, and host responses to larvae. The present results clearly demonstrated that active parasitic females in the small intestine cause sudden cardiac death in lambs regardless of the presence or absence of migratory larvae. Components that make up the cytoskeleton of parasitic females are probably excluded from causative substance(s) for the disease, since the inoculation of homogenized worms failed to produce cardiac arrest in lambs. The following are potential factors which are probably responsible for the cardiac dysfunctions in *S. papillosus* infection: (1) mechanical stimuli by parasitic females which provoke intestinal reflexes, resulting in some effects on the cardioregulatory system; (2) products of parasitic females which act directly or indirectly upon the heart or upper regulatory systems. Active parasitic females are considered to release various kinds of secretory substances in the small intestine. Such substances may include a cardioactive compound or its precursor.

Sinus tachycardia began before worms started laying eggs in the cases of percutaneous larval infection, from day 5 in two calves (Tsuji et al., 1992) and from day 6 in Lamb No. 401 (see Chapter 1). Migratory larvae are first detected in the small intestine of infected lambs at 88 h after percutaneous infection, and thereafter the number of the intestinal worms increases (Turner et al., 1960). Thus, migratory larvae in somatic tissues and/or immature worms in the small intestine could still be suspicious stages associated with the cardiac dysfunctions in infected calves and lambs. However, research targets should be focused on parasitic females to elucidate the developmental mechanism of sudden cardiac

death-type strongyloidosis.

In this study, I proved the hypothesis that parasitic females of *S. papillosus* in the small intestine are the developmental stage responsible for sudden cardiac death-type strongyloidosis.

Tables and Figures

Table 7. Lambs used in the experiments on intraduodenal inoculation with *Strongyloides papillosus.*

Lamb No. [1)]	Age (month)	Body weight (kg)	Inoculum [2)]	Inoculation dose (x 10^3)	
				per kg body weight	per animal
501	9	23.0	control	-	-
502	6	23.8	LPF	2.5	60
503	9	17.3	LPF	10.0	170
504	4	12.0	LPF	22.5	270
505	9	22.8	HPF	13.2	300
506	9	19.0	HPF	15.8	300

[1)] All animals were male.

[2)] control: Hanks' solution (vehicle) only; LPF: live parasitic females; HPF: homogenized parasitic females.

Table 8. Survival time and body weight at necropsy following intraduodenal inoculation with *Strongyloides papillosus.*

Lamb No. [1]	Survival time (days)	Body weight at necropsy		
		Body weight (kg)	Weight gain (kg) [3]	Weight gain (%) [4]
501	21 [2]	26.8	3.8	117
502	9	22.1	-1.7	93
503	7	16.7	-0.6	97
504	2	12.7	0.7	106
505	14 [2]	25.6	2.8	112
506	14 [2]	21.6	2.6	114

[1] See Table 7 for inoculum.

[2] Killed.

[3] Calculated as (body weight at necropsy) - (body weight at the time of inoculation shown in Table 7). Negative values indicate weight loss during the experimental period.

[4] Calculated as (body weight at necropsy) / (body weight at the time of inoculation shown in Table 7) x 100.

Table 9. Fecal egg output and worm recovery from the small intestine following intraduodenal inoculation with *Strongyloides papillosus.*

Lamb No. [1]	Fecal egg output			Worm recovery	
	Day of first detection [2]	Maximum count per gram of feces [3]	Day of maximum count [4]	Parasitic females recovered	Worm recovery (%) [5]
501	Not tested	Not tested	Not tested	Not tested	Not tested
502	2	41,600 [a]	8	19,219	32.0
503	2	57,800 [b]	7	21,969	12.9
504	ND	155,000 [b]	2	81,875	30.3
505	ND	0	-	0	-
506	ND	0	-	0	-

[1] See Tables 7 and 8 for inoculum and survival time, respectively.

[2] Number of days from inoculation until the first eggs were detected in rectal feces. ND: not detected. Lamb No. 504 had no egg output in rectal feces but had eggs in cecum feces at necropsy on day 2 after inoculation.

[3] a: Detected in rectal feces; b: Detected in cecum feces.

[4] Number of days from inoculation until the maximum count was obtained.

[5] Calculated as (number of parasitic females recovered) / (number of parasitic females inoculated per animal shown in Table 7) x 100.

Table 10. Electrocardiogram findings following intraduodenal inoculation with *Strongyloides papillosus.*

Lamb No. [1)]	Occurrence of arrhythmia [2)]		
	Sinus tachycardia	Other arrhythmias	Final pattern
501	9~33 min pi only	Not observed	Normal
502	1 h pi ~ until death	VT (5 days pi ~) 2nd AVB (6 days pi ~) VPB (6 days pi ~)	VT → VF
503	6 min pi ~ until death	2nd AVB (9 h pi ~) VPB (20 h pi ~)	VT → VF
504	5 min pi ~ until death	ST depression (18 h bd ~) VT (16 h bd ~) 2nd AVB (15 h bd ~) VPB (10 h bd ~)	VPB → VF
505	6~16 min pi only	Not observed	Normal
506	8~25 min pi only	Not observed	Normal

[1)] See Tables 7 and 8 for inoculum and survival time, respectively.

[2)] pi: post-inoculation; bd: before death; 2nd AVB: second degree atrioventricular block; ST: the ST segment of electrocardiogram; VF: ventricular fibrillation; VPB: ventricular premature beat; VT: ventricular tachycardia.

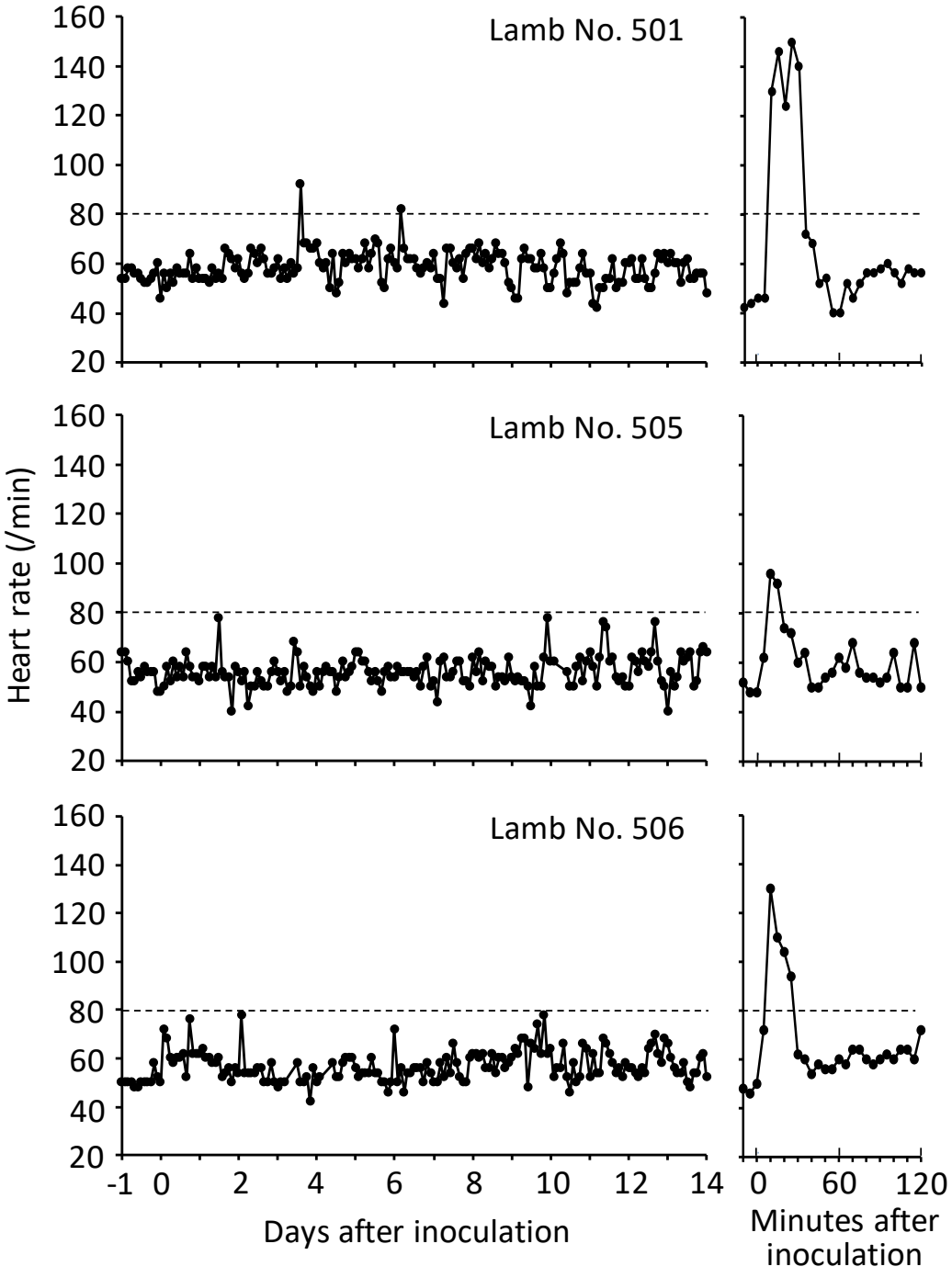

Figure 6a. Alterations of heart rate following intraduodenal inoculation. Lamb No. 501 was inoculated with Hanks' solution (control). Lambs No. 505 and No. 506 were inoculated with homogenized parasitic females of *Strongyloides papillosus* (300,000

worms per animal equivalent). A heart rate of more than 80/min (dotted line) was considered tachycardia. Left panels show heart rate plotted every 2 h from 1 day before to 14 days after inoculation. Right panels show heart rate plotted every 5 min from 10 min before to 120 min after inoculation.

