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Report of an NIAID Workshop on Dengue Animal Models

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Abstract

Dengue is a mosquito-borne viral disease of humans that has re-emerged in many parts of the world and has become an important international public health threat. Dengue incidence and geographical spread has dramatically increased in the last few decades and is now affecting most tropical and subtropical regions of the world. Despite extensive research efforts for several decades, no vaccines or therapeutics are currently available to prevent and treat dengue infections. One of the main obstacles to the development of countermeasures has been the lack of good animal models that recapitulate dengue pathogenesis in humans and reliably predict the safety and efficacy of countermeasures against dengue. In September 2008, the National Institute of Allergy

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and Infectious Diseases (NIAID) held a workshop to consider the current state-of-the-art developments in animal models for dengue and discuss strategies to accelerate progress in this field. This report summarizes the main discussions and recommendations that resulted from the meeting.

Keywords

dengue; animal; models

Introduction

Dengue is the one of the most common mosquito-borne viral diseases of humans and it affects most tropical and sub-tropical regions of the world (1). Its incidence and geographical distribution have dramatically increased during the last 50 years and the WHO estimates that dengue viruses causes an estimated 50–100M infections per year worldwide and that about two-fifths of the world population are at risk for dengue infection (2). This expanded incidence is largely the result of factors that increase exposure to mosquitoes such as increased urbanization and travel, climate changes, and decreased vector control.

Dengue is caused by four serotypes of dengue virus, a positive-strand RNA virus that belongs to the *Flaviviridae* family (3). Other members of this virus family include important human pathogens such as yellow fever, Japanese encephalitis and West Nile virus. Dengue infection is transmitted to humans by two main mosquito vectors, *Aedes aegypti* and *Aedes albopictus*, which are day-biting mosquitoes that thrive in urban and semi-urban environments.

Human infection with any of the dengue viruses (DENVs) can result in a milder, self-limiting flu-like illness, dengue fever (DF), or in more severe forms of the disease, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which cause significant morbidity and mortality especially in young children. While the risk factors for the more severe forms of the diseases are not totally understood, epidemiological evidence indicates that pathogenic dengue-specific immune responses play an important role (see section on animal models to study dengue for pathogenesis). Although manufacturers have been trying to develop a vaccine for dengue for several decades, we currently have no licensed vaccines or therapeutics to prevent or treat human DENV infections. One of the main obstacles to the development of therapies and vaccines for dengue has been the lack of animal models that faithfully recapitulate pathogenesis in humans and predict which immune responses confer protection from disease or result in enhanced disease. In September 2008, the NIAID, with the co-sponsorship of the National Institute of Health (NIH) Office of Rare Diseases, held a workshop involving experts from the government, academia and private sector who use animal models to study dengue pathogenesis and to evaluate dengue vaccines and antiviral therapies. The goals of this workshop were to evaluate the animal models currently available for dengue; identify the most appropriate models to address specific scientific questions; discuss strategies to facilitate the development of the most promising models; and foster collaborations between researchers in the field. The animal models discussed in this workshop are summarized in Table 1.

The workshop began with a review of the dengue research program and research resources at NIAID. The dengue research portfolio at NIAID consists of basic and translational research projects supported through grants and contracts by both the extramural and the intramural divisions. In addition, NIAID supports several research resources that are made available to the scientific community to facilitate research on infectious diseases, including

dengue. A list of resources supported by the Division if Microbiology and Infectious Diseases (DMID) at NIAID can be found at: http://www3.niaid.nih.gov/LabsAndResources/resources/dmid/. Resources that could be of interest to the dengue research community include (1) large collections of reagents available through the NIAID BEI repository (www.beiresources.org) and the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA); (2) genomic, proteomic and bioinformatics tools, including the Microbial Sequencing Centers and the Viral Pathogen Database and Resource (www.viprbrc.org); and (3) contracts to develop new animal models and perform in vivo and in vitro antiviral drug screening.