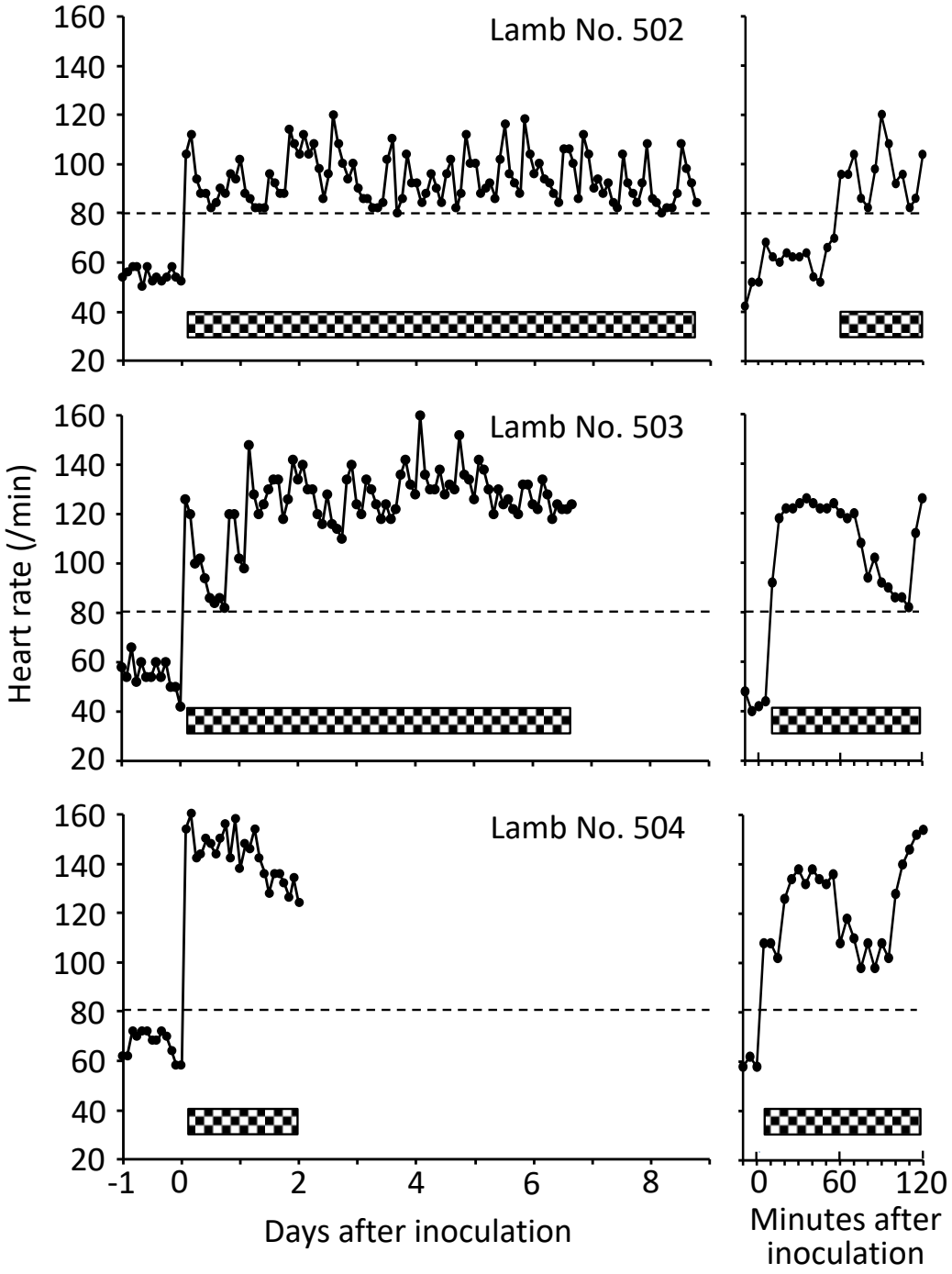

Figure 6b. Alterations of heart rate following intraduodenal inoculation. Lambs Nos. 502, 503 and 504 were inoculated with 60,000, 170,000 and 270,000 live parasitic females of *Strongyloides papillosus* per animal, respectively. A heart rate of more than 80/min

(dotted line) was considered tachycardia. Tachycardia that lasted over 12 h was considered continuous (checkered bar). Left panels show heart rate plotted every 2 h from 1 day before inoculation to the time of death. Right panels show heart rate plotted every 5 min from 10 min before to 120 min after inoculation.

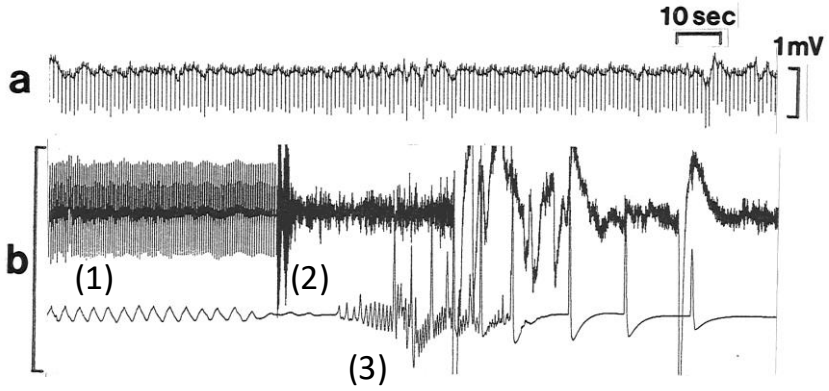

Figure 7. Electrocardiogram and pneumogram changes in Lamb No. 502 following intraduodenal inoculation with 60,000 parasitic females of *Strongyloides papillosus*.

a: Normal cardiac rhythm before inoculation; **b**: (1) continuous sinus tachycardia, (2) onset of ventricular fibrillation (upper strip) and (3) transient accelerated respiration (lower strip) at the time of death on day 9 after inoculation.

Chapter 3

Recovery from Arrhythmias in Lambs Infected with *Strongyloides papillosus* Following Worm Elimination

Summary

Calves and lambs heavily infected with *Strongyloides papillosus* develop continuous sinus tachycardia and finally die of sudden cardiac arrest by ventricular fibrillation (VF). In the present study, cardiac rhythms following anthelmintic treatment were investigated in lambs infected with *S. papillosus* to ascertain whether the cardiac disorders due to the infection are based on a reversible and curable change. Eight lambs were percutaneously infected with a lethal dose of *S. papillosus*. Five of the animals were injected with ivermectin when they developed continuous sinus tachycardia accompanied by prolongation of the PQ interval (the portion of the electrocardiogram between the P wave and the QRS complex), between 240 h (day 10.0) and 323 h (day 13.5) after infection. The other three animals served as untreated controls. In the treated animals, the elevated heart rates and PQ intervals began to decrease between 10 h and 21 h, then returned to normal levels within 39 h of treatment. No arrhythmias were detected after the disappearance of sinus tachycardia. Fecal egg counts became negative within 61 h of treatment. At necropsy, only a few worms were recovered from the small intestine. The control animals developed VF by 349 h (day 14.5) after infection, having high fecal egg counts and intestinal worm burdens. The absence of any effects of ivermectin upon cardiac rhythms was confirmed in an additional uninfected animal given ivermectin. These results indicated that the cardiac disorders generated by *S. papillosus* infection are reversible and curable following worm elimination. The disturbance of cardiac regulation in sudden cardiac death-type strongyloidosis is more likely to be functional rather than organic lesion-based.

Introduction

Previous studies demonstrated that heavy infection with *Strongyloides papillosus* causes lethal arrhythmias in calves (Tsuji et al., 1992; Ura et al., 1993b) and in lambs (see Chapter 1). Animals percutaneously infected with lethal doses of infective larvae develop continuous sinus tachycardia which finally results in sudden cardiac arrest by the onset of ventricular fibrillation (VF) after the establishment of the patent infection. In calves, continuous sinus tachycardia following infection is accompanied by prolongation of the PQ interval, the portion of the electrocardiogram (ECG) between the P wave and the QRS complex (Tsuji et al., 1992). Sudden cardiac death is also caused by the direct inoculation of parasitic females, the parthenogenetic adult stage of *S. papillosus*, into the duodenum of recipient lambs through a sequence of arrhythmias identical to that observed in percutaneous larval infection, indicating that parasitic females in the small intestine are responsible for the development of cardiac disorders following infection (see Chapter 2).

In the previous experiments, animals that developed continuous sinus tachycardia failed to recover normal cardiac rhythms and died of sudden cardiac arrest following heavy infection or inoculation with *S. papillosus*. Animals exposed to lower larval doses survived, developing neither continuous sinus tachycardia nor any other arrhythmias including VF. There were no animals whose cardiac rhythms spontaneously returned to normal after continuous sinus tachycardia had been established (Tsuji et al., 1992; see also Chapters 1 and 2). Therefore, it remains unknown whether the cardiac disorders developed in the disease are based on a reversible and curable change or a progressive and unrecoverable change.

The purpose of this Chapter was to clarify the reversibility of the cardiac disorders generated in sudden cardiac death-type strongyloidosis. I hypothesized that the cardiac disorders would be reversible and curable if the causative factor were to be eliminated before the terminal stage of infection. The hypothesis was derived from several field reports on the successful prevention of subsequent occurrence of lethal strongyloidosis by anthelmintic treatment of a group of calves in farms that suffered from frequent outbreaks of the disease (Ito, 1991; Matsutani et al., 1991; Taira and Ura, 1991a; Taira and Ura, 1991b; Tomishita et al., 1991; Almeida et al., 2005). However, none of the reports described the effects of anthelmintic treatment on the cardiac rhythms in individual animals because none included ECG monitoring before and after treatment. To verify the hypothesis, I performed anthelmintic treatment on lambs during the middle stage of lethal percutaneous infection, when continuous sinus tachycardia had already been established, and analyzed the effects of the resulting worm elimination on the disease course using ECG monitoring.

Materials and Methods

Animals

Nine helminth-free male Suffolk lambs were employed in this study. Their groups and body weights are listed in Table 11. The age of the animals was unknown because their birth records were not available. However, their weight ranges suggested that the animals were approximately the same age as those in the experiments in Chapters 1 and 2. They were fed a diet of hay and concentrate twice a day. Water was provided *ad libitum*. The animals were loosely restrained by an individual neck stanchion on a drainboard in a recording room as described in Chapter 1. Specific pathogen-free male Japanese-White rabbits weighing 2-3 kg were used for the passage of *S. papillosus*. They were kept separately in stainless steel wire cages with a drainboard and a feces tray. Standard pelleted food and water were provided *ad libitum*. All experiments using animals were approved by the Ethics Committee of the National Institute of Animal Health in Japan. Based on the results described in the previous Chapters, eventual occurrence of sudden cardiac death would be predicted if lambs developed continuous sinus tachycardia. In these experiments, however, the untreated control animals were neither treated nor euthanized in order to confirm the entire course of the disease. The control animals showed almost no significant symptoms and abnormal behavior until the final onset of VF, suggesting they experienced little or no pain and suffering throughout the experiment.

Parasites

The Himeji strain of *S. papillosus* (Taira et al., 1991; Taira et al., 1992a) was maintained by serial passages in rabbits as previously described (Nakamura et al., 1994). Infective larvae were obtained as described in Chapter 1.

Percutaneous infection of lambs

Eight lambs were percutaneously exposed to 10,000 infective larvae per kg body weight by attaching cotton wool pads containing the required number of larvae as described in Chapter 1. The remaining lamb was not infected. In this study, the time course of experiments was recorded to the nearest hour and in days in order to ensure accurate time intervals between events, such as the interval from infection to the establishment of continuous sinus tachycardia. The time of the initial larval exposure was defined as time 0 of infection.

Electrocardiogram recording

Continuous ECG recording was carried out on all the animals from 7 days before infection as described in Chapter 1. Heart rates and PQ intervals were measured at least once per hour when an animal was at rest and the recording baseline was stable, excluding the period of 1 h after the start of feeding. The normal heart rate was 56±5/min (mean ±standard deviation (SD)). Relative PQ intervals were calculated as (PQ interval) / (RR interval), i.e., as (PQ interval) x (heart rate) / 60. The normal relative PQ interval was 0.102±0.021 (mean±SD). A heart rate of more than 80/min was considered tachycardia (Detweiler, 1993). A relative PQ interval of more than 0.165 (the mean+3SD of the normal value) was arbitrarily defined as a prolonged PQ interval. Tachycardia and

prolongation of the PQ interval which lasted over 12 h were considered continuous.

Anthelmintic treatment

The uninfected animal (Lamb No. 601) was subcutaneously injected with ivermectin (Ivomec; MSD Agvet, New Jersey, USA) at a dose rate of 1 mg/kg body weight (1ml/10kg body weight, of a 1 % solution) to observe the adverse effects of ivermectin on normal cardiac rhythms over a period of 14 days after injection. Five of the eight infected animals (Lambs Nos. 801 to 805: treated animals) received ivermectin in the same manner as Lamb No. 601 when the animals developed continuous sinus tachycardia and prolongation of the PQ interval. The time of ivermectin injection was defined as time 0 of treatment. The other three infected animals (Lambs Nos. 701, 702 and 703: control animals) remained untreated.

Fecal egg counts

Rectal feces were collected from the eight infected animals every 12 h from 192 h (day 8.0) after infection to the time of death or necropsy, to determine the number of eggs per gram of feces (EPG) as described in Chapter 1.

Necropsy

The control and the treated animals were necropsied within 3 h of death or killed 504 h (day 21.0) after infection by intravenous injection with sodium pentobarbital (Somnopentyl, 200 mg/kg; Kyoritsu Seiyaku, Tokyo, Japan). Organs were examined for gross lesions. Worms in the small intestine were recovered and counted as described in

Chapter 1. Necropsy was not performed on the uninfected treated Lamb No. 601.

Results

Fate of the lambs

The survival time is shown in Table 12. The first eggs were detected in rectal feces of the eight infected animals between 216 h (day 9.0) and 264 h (day 11.0) after infection (Table 13). No diarrhea was observed in the animals throughout the experiment. The infected animals developed continuous sinus tachycardia, which began between 191 h (day 8.0) and 290 h (day 12.1) after infection (Table 14). Prolongation of the PQ interval was established in the animals within 26 h of developing sinus tachycardia (Table 14). All the control animals died of sudden cardiac death, whereas all the treated animals recovered from the arrhythmias and survived. The uninfected, treated animal did not develop any adverse reactions to ivermectin injection, and showed no arrhythmias over the 14-day observation period after treatment (Figure 8a).

The control animals

The heart rates and relative PQ intervals of the control animals increased gradually up to 95-115/min and 0.207-0.264, respectively (Figures 8a and 8b). Other arrhythmias were recorded before the onset of VF, including second degree atrioventricular (AV) block, ventricular premature beat and ventricular tachycardia (Table 14). All three animals developed VF between 297 h (day 12.4) and 349 h (day 14.5) after infection, having high fecal egg counts (Tables 13 and 14, Figures 8a and 8b). The animals had a good appetite until the day before death. At necropsy, scarring due to petechial hemorrhages was sporadically observed in the lungs of the three animals. All the animals had high worm

burdens in the small intestine (Table 13). Lamb No. 702 had congestion in the duodenum and jejunum.

The treated animals

The treated animals received ivermectin between 240 h (day 10.0) and 323 h (day 13.5) after infection (Table 12). Heart rates and relative PQ intervals were 95-110/min and 0.192-0.224, respectively, at the time of ivermectin treatment (Figures 8c, 8d and 8e). No adverse reactions to ivermectin treatment were noted in any of the animals. Heart rates began to decrease between 10 h and 18 h after treatment and were less than 80/min between 29 h and 39 h after treatment (Table 14, Figures 8c, 8d and 8e). Relative PQ intervals also began to decrease between 12 h and 21 h after treatment and were less than 0.165 between 26 h and 35 h after treatment. Second degree AV block was observed three times in Lamb No. 801 between treatment and the disappearance of sinus tachycardia (Table 14). No arrhythmias were detected in any of the animals, including Lamb No. 801, after the disappearance of sinus tachycardia.

Fecal egg counts of the animals reached maximum values between 264 h (day 11.0) and 336 h (day 14.0) after infection (Table 13, Figures 8c, 8d and 8e). The counts then decreased to undetectable levels by 384 h (day 16.0) after infection, i.e., by 61 h (day 2.5) after treatment. The animals kept a good appetite throughout the experiment. At the necropsy, which was performed at 504 h (day 21.0) after infection, only a few worms were recovered from the small intestine (Table 13). No gross lesions were observed in any of the animals.

Discussion

Ivermectin is a potent anthelmintic with a broad spectrum of activity against nematode and arthropod parasites, and has been used worldwide since it was first marketed in 1981 (Campbell and Benz, 1984; Bennett, 1986). Ivermectin generally has a high efficacy of greater than 99% for the reduction of fourth-stage larvae, and immature and mature parasitic females of *S. papillosus* in the small intestine (Yazwinski et al., 1983; Swan et al., 1984; Bennett, 1986; Tassi et al., 1990). However, ivermectin disappointingly shows low efficacy against infective and migratory larvae in the third-stage (Swan et al., 1984; Taira and Ura, 1991b; Rebollo et al., 2003). The dose rate of ivermectin in the present study was 5-fold higher than the recommended dose rate of 0.2 mg/kg for cattle in order to eliminate worms from the small intestine as completely and quickly as possible. Petersen et al. (1996) reported that the incomplete efficacy of the recommended dose rate of ivermectin against *Oesophagostomum dentatum* in pigs was improved by administering higher dose rates. In the present experiments, a subcutaneous dose rate of 1 mg/kg had no adverse effects on either the clinical conditions or cardiac rhythms of lambs. Indeed, ivermectin has not been reported to induce adverse reactions in cattle and sheep until a dose rate of at least 4 mg/kg (Campbell and Benz, 1984).