Animal Models to Study Dengue Pathogenesis

Dengue disease in humans

The first session of the workshop focused on dengue animal models currently available to study the mechanisms of dengue pathogenesis, including the phenomenon of immune enhancement. The first presentation by Alan Rothman provided a review of dengue disease in humans reminding the workshop participants of the clinical symptoms that animal models should reproduce. DENV is delivered into the skin through the bite of an infected mosquito and it is believed to first infect subdermal dendritic cells and macrophages (4). Virus-infected cells then migrate to the regional lymph nodes, where further replication results in viremia and dissemination to distal sites in the body. The clinical signs and symptoms of dengue include fever, rash, thrombocytopenia, plasma leakage, bleeding and elevation of hepatic enzymes (ALT/AST). Viral clearance is associated with the end of the febrile period. Severe cases of dengue disease are usually associated with higher levels of viremia (5).

The risk factors that result in severe disease have not been completely defined; however, epidemiological studies have shown that the majority of DHF/DSS cases occur in young infants born to DENV antibody-positive mothers or in response to a secondary infection with a heterotypic DENV serotype. One theory is that pre-existing, serotype-crossreactive non-neutralizing antibodies (or serotype-crossreactive neutralizing antibodies at sub-neutralizing concentrations) raised against the first infecting DENV serotype *(e.g.*, DENV2) can “enhance” replication of a second infecting DENV serotype (*e.g.*, DENV1) upon subsequent infection. This hypothesized immune enhancement of replication is thought to be the result of virus-antibody complexes infecting Fc-receptor bearing cells. These “enhancing” antibodies can be acquired either from a DENV-immune mother through transplacental transfer, or through previous infection of the individual. This phenomenon is called antibody-mediated enhancement or antibody-dependent enhancement (ADE) (6). A second theory associates enhanced DENV pathogenesis with increased production of cytokines by either DENV-infected cells (*e.g.*, macrophages) or cross-reactive memory T-cells from the primary infection that are preferentially activated during the secondary infection.

In this hypothesis, these T-cells produce cytokines associated with vascular leakage, but are less able to clear the virus due to sub-optimal affinity for the peptide epitopes from the virus that causes the second infection (7). It is likely that both antibody and T cell responses are involved in the development of severe disease during secondary infection. Other factors that may contribute to disease severity are genetic diversity in the viruses and the host (*e.g.*, HLA types) and increased production of pro-inflammatory cytokines (e.g., TNFα and IFNγ).

Immune-competent mouse models

Most human DENV viral isolates do not replicate well in immune-competent mice. However some groups have reported that infection of immune competent mice with DENV can result in some clinical signs (8–9).

The first presentation by Alan Rothman summarized research conducted in BALB/c mice infected with DENV (10). When BALB/c mice are infected with DENV, they do not display any disease or measurable viremia. However T-cell immune responses are detected and upon heterologous secondary infections, T-cell responses against the original infecting strain were enhanced compared with the acute or memory response after primary infection (a phenomenon known as T cell “original antigenic sin”). It was suggested that this animal model can be used to study the effect of secondary infections on T-cell repertoires and responses against DENV. Since it is difficult to obtain DENV replication and pathology in immune competent mice, different mouse models have been developed to study DENV-induced disease as described in the following sections.

Humanized mouse models

Humanized mouse models to study dengue pathogenesis were reviewed by both Rebecca Rico-Hesse and Ramesh Akkina. Three types of humanized mouse models to study dengue were summarized: humanized non-obese diabetic/severe combined immunodeficiency (hNOD/SCID) mice, humanized NOD/SCID/IL2Rγ^{null} (hNSG) mice and humanized RAG2$^{-/-}$ $\gamma_c{}^{-/-}$ (RAG-hu) mice. Humanized mice are generated by irradiating immunocompromised, newborn mice to destroy the remaining immune cells followed by partial immune reconstitution with engrafted human hematopoietic stem cells (*e.g.* CD34$^+$ cells). Within a few weeks of engraftment, a variable percentage of the mice produce functional human B cells, T cells, monocytes, dendritic cells and cytokines.

NOD/SCID mice have several immunological defects, including lack of B and T lymphocytes, which are partially reconstituted with CD34 engraftment. Infection of humanized NOD/SCID mice subcutaneously (SC) with low passage dengue human isolates resulted in measurable viremia, fever, rash, thrombocytopenia, and elevated liver enzymes, which are some of the hallmarks of DF in humans (11). Human cytokines IL1β, IL-8, IFNγ and IFNα were detected in spleen, liver and bone marrow of NOD/SCID mice. However, very few mice developed human anti-dengue antibodies as detected by ELISA.

NOD/SCID/IL2Rγ^{null} (hNSG) mice are NOD/SCID mice that lack the IL-2 receptor common γ chain. These mice develop a higher level of engraftment of human cells than other humanized mice (12). When infected with DENV, hNSG mice replicate virus and develop rash, fever, thrombocytopenia, and some evidence of IgG specific for dengue (13). Secondary infection of hNSG mice resulted in no viremia and little thrombocytopenia, suggesting that these animals were protected from challenge.

RAG2$^{-/-}$ $\gamma_c{}^{-/-}$ mice do not produce B, T or NK cells. After immune reconstitution by engraftment, RAG-hu mice have a longer life span than other humanized mice. When RAG-hu mice are infected with DENV2, they develop viremia, fever and DENV-specific human antibody responses (IgM and IgG) (14). Some of the antibodies from infected RAG-hu mice neutralized DENV2 virus in vitro. Secondary infection in the RAG-hu model, in the presence of pre-existing DENV antibodies, resulted in evidence of hemorrhage and shock. Humanized mouse models are attractive because they generate human antibody immune responses and reproduce some of the important clinical characteristics of dengue disease with non-mouse adapted DENV strains. However, the relatively high cost and labor-intensive procedure to prepare engrafted mice has limited the extensive characterization of this model and its wide use by the research community. Additionally, each set of engrafted

mice are unique because they are derived from individual stem cell donors. As such, these mice are “outbred” and this could result in mouse-to-mouse variations in immune responses.

Since humanized mice have the potential to help elucidate important aspects of dengue pathogenesis in the context of a humanized immune response, however, several workshop participants felt that additional resources are needed to further develop this model. These resources should aim at increasing the number of infected mice studied, further characterizing the immune and pathogenic responses, optimizing protocols to obtain more consistent levels of engraftment and standardizing the source of hematopoietic stem cells (*e.g.*, cord blood expansion ex vivo or cell line) for the engraftment.

Intereferon response-deficient mouse model

Interferon (IFN) receptor-deficient mouse models (AG129) were reviewed by Eva Harris and Sujan Shresta (. The AG129 mouse strain (15–16) lacks the receptors for IFN α/β and γ, which permits all 4 DENV serotypes to replicate in this model after peripheral inoculation. The tissue and cellular tropism of DENV in these mice is similar to that in humans in that the virus targets lymph nodes, the spleen and a subset of leukocytes. Infection of AG129 mice with DENV2 virus typically results in a paralysis phenotype (17). Although encephalitis associated with DENV infection in humans is being increasingly recognized, the vast majority of dengue infections in humans are viscerotropic. In order to adapt DENV to cause a viscerotropic infection in this mouse model, DENV2 virus was passaged ten times between AG129 mice (serum) and mosquito cell lines (18,19). The resulting adapted DENV 2 strain, D2S10, had a more viscerotropic phenotype, causing thrombocytopenia and vascular leakage in infected mice. The phenomenon of ADE was also observed in AG129 mice following D2S10 infection (20,21). AG129 mice injected with anti-DENV 1 antibodies and then infected with D2S10, showed higher levels of viremia, pro-inflammatory cytokines, thrombocytopenia and mortality. This phenomenon was also observed when mice were treated with monoclonal antibodies (mAbs). When the Fc fragment of the antibodies was eliminated by proteolysis or modified genetically, the antibodies could no longer enhance DENV infection *in vitro* and *in vivo*, suggesting that in this model ADE contributes to severe dengue disease.

It was also observed that these mice mount a CD8+ T-cell response to viral infection against several viral proteins that contributes to protection. AG129 mice that were immunized with a vaccine consisting of a pool of 4 DENV-specific CD8 epitopes showed decreased viral load upon viral challenge (22). This model is attractive because the virus appears to infect the same target cells as in humans (DC and macrophages), recapitulates some of the clinical features of severe dengue disease and allows for the study of ADE as well as the role of T-cells and other immune responses in protection. In addition, since these mice are commercially available and relatively easy to breed they are amenable to large-scale experimentation. However, this model is limited because viral infection does not induce the full-spectrum of immune responses since the host lacks a normal IFN response. In addition DENV2 infection induces a physiologically-relevant disease only after viral adaptation. Additional work needs to be done to adapt the other DENV serotypes to induce a dengue-like pathology. It would also be desirable to develop similar animal models in less immunocompromised hosts.