The plasma ivermectin concentration becomes maximal approximately 36 h after subcutaneous injection into sheep and remains high until day 5 (Marriner et al., 1987; Bogan and McKellar, 1988). Concentrations of ivermectin in the mucus of the small intestine are correlated with those in plasma (Marriner et al., 1987). The continuous sinus tachycardia and prolongation of the PQ interval that developed in the treated animals

began to recover from 10 h, and then disappeared completely by 39 h after treatment. The recovery course of the cardiac rhythms appeared to be accompanied by a reduction of fecal egg output following ivermectin treatment. Fecal egg counts became negative within 61 h of treatment. Only a few worms, representing approximately 0-0.03% of the total larval infection dose, were recovered from the small intestine of the treated animals at necropsy. These findings in the treated animals indicated that the worm elimination from the small intestine was achieved in a relatively short time and was almost complete. The recovery from these arrhythmias must have resulted from the inactivation and elimination of parasitic females by the action of ivermectin. Once recovering from sinus tachycardia, none of the treated animals developed sinus tachycardia with prolongation of the PQ interval again, and none developed any ventricular arrhythmias. No AV blocks appeared after the disappearance of sinus tachycardia in the treated animal, Lamb No. 801, in which the blocks had been recorded among patterns of sinus tachycardia. As a result, all the treated animals survived with no occurrence of fatal VF.

Together with the results described in Chapter 2, these results indicated that parasitic females in the small intestine were responsible for not only the initiation of, but also the maintenance and progress of cardiac disorders following heavy infection with *S. papillosus*. The present study clearly demonstrated that the cardiac disorders generated by *S. papillosus* infection are based on a reversible and curable change and are successfully resolved by worm elimination before the occurrence of ventricular arrhythmias.

Cardiac disorders with a pathological lesion or morphological abnormality, such as myocardial infarction, cardiomyopathy and ventricular hypertrophy, have a higher risk of

developing to heart failure than those with no lesion or abnormality (Tsutsui, 2018). Cardiac lesions generally hindered the recovery of regular cardiac functions (Robinson and Maxie, 1985; Nagai and Yazaki, 1990; Tsutui, 2018). In particular, cardiac disorders that induce ventricular arrhythmias tend to become severe if a patient has organic heart disease (Spielman et al., 1985; Moss et al., 1996; Harada and Akashi, 2016). Therefore, functional disorders of the heart with few or no lesions are more likely to be associated with the reversible and curable arrhythmias in sudden cardiac death-type strongyloidosis rather than cardiac disorders with lesions. The absence of overt histological changes in calves that died of strongyloidosis (Nakanishi et al., 1993) supports this possibility.

In a frequently observed process known as reperfusion injury, the recovery of blood circulation causes severe damage to ischemic heart tissue, ultimately resulting in heart failure and lethal arrhythmias (Hearse, 1977; Manning and Hearse, 1984; Kodama and Toyama, 2001). It was unlikely that the cardiac disorders in strongyloidosis were associated with the ischemia of heart tissue. If the cardiac disorders had been associated with ischemia due to *S. papillosus* infection, reperfusion injury should have developed in the treated animals upon the recovery of circulation following ivermectin treatment. However, no findings suggestive of reperfusion injury were observed in any of the animals after treatment in the present study.

In this study, I demonstrated that the cardiac disorders generated in sudden cardiac death-type strongyloidosis are essentially both reversible and curable via anthelmintic treatment to eliminate parasitic females from the small intestine.

Tables and Figures

Table 11. Lambs used in the experiments on ivermectin treatment following percutaneous infection with *Strongyloides papillosus.*

Lamb No. [1)]	Group [2)]	Body weight (kg)	Infection dose per kg body weight
601	Uninfected	19.0	-
701	Control	22.0	10,000
702	Control	24.5	10,000
703	Control	29.5	10,000
801	Treated	18.5	10,000
802	Treated	22.4	10,000
803	Treated	23.3	10,000
804	Treated	25.8	10,000
805	Treated	27.7	10,000

[1)] All animals were male. The age of animals was unknown.

[2)] Uninfected: uninfected and treated with ivermectin; Control: infected and untreated; Treated: infected and treated with ivermectin. See Table 12 for the time of ivermectin treatment.

Table 12. Time of ivermectin treatment and survival time following percutaneous infection with *Strongyloides papillosus.*

Lamb No.	Time of ivermectin treatment		Survival time		
	Hours	(Days)	Hours	(Days)	
601	Uninfected, treated animal				
701	Not treated		349	(14.5)	
702	Not treated		310	(12.9)	
703	Not treated		297	(12.4)	
801	264	(11.0)	504	(21.0)	1)
802	264	(11.0)	504	(21.0)	1)
803	288	(12.0)	504	(21.0)	1)
804	323	(13.5)	504	(21.0)	1)
805	240	(10.0)	504	(21.0)	1)

1) Killed.

Table 13. Fecal egg output and worm recovery from the small intestine following percutaneous infection with *Strongyloides papillosus*.

Lamb No. [1]	Period of egg output [2]			Maximum egg output		Parasitic females recovered at necropsy
	Time of first detection	Time of last detection	Time of disappearance	Eggs per gram of feces	Time of maximum count [2]	
601	Uninfected, treated animal: not determined					
701	264 (11.0)	Time of death	No disappearance	39,600	336 (14.0)	25,180
702	216 (9.0)	Time of death	No disappearance	60,600	310 (12.9)	62,840
703	252 (10.5)	Time of death	No disappearance	22,400	297 (12.4)	49,120
801	228 (9.5)	300 (12.5) [36]	312 (13.0) [48]	18,000	288 (12.0) [24]	12
802	240 (10.0)	300 (12.5) [36]	312 (13.0) [48]	8,600	276 (11.5) [12]	13
803	240 (10.0)	336 (14.0) [48]	348 (14.5) [60]	27,800	288 (12.0) [0]	2
804	264 (11.0)	372 (15.5) [49]	384 (16.0) [61]	26,200	336 (14.0) [13]	79
805	228 (9.5)	288 (12.0) [48]	300 (12.5) [60]	10,800	264 (11.0) [24]	0

[1] See Table 12 for the time of ivermectin treatment and survival time.

[2] Numbers indicate hours after infection. Numbers in parentheses indicate days after infection. Numbers in brackets indicate hours after ivermectin treatment.

Table 14. Electrocardiogram findings following percutaneous infection with *Strongyloides papillosus*.

Lamb No. [1]	Occurrence of arrhythmia [2]			
	Sinus tachycardia	Prolongation of the PQ interval	Ventricular fibrillation	Other arrhythmias [3]
601	Not observed	Not observed	Not observed	Not observed
701	290 (12.1) ~ Until death	308 (12.8) ~ Until death	349 (14.5)	VPB: 346 (14.4)
702	245 (10.2) ~ Until death	253 (10.5) ~ Until death	310 (12.9)	VT: 308 (12.8)
703	191 (8.0) ~ Until death	217 (9.0) ~ Until death	297 (12.4)	2nd AVB: 275 (11.5) ~ frequently observed; VT: 276 (11.5)
801	241 (10.0) ~ 295 (12.3) [31]	249 (10.4) ~ 294 (12.3) [30]	Not observed	2nd AVB: 277 (11.5), 287 (12.0), 292 (12.2)
802	243 (10.1) ~ 301 (12.5) [37]	251 (10.5) ~ 293 (12.2) [29]	Not observed	Not observed
803	260 (10.8) ~ 324 (13.5) [36]	274 (11.4) ~ 320 (13.3) [32]	Not observed	Not observed
804	286 (11.9) ~ 362 (15.1) [39]	312 (13.0) ~ 358 (14.9) [35]	Not observed	Not observed
805	220 (9.2) ~ 269 (11.2) [29]	229 (9.5) ~ 266 (11.1) [26]	Not observed	Not observed

[1] See Table 12 for the time of ivermectin treatment and survival time.

[2] Numbers indicate hours after infection. Numbers in parentheses indicate days after infection. Numbers in brackets indicate hours after ivermectin treatment.

[3] 2nd AVB: second degree atrioventricular block; VPB: ventricular premature beat; VT: ventricular tachycardia.