Non-human primate models

A third approach using non-human primates (NHPs) was discussed by Anna Durbin and Ana Goncalvez. NHPs and mosquitoes are the only other natural hosts for DENV besides humans (23). NHPs can be infected in the wild by sylvatic DENV strains (24). Human clinical isolates replicate in NHPs without the need for adaptation, but infection does not

result in overt disease that resembles DF, DHF or DSS (25–28). NHPs have been used to evaluate the replication of live vaccine candidates and the immune responses to vaccines (29–30). Recent progress in the development of the macaque and chimpanzee models for dengue was reviewed. Primary infection (SC or intradermal-ID) of macaques with clinical DENV isolates resulted in moderate lymphadenopathy and robust immune responses. Secondary infection of macaques with a heterotypic DENV strain (12–20 months after primary infection) resulted in some signs of disease including lymphadenopathy, splenomegaly, hepatomegaly and mild dehydration. Some of the infected animals also developed a mild rash, subcutaneous bleeding and elevated liver enzymes, which, again, are features of DF in humans.

Chimpanzees infected with DENV developed high titers of neutralizing anti-DENV antibodies (28,31). Since chimpanzee immunoglobulins are ~98% homologous to those of humans, they make a good source of mAbs for prophylaxis of DENV (32,33). Rhesus monkeys that were transfused with a chimpanzee-derived highly neutralizing mAb were protected against DENV4 challenge, as indicated by the absence of viremia and lack of seroconversion (34). In contrast, pretreatment of rhesus monkeys with a humanized weakly cross-neutralizing mAb did not protect monkeys against DENV4 challenge. Furthermore, this flavivirus cross-reactive mAb enhanced DENV4 replication upon challenge, thus providing evidence of ADE in this system (35). Interestingly, a nine amino acid deletion at the N-terminus of the Fc region of the mAb that eliminated its capacity to bind to Fc receptors abrogated the enhancing activity of this antibody (35).

Because DENV replicates in NHPs without the need for virus adaptation, the NHP model for dengue is useful to measure protection conferred by vaccination or passively acquired antibody. However this animal model is limited by the lack of overt disease and by the high cost and limited accessibility to the research community. In order to limit costs, workshop participants suggested utilizing flavivirus-negative NHPs that have already been used in other studies. Further characterization of this model and the detailed evaluation of its immune responses to DENV are needed. To this end, additional reagents including new DENVs strains (both human and sylvatic isolates) that replicate at high titers in NHPs and immunological reagents should be developed.

Miniature swine model

Finally, a novel model of dengue in Yucatan miniature swine was described by Tim Burgess (manuscript in preparation). Pigs have several features that make them attractive as animal models. These include their physiological similarities to humans, the availability of a large amount of immunological reagents generated for xenotransplantation research and their significantly lower acquisition cost compared with NHPs. The pigs were infected subcutaneously (SC) or intravenously (IV) with different doses of DENV1 virus. SC infection resulted in detectable viremia and production of DENV neutralizing antibodies. This immune response protected the pigs from subsequent challenge. IV infection induced production of neutralizing antibodies with no detectable viremia. When the SC-infected pigs were subsequently re-infected with DENV1, they developed skin rash and dermal edema. DENV-containing immune complexes were found in the serum of these pigs, suggesting the possibility of an ADE-like phenomenon. When pigs were vaccinated with a DENV1 DNA vaccine construct, they developed a robust neutralizing immune response against DENV. There was general consensus that the preliminary data are promising but more work should be done to further characterize the disease phenotype and immune response in this model.