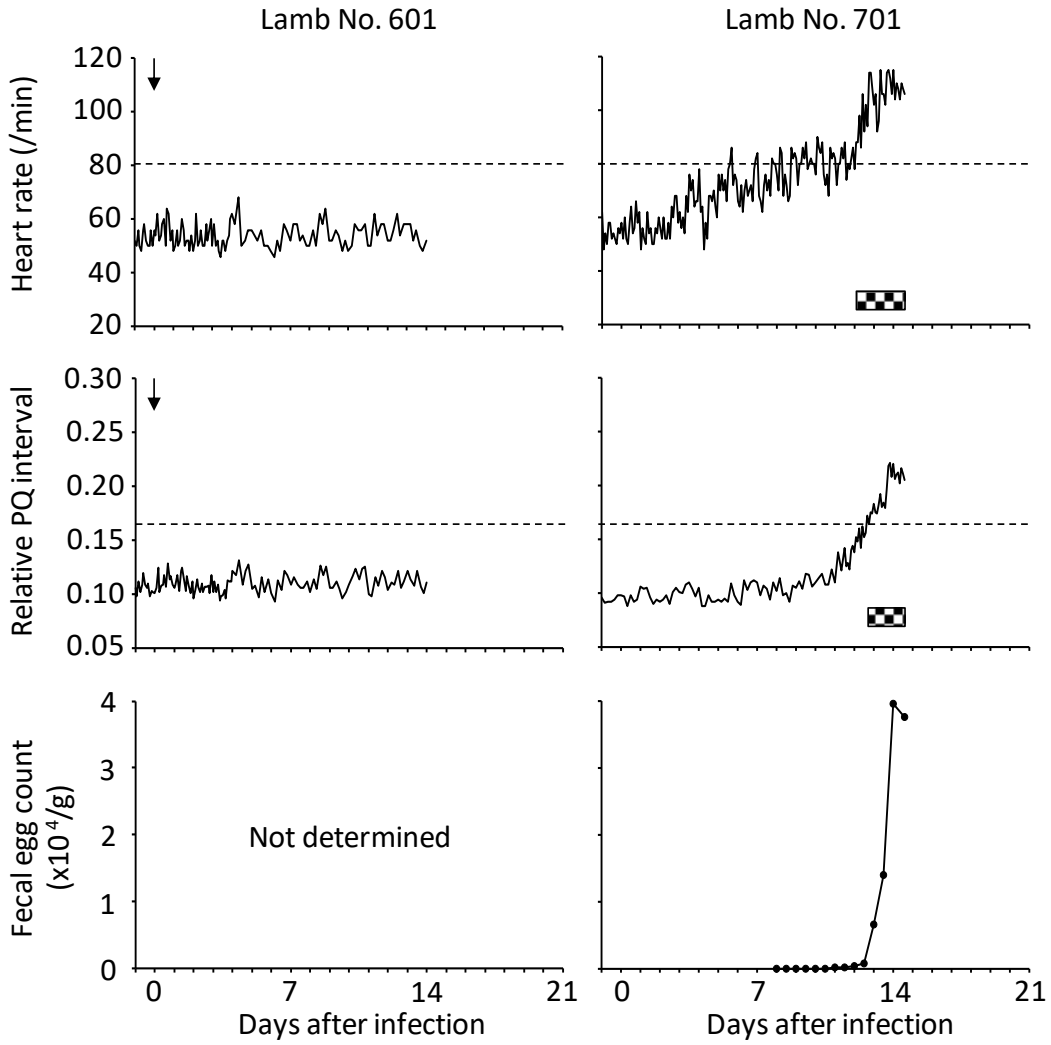

Figure 8a. Alterations of heart rate (top), relative PQ interval (middle) and fecal egg count (bottom) in Lamb No. 601 that remained uninfected (left) and Lamb No. 701 following percutaneous infection with 10,000 infective larvae of *Strongyloides papillosus* per kg body weight (right). Lamb No. 601 was treated with ivermectin at the time indicated by an arrow, while Lamb No. 701 remained untreated. A heart rate of more than 80/min and a relative PQ interval of more than 0.165 were considered tachycardia and prolonged, respectively (dotted line). Tachycardia and prolongation of the PQ interval which lasted over 12 h were considered continuous (checkered bar).

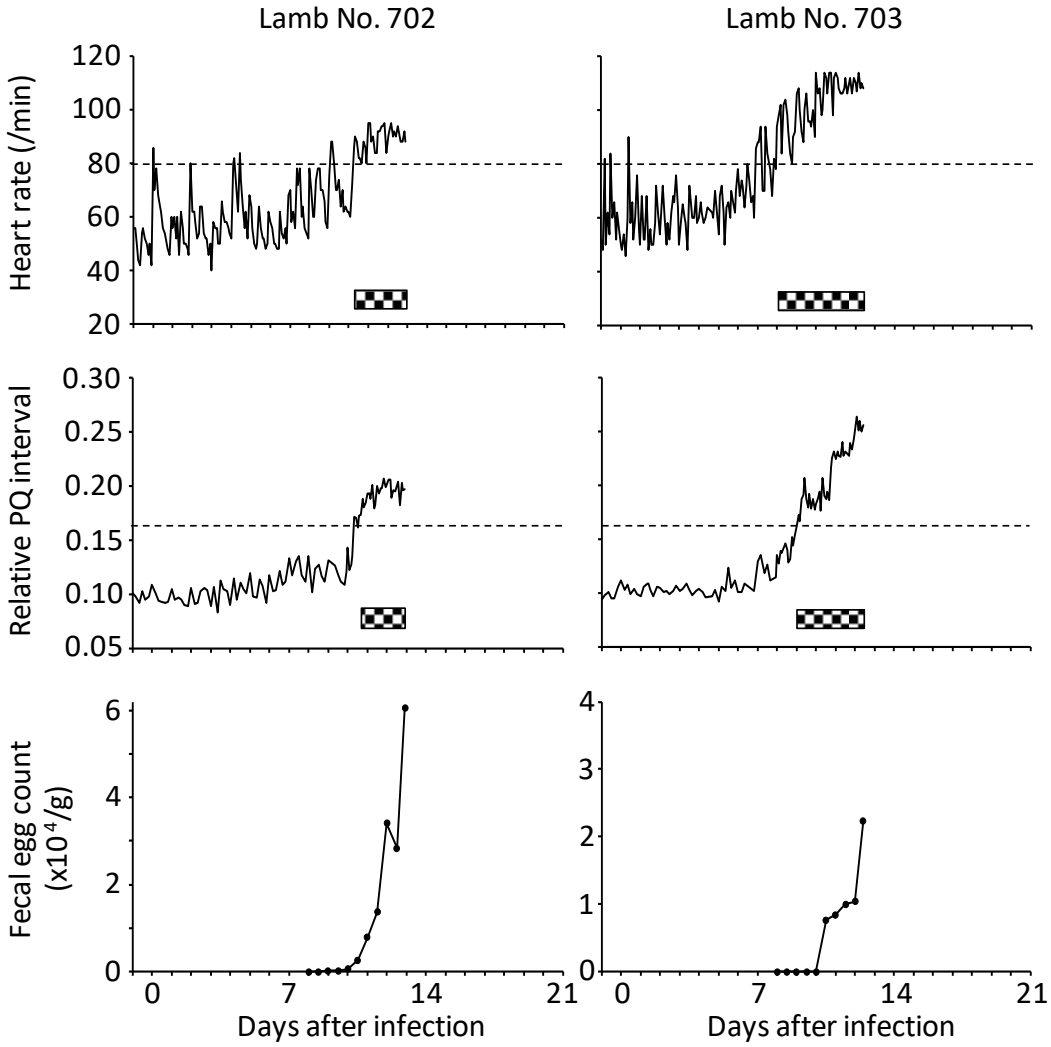

Figure 8b. Alterations of heart rate (top), relative PQ interval (middle) and fecal egg count (bottom) in Lambs No. 702 (left) and No. 703 (right) following percutaneous infection with 10,000 infective larvae of *Strongyloides papillosus* per kg body weight. These animals remained untreated with ivermectin. A heart rate of more than 80/min and a relative PQ interval of more than 0.165 were considered tachycardia and prolonged, respectively (dotted line). Tachycardia and prolongation of the PQ interval which lasted over 12 h were considered continuous (checkered bar).

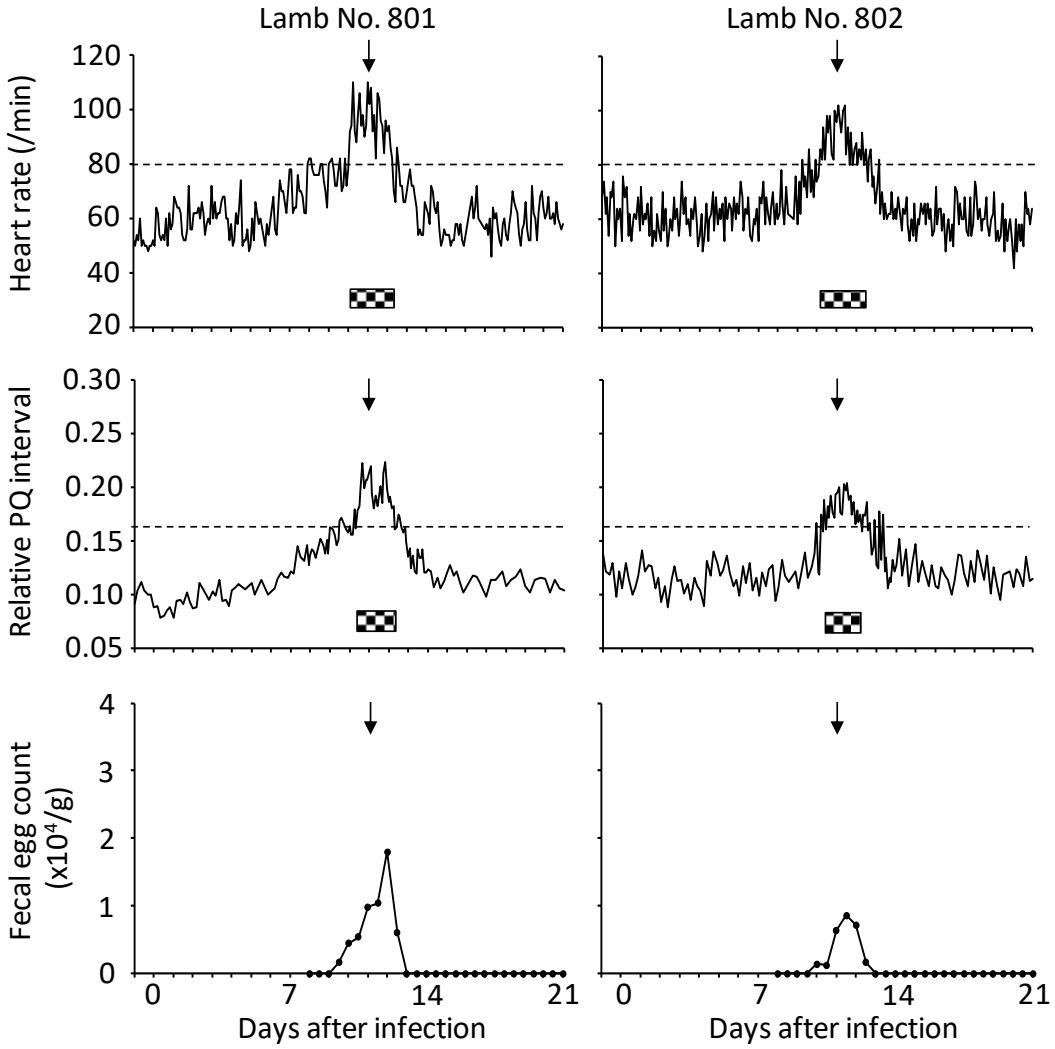

Figure 8c. Alterations of heart rate (top), relative PQ interval (middle) and fecal egg count (bottom) in Lambs No. 801 (left) and No. 802 (right) following percutaneous infection with 10,000 infective larvae of *Strongyloides papillosus* per kg body weight. These animals were treated with ivermectin at the time indicated by an arrow. A heart rate of more than 80/min and a relative PQ interval of more than 0.165 were considered tachycardia and prolonged, respectively (dotted line). Tachycardia and prolongation of the PQ interval which lasted over 12 h were considered continuous (checkered bar).