Animal Models to Evaluate Dengue Vaccines and Therapeutics

The second session of the workshop focused on the review of models that are currently used to evaluate vaccines and therapeutics for dengue. The animal models appropriate for evaluating vaccines were reviewed by Stephen Whitehead, Thierry Decelle, Sandra Giannini and Kenneth Eckels. One of the challenges of dengue vaccine development is that vaccines that don't induce neutralizing antibodies against all 4 serotypes of the virus could theoretically induce enhanced disease in vaccine recipients that are subsequently exposed to wild type infection. Therefore dengue vaccines must produce sufficient neutralizing antibodies against all serotypes to prevent breakthrough infection and disease. Tetravalent live attenuated dengue vaccine candidates are being developed that should induce strong immune responses to all four serotypes with minimal reactogenicity. DENV vaccines should be evaluated pre-clinically in animal models that support viral replication, induce measurable immune responses and that are accessible and easy to use to allow for the evaluation of dozens of different vaccine candidates. Each step of DENV vaccine development requires animal models with different characteristics. For example, to evaluate the attenuation of vaccine strains, wild type viruses need to replicate in the animal host at high titers. The reduction in replication of the vaccine candidate is then a measurable correlate of attenuation. The classical animal model used to measure dengue virus attenuation is non-human primates, since they allow dengue viruses' replication at high titers (29,36). In 2002, a mouse model, the SCID-HuH7, was developed to measurevaccine attenuation. This model consists of SCID mice injected intraperitoneally (IP) with the human hepatoma cell line Huh7, which is then allowed to grow to a visible tumor in a few weeks. All 4 serotypes of wild type (wt) DENV replicated to high titers when injected directly into the tumor while the attenuated strains replicated to significantly lower titers (37,38). Since titers of the different viral strains in the SCID-HuH7 mouse model correlated quite well with the ones in NHPs (39), this model is used by some groups to evaluate vaccine attenuation prior to clinical evaluation. Although this model is limited by the lack of any dengue-like pathology and immune responses, it is very reproducible.

Early-stage evaluation of vaccine immunogenicity is generally conducted in small animal models. Indeed the AG129 mouse model for DENV was first developed as a small animal model for dengue vaccine testing (17). These mice can be infected and generate neutralizing immune responses against all 4 DENV serotypes. In addition, lethal challenge studies using DENV1 and DENV2 can demonstrate protection. Later-stage preclinical evaluation of vaccine immunogenicity and efficacy is generally performed in NHP models like rhesus macaques and cynomolgus monkeys, as these animals are fully immune-competent and develop measurable viremia and humoral and cellular immune responses at physiologically low inoculation levels of DENV (29,30,40–42). NHPs are also used by some groups to study how the immune responses against one strain can interfere with the responses against the other 3 viral strains in a tetravalent vaccine formulation (the phenomenon called inter-serotypic interference) and to optimize immunization schedules.

Although NHPs are considered the best model to study the immunogenicity and efficacy of vaccines, these animals have not been able to fully predict vaccine immunogenicity and reactogenicity in humans. In order to evaluate the potential toxicity of live-attenuated dengue vaccines, an animal model has to support replication by all viral serotypes and induce measurable adaptive immune responses. Both NHP and the AG129 mouse models have these characteristics and therefore have been used by different groups to study the reproductive and systemic toxicity of vaccine candidates. Since DENV infection has been sporadically associated with CNS disease in humans, neurovirulence toxicology studies were discussed. The most sensitive system to study dengue neurovirulence appears to be

intracranial injection of suckling mice. NHPs have also been used to study neurovirulence but they display very little neuropathology.

Models used to evaluate therapeutics were reviewed by Wouter Schul. Several characteristics were cited during the workshop as being desirable in an animal model to evaluate drug candidates for anti-DENV activity. The model should be affordable, easy to acquire and scalable to allow for the evaluation of large numbers of compounds. It should support replication with all 4 strains of DENV, display simple markers of protection such as reduction of viremia and/or death and should be relevant to human disease (*i.e.*, display components of both DF/DHF). The AG129 mouse model has several of these characteristics and is currently being used by drug developers to evaluate the *in vivo* efficacy of promising DENV therapeutics (43,44). One of the drawbacks of this model for evaluating therapeutics is that viremia is transient making it difficult to evaluate the effect of drugs at different times post-infection. Also, since evidence of DF/DHF is observed only after infection with the mouse-adapted DENV2 strain, this model cannot yet be utilized to evaluate the effect of antiviral drugs on disease caused by the other DENV strains. Additional work needs to be done to develop better models for in vivo drug testing.