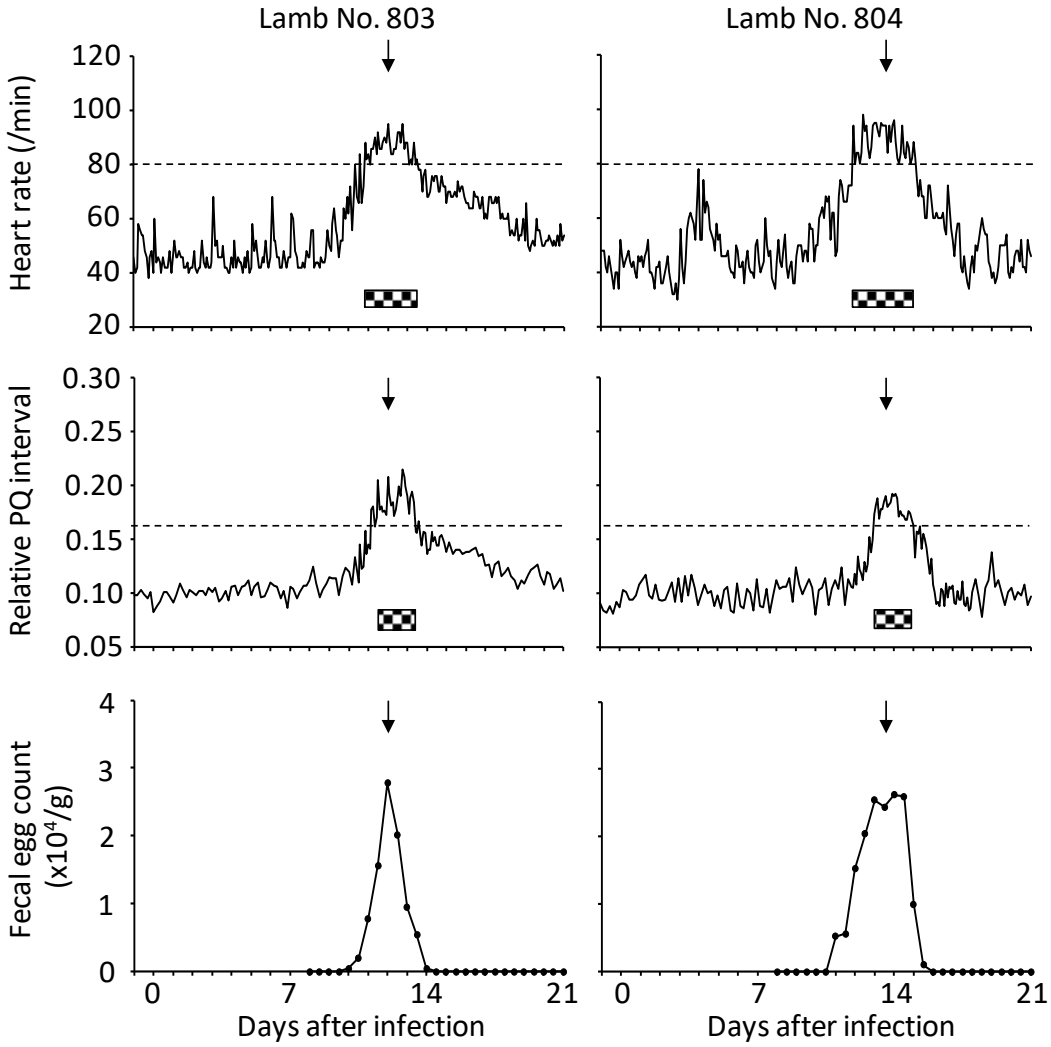

Figure 8d. Alterations of heart rate (top), relative PQ interval (middle) and fecal egg count (bottom) in Lambs No. 803 (left) and No. 804 (right) following percutaneous infection with 10,000 infective larvae of *Strongyloides papillosus* per kg body weight. These animals were treated with ivermectin at the time indicated by an arrow. A heart rate of more than 80/min and a relative PQ interval of more than 0.165 were considered tachycardia and prolonged, respectively (dotted line). Tachycardia and prolongation of the PQ interval which lasted over 12 h were considered continuous (checkered bar).

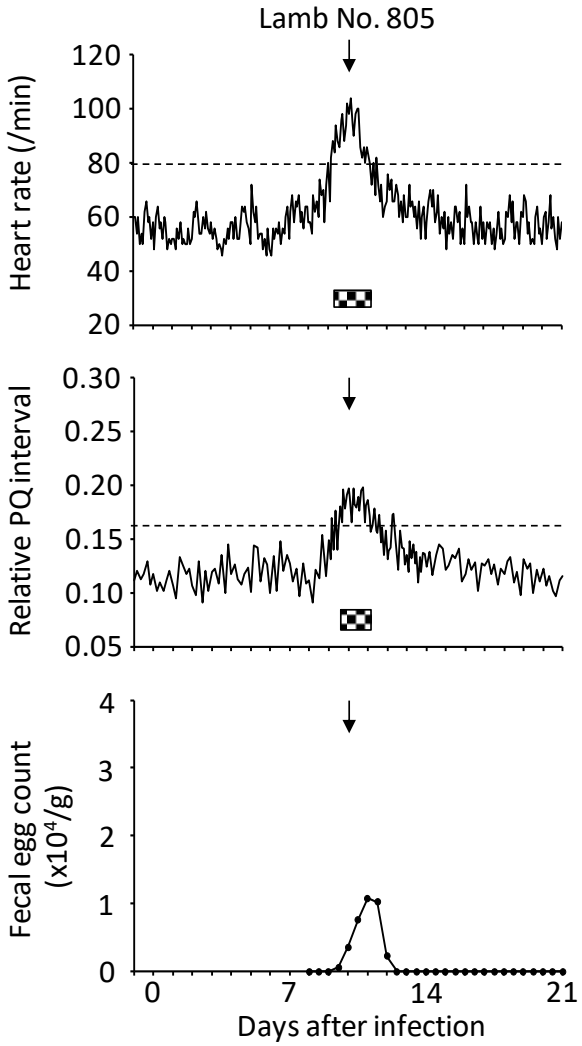

Figure 8e. Alterations of heart rate (top), relative PQ interval (middle) and fecal egg count (bottom) in Lamb No. 805 following percutaneous infection with 10,000 infective larvae of *Strongyloides papillosus* per kg body weight. The animal was treated with ivermectin at the time indicated by an arrow. A heart rate of more than 80/min and a relative PQ interval of more than 0.165 were considered tachycardia and prolonged, respectively (dotted line). Tachycardia and prolongation of the PQ interval which lasted over 12 h were considered continuous (checkered bar).

General Discussion

In Chapter 1, I demonstrated that lambs develop sudden cardiac death-type strongyloidosis through a course and in a manner identical to those in calves following heavy infection with *S. papillosus*. An ovine model was thus established for experimental studies on the disease. I also revealed that the acute shock-like state in the terminal stage of sudden cardiac death-type strongyloidosis is not associated with the circulatory response of inflammatory cytokines. The disease in calves and lambs is characterized by a series of arrhythmias in the intestinal phase of infection, without significant premonitory signs and lesions to be the cause of death. Animals die of sudden cardiac arrest by ventricular fibrillation (VF) approximately 2-3 weeks after percutaneous infection with the minimum lethal dose of 3,200 larvae per kg body weight. In addition, Tetsuka et al. (1993) reported the group death of young goats due to heavy strongyloidosis with no diarrhea on a farm in Kagoshima Prefecture. Yoshifuji et al. (1994) confirmed that goats develop sudden death in a manner similar to calves following experimental heavy infection with the parasite. Although Yoshifuji et al. (1994) did not perform electrocardiogram (ECG) monitoring of the animals, it is most likely that all ruminant host animals develop sudden cardiac death following heavy infection with *S. papillosus*.

Bovine heavy strongyloidosis is sometimes categorized into two types, sudden death-type and emaciation death-type, based on the absence and presence, respectively, of clinical symptoms such as diarrhea and pneumonia at the time of death (Taira, 1991; Taira and Ura, 1991b; Taira and Nakanishi, 1995; Taira and Ura, 2005). Field reports on heavy strongyloidosis in calves have described not only the occurrence of sudden death with no

clinical signs but also the occurrence of death accompanied primarily by persistent diarrhea or occasionally by pneumonia (Yachi et al., 1987; Amahashi et al., 1991; Ito, 1991; Taira and Ura, 1991b; Ideguchi et al., 1992). One field report described the group death of lambs showing diarrhea and emaciation following heavy natural infection with the parasite (Taira and Kato, 1986).

However, no lambs in the present studies developed diarrhea or pneumonia throughout the course of experimental heavy infection. Among 11 calves that died in the previous study, all animals but one developed sudden death following experimental heavy infection (Taira et al., 1992a). One special case, Calf No. 209, was categorized as an animal that developed emaciation death-type strongyloidosis due to showing persistent diarrhea. However, according to that report, it had a weight gain of 9.4 kg during the experimental period, indicating that the animal had not really become emaciated. Although ECG monitoring was not performed in Calf No. 209, the experimental data from the present and previous studies strongly suggest that cardiac dysfunction finally resulting in sudden cardiac arrest is the inevitable pathophysiology developing in all animals with the fatal heavy strongyloidosis irrespective of the disease-type categorization. In farms equipped with small pens, sawdust bedding contaminated with a huge number of *S. papillosus* infective larvae provides a steady supply of larval infection to the animals. The skin of the animals is continuously irritated by such repeated larval invasion. In farms with poor management, the animals in these pens would also be exposed to continual stress due to the unsuitable environment, malnutrition, and possible secondary infections, which would easily develop into the diarrhea or pneumonia that causes the unhealthy appearance at the time of death, and potentially masks the true cause of death, i.e., cardiac disorders are the

real cause of their death.

The course of heavy strongyloidosis is significantly different between rabbits and ruminant animals. Rabbits develop a wasting condition characterized by significant anorexia, anemia, weight loss and subsequent death in the intestinal phase of heavy infection, but never develop diarrhea and cardiac disorders (Chomicz, 1967; Nakamura et al., 1994). If the peak stage of infection is overcome, surviving rabbits recover from the wasting condition as fecal egg output decreases (Nakamura et al., 1994). The wasting condition in infected rabbits is a real state of emaciation causing death, which is distinct from the clinical conditions of emaciation death-type strongyloidosis in calves discussed above. The systemic emaciation in infected rabbits is similar in clinical appearance to a state of cachexia usually induced by inflammatory cytokines (Grunfeld and Feingold, 1991). However, it has also been indicated that the pathophysiology of heavy strongyloidosis in rabbits is not associated with cachexia due to cytokine responses (Nakamura and Motokawa, 2000). Instead, rabbits with heavy strongyloidosis develop a hepatic disorder characterized by defects in lipid synthesis (Nakamura and Motokawa, 2000) and gastrointestinal motor disturbance probably due to paralytic ileus (Kobayashi and Horii, 2008; Kobayashi et al., 2009). It has remained unknown what causes the different pathophysiology of heavy strongyloidosis between ruminant animals and rabbits.

In Chapter 2, I demonstrated that active parasitic females in the small intestine are responsible for the fatal cardiac disorders of sudden cardiac death-type strongyloidosis. Two reports have been published on human strongyloidosis associated with the cardiac arrest of patients (da Silva et al., 1981; Kane et al., 1984). In both cases, it was speculated that the cardiac arrest resulted from hypokalemia due to persistent severe diarrhea

following *S. stercoralis* infection. Prior to the cardiac arrest, one of the patients showed respiratory failure due to respiratory muscle paralysis, and the other was accompanied by myocardial infarction. These symptoms and courses were quite distinct from those observed in sudden cardiac death-type strongyloidosis of calves and lambs. It has also been reported that pigs develop sudden death following heavy infection with *S. ransomi* (Spindler and Hill, 1942; Spindler, 1944). However, these were likely rare accidental cases caused by mechanical injury to the heart tissue by the invasion of migratory larvae. Ura et al. (1993a) reported that no sudden death was developed in pigs following experimental heavy infection with *S. ransomi*.

There have been no reports in which parasites living in the gastrointestinal tract induced fatal cardiac dysfunction in animals or humans in the absence of accompanying symptoms or other diseases. In the present experiments, there were absolutely no migratory larvae invading the heart and the central nervous system of lambs intraduodenally inoculated with parasitic females of *S. papillosus*. It was impossible for the parasite to provide any mechanical injury to the heart or its regulatory center of the host. Thus, the present studies clearly proved that parasitic females in the small intestine have a fatal effect directly or indirectly on the heart or its upper center in calves and lambs. The present studies thus contribute new and unexpected knowledge to the field of parasitology.

The present studies did not clarify how parasitic females generate the cardiac disorders in fatal strongyloidosis of calves and lambs. It has been reported that the excretory and secretory (ES) products of gastrointestinal nematodes modulate or alter the physiological conditions of host animals. The ES products activate the host immune system to

contribute to worm expulsion and protection against reinfection (Mimori et al., 1987; Savin et al., 1990; Emery et al., 1993; Griffiths and Pritchard, 1994). Some nematodes release acetylcholinesterase or vasoactive intestinal polypeptide-like protein to reduce the contraction and inflammation of the alimentary tract, and others release unidentified chemicals to inhibit the acid secretion of the abomasum, for their longer stay in the preferred site (Eiler et al., 1981; Lawton et al., 1996; Lee, 1996; Hertzberg et al., 2000). There is a possibility that parasitic females of *S. papillosus* in the small intestine excrete or secrete a cardioactive substance or its precursor capable of modulating the regulation of cardiac rhythms. No cardiac disorders were generated by injection with the homogenate prepared from a sufficient number of parasitic females which was greater than the lethal dose for the inoculation with live worms. The effects of ES products released from live parasitic females upon cardiac regulation should be investigated to understand the pathophysiology of sudden cardiac death-type strongyloidosis.

The normal cardiac rhythm is initialized by the spontaneous and cyclic depolarization of the sinoatrial node, which locates in the superior portion of the right atrium (Barrett, 2016). The excitation first spreads radially through the atria (the P wave on ECG) to start atrial systole, and then is transmitted to the ventricles through the atrioventricular (AV) node and the His-Purkinje system (the QRS complex) to launch ventricular systole. The cardiac cycle is completed by the repolarization of the ventricles (the T wave). In addition to its spontaneous activity, the heart receives reciprocal innervation from the sympathetic and parasympathetic (vagal) nerves (Ardell and Randall, 1986; Ardell et al., 1988; Barrett et al., 2016; Westfall et al., 2018). The activation of the sympathetic tone increases the heart rate, conduction velocity and contractility mainly via β_1 adrenergic

receptors in the heart; conversely, that of the vagal tone shows suppressive effects mainly via M_2 muscarinic receptors.

The unusual course of sudden cardiac death-type strongyloidosis is associated with cardiac disorders showing three elements of arrhythmias: sinus tachycardia, prolonged PQ interval (the portion of ECG between the P wave and the QRS complex) and VF (Tsuji et al., 1992; see also Chapter 3). Prolonged PQ interval, which is also called first degree AV block (Nakata, 2001; Josephson, 2008), is established a little bit after the appearance of sinus tachycardia and later occasionally progresses to second degree AV block. The combination of sinus tachycardia and accompanying prolonged PQ interval is most likely to be the primary and fundamental pathophysiology for the disease, since this combined arrhythmia appears in the earlier stage of infection rather than ventricular arrhythmias and continues until the onset of VF.

Sinus tachycardia is often generated as a physiological phenomenon when the excitation cycle of the sinoatrial node is accelerated either by the stimulation of sympathetic tone or by the suppression of vagal tone (Kodama and Toyama, 2001; Barrett, 2016). If parasitic females provide the heart with a potent β_1 receptor agonist or M_2 receptor antagonist, sinus tachycardia itself can be generated. However, the primary cause of AV block, including prolonged PQ interval, is the disturbance of conduction in the AV node by excessive vagal stimulation which results in inducing bradycardia (Billman et al., 1989; Kanda and Yoshioka, 2016). In physiological tachycardia, the PQ interval usually shortens as the heart rate increases (Akhtar, 1984; Castellanos et al., 1984; Warner et al., 1986; Nakata, 2001). Therefore, a simple unbalance of the autonomic nerves or their receptor signalings can hardly account for the appearance of sinus

tachycardia accompanied by prolonged PQ interval. If a factor derived from parasitic females causes the stimulation of sympathetic tone or the suppression of vagal tone to establish sinus tachycardia, the PQ interval must become shortened. Instead, under abnormal situations or by pharmaceutical effects, enhanced automaticity is evoked in cardiac muscle fibers when they have their membrane potentials are reduced enough to trigger quick repetitive discharges (Wit and Rosen, 1984; Kato and Sotohata, 2001; Kodama and Toyama, 2001; Barrett, 2016). During the automatic atrial tachycardia in which atrial muscles show enhanced automaticity, the PQ interval often becomes longer as the rate of tachycardia increases (Benditt et al., 1984; Kato and Sotohata, 2001; Josephson, 2008).

Here, one hypothesis may be proposed to account for the combined arrhythmia in this disease (Figure 9). Namely, a key factor derived from parasitic females could elicit enhanced automaticity in the atria via a route independent of the autonomic nervous system; such abnormal automaticity would lead to an alteration in the pacing rate of the sinoatrial node; and finally, the altered pacing could lead to tachycardia accompanied by a prolonged PQ interval that occasionally progresses to second degree AV block. If this hypothesis is correct, the administration of β_1 blocker or M_2 agonist would fail to decrease the elevated heart rate in the disease due to the lack of an association between the tachycardia and autonomic nervous regulation. Experiments are required to ascertain the effects of such drugs on the heart rate of animals in heavy strongyloidosis. In addition, if this hypothesis is correct, mechanical stimulation to the intestinal mucous membrane by parasitic females could be excluded as a potential cause of the cardiac disorders, since the mechanism of such mechanical stimulation is likely to involve an organ-heart reflex

controlled by the autonomic nervous system (Sato, 1980). The main mechanism of VF and other ventricular arrhythmias is the generation of chaotic excitations from ectopic foci and the formation of reentry circuits in the ventricles due to the loss of normal rhythm conduction (Barrett, 2016). Even in sudden cardiac death-type strongyloidosis, it is most likely that VF occurs secondarily without being able to control the disorganized activity of the ventricles due to the dissociation of normal conduction from the atria. Further studies are required to clarify the factors associated with the mechanism underlying the arrhythmias induced by parasitic females. The same factor of parasitic females may cause the different pathophysiology of heavy strongyloidosis in rabbits, targeting an effector different from the heart.

In Chapter 3, I demonstrated that sinus tachycardia with prolonged PQ interval in heavy strongyloidosis is based on reversible dysfunction of the heart, and that the affected animals can recover from the supraventricular arrhythmias to survive following worm elimination. In several farms, the subsequent occurrence of sudden death was empirically prevented by anthelmintic treatment to their calves, after the disease had been suggested to be associated with heavy strongyloidosis (Ito, 1991; Matsutani et al., 1991; Taira and Ura, 1991a; Taira and Ura, 1991b; Tomishita et al., 1991; Almeida et al., 2005). The present studies provided clear scientific evidence that worm elimination is effective for recovery from the cardiac dysfunction of the disease. It is impossible to monitor ECG on all calves in any farm. However, the risk of sudden cardiac death can be evaluated by counting *S. papillosus* egg output in fecal samples. When high egg counts on the order of 10^4 or more are detected, both the animal with the high egg output and the other animals being reared in the same group will be at high risk for sudden cardiac death. However,

disease occurrence can be prevented in this group by immediate treatment with anthelmintics. By following the above preventive and treatment measures, together with appropriate hygiene control including the regular exchange of sawdust bedding, farms can remain free of heavy strongyloidosis.

The present studies were carried out from 1992 to 1999 following the previous studies using calves described in the General Introduction. The new knowledge disclosed and obtained in the present studies was enlightened to cattle farms nationwide through a network formed by the National Institute of Animal Health and the Prefectural Livestock Hygiene Service Centers that control livestock farms. A total of 1,689 calves were officially recorded to have developed strongyloidosis in 42 Prefectures in Japan from 1985 to 2018 (Ministry of Agriculture, Forestry and Fisheries, 1985-2020: note that no public records were available before 1985, and that farms have no obligation to report the disease). The annual occurrence rate of the disease has been declining since 2003, and is currently less than 50 calves per year (Figure 10). The present studies, together with the previous studies, have made a major contribution to solving a serious problem in the field of livestock husbandry in Japan.

On the other hand, the occurrence of sudden cardiac death-type strongyloidosis has not yet been reported in the main livestock-producing countries of the world where most cattle graze in pastures free from the dense contamination of *S. papillosus* larvae. There have been only a few exceptions, such as the sudden death of calves due to heavy strongyloidosis in a farm using a straw bedding cowshed in the Czech Republic (Kváč and Vítovec, 2007) and the sublethal heavy infection of calves with *S. papillosus* in two small farms in Thailand (Chompoochan et al., 1998). Due to the reason mentioned above,

to my knowledge there have been no experimental studies on sudden cardiac death-type strongyloidosis following the present studies.

Figures

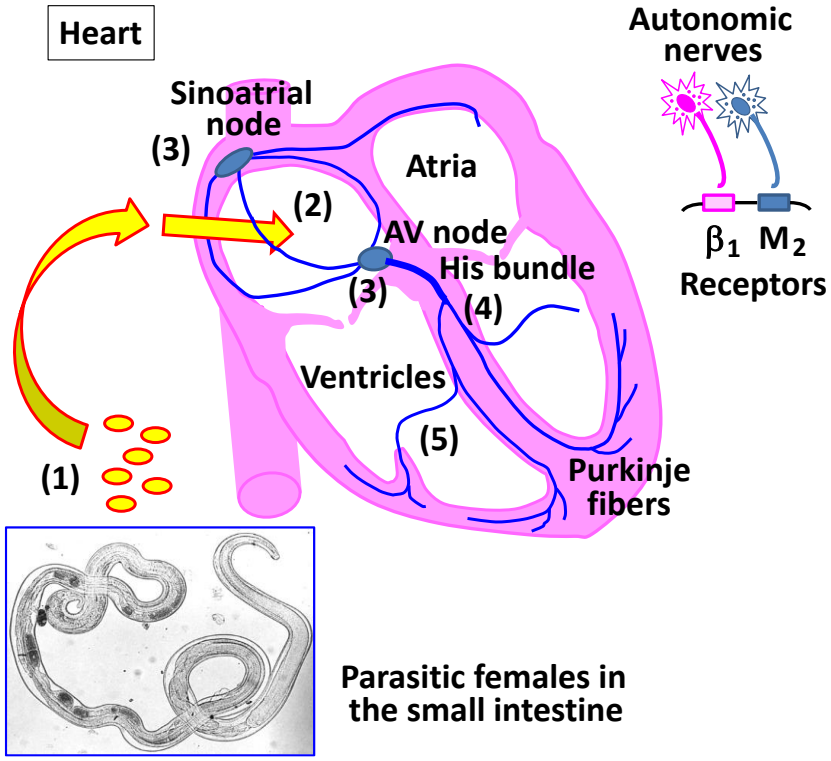

Figure 9. Hypothesized mechanism underlying the development of cardiac disorders in sudden cardiac death-type strongyloidosis. (1) Parasitic females in the small intestine excrete or secrete a cardioactive substance or its precursor. (2) The substance enhances the automaticity of atrial muscles via a route independent of the autonomic nervous system. (3) The abnormal automaticity accelerates the pacing rate of the sinoatrial node and reduces the conduction velocity of the atrioventricular (AV) node, resulting in sinus tachycardia accompanied by prolonged PQ interval. (4) The ventricle muscles fail to receive normal rhythm conduction. (5) Chaotic excitations are generated in the ventricle muscles, ultimately resulting in ventricular fibrillation.

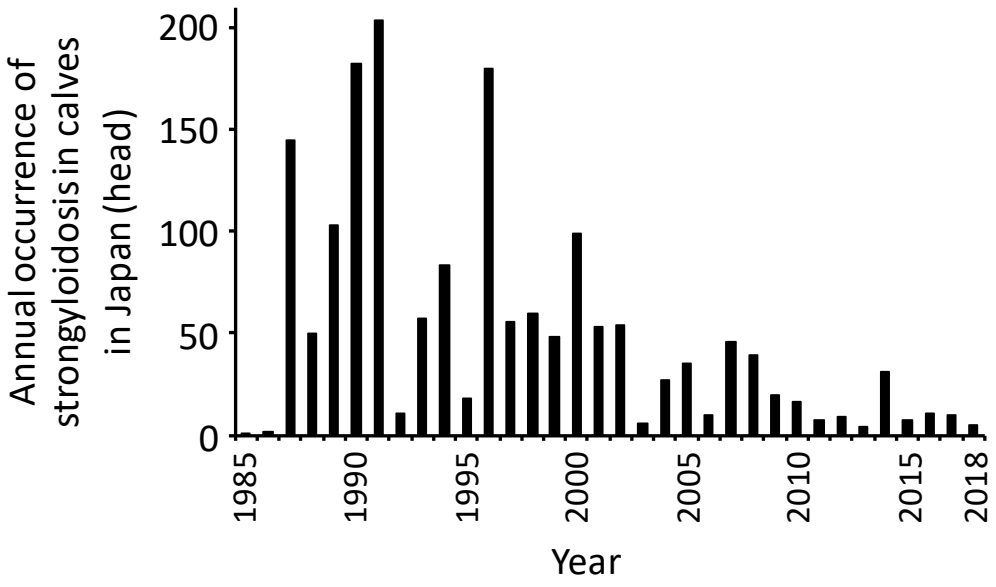

Figure 10. Annual occurrence of strongyloidosis in calves between 1985 and 2018 in Japan based on official reports from the Prefectures to the Ministry of Agriculture, Forestry and Fisheries. The number of animals includes dead, killed, and recovered cases. Note that no public records were available before 1985, and that farms have no legal obligation to report the disease to their Prefecture. (Data source: Ministry of Agriculture, Forestry and Fisheries, 1985-2020)

Acknowledgements

I would like to express my deep gratitude to Associate Professor Kazuichi Sakamoto, University of Tsukuba, for his profound guidance and encouragement as being in charge of this dissertation.

I would also like to express my sincere gratitude to Professor Chikafumi Chiba, Associate Professor Kyoichi Sawamura, and Associate Professor Hidekazu Kuwayama, University of Tsukuba, for their valuable discussions during the preparation of this dissertation.

I am very thankful to Drs. Noriyuki Taira, Naotoshi Tsuji, and Hisashi Hirose, the National Institute of Animal Health (at the time of conducting the experiments), and Dr. Chihaya Ooba, Hokkaido Prefectural Nemuro Livestock Hygiene Service Center, for their great contributions to the present studies.

I am also thankful to the members of Experimental Animal Services, the National Institute of Animal Health (Tsukuba and Shichinohe), for their excellent technical assistance on the experiments using animals.

I am very grateful to Honorary Professor James C. Williams, Louisiana State University, for his helpful advice and suggestions to the present studies.

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Appendix

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