The last presentation by Lewis Markoff reviewed dengue animal models from the regulatory perspective. Different animal models should be employed to answer particular scientific questions about the vaccines or drugs being evaluated, such as vaccine attenuation, efficacy and safety. None of the dengue animal models currently available are sufficiently comprehensive to be used by themselves in the regulatory process but in combination they should be able to support the clinical evaluation of products in humans.

Summary and recommendations

The last session of the workshop was dedicated to an open discussion with meeting participants to identify scientific gaps and strategies to move the field forward.

There was general consensus that significant progress has been made with many of the animal models for dengue but that more work is required to complete the characterization of these models including viral replication, cellular and tissue tropism, immune responses and mechanisms of pathogenesis. Additional immunocompetent models should be generated to try to reproduce the full spectrum of dengue disease.

Until more clinically relevant models are developed, clinical studies will remain essential to answer important questions about the immune responses and pathogenesis of dengue in humans.

There was agreement that none of the models alone will ever address all the scientific questions about pathogenesis and the efficacy of drugs and vaccines. Rather, each model can address a different subset of scientific questions and a combination of models should be used to evaluate drugs and vaccines for efficacy and safety before they are tested in humans.

A limiting factor for many researchers who use non-human primates to study dengue is the very high cost and limited accessibility of these animals. It was suggested that the dengue community coordinate with researchers from other areas to enroll flavivirus-negative animals previously employed in other nonterminal studies.

It was pointed out that many of the groups working with the same animal models use different reagents and unit measures and that this has complicated the comparison of data across groups. For example, different groups measure virus challenge doses using particle-forming units, infectious doses or genome equivalents. It was suggested that standardized

sets of reagents and protocols be developed and made available to the research community as has been done in other fields (*e.g.* HIV). Some of the reagents that were suggested are new DENV strains (that produce high viremia) with matching probes, NHP primate reagents (including new sylvatic DENV strains and immunological reagents) and stem cell lines to develop humanized mouse models.

As many of the unsuccessful animal model development efforts are neither published nor disseminated to the community, some participants suggested that an internet-based forum where experts in the field can share their experiences and unpublished data, including well-controlled negative results should be established.

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Table 1

List of dengue animal models summarized in this report

Species	Immune status	Name	Routes of infection[a]	Dose of infection[b]	Detectable Viremia[c, d]	Anti-DENV immune responses[d]	DENV induced disease[e]	References
Mouse	Immunecompetent	Balb/C	IP	10^5	−/+	Ab (−/+), T-cell	-	10
	Humanized	hNOD/SCID	SC	10^4	+	Ab (−/+)	Fever, rash, thrombocytopenia	11
		hNOD/SCID/IL2Rγ^{null}	SC	10^6	+	Ab (−/+)	Fever, rash, thrombocytopenia	13
		hRAG2$^{-/-}$ γc $^{-/-}$	IP, SC	10^6	+	Ab	Fever, rash, shock	14
		SCID-HuH7	IP	10^4	+	-	-	30,37
	IFN-deficient	AG129 + DENV	IP	10^6	+	Ab	Paralysis	17
		AG129 + D2S10	IV	10^7	+	Ab, T-cells	Vascular leakage, thrombocytopenia, ADE	18_22
Swine	Immunecompetent	Yucatan miniature pig	SC, IV	10^7	+ (SC)	Ab	Rash (+/−)	-
Non- human primate	Immunecompetent	Rhesus macaque	SC, ID	10^5	+	Ab, T-cells	lymphadenopathy, splenomegaly, hepatomegaly, mild dehydration, mild rash, increased viremia (+/−)	24_27,29_30
	Immunecompetent	Chimpanzee	SC, ID	10^3–10^6	+	Ab	-	28,31

[a] IP: intraperitoneal injection; SC: subcutaneous injection; IV: intravenous injection.

[b] Doses expressed in plaque-forming units (PFU)

[c] Viremia detected by quantitative real-time PCR or by plaque assay.

[d] (−/+): these responses have not been detected consistently. Only immune responses that have been reported are listed here. It is possible that additional immune responses are induced in these animals but have not been reported.

[e] (−/+): these clinical signs have not been detected consistently. This is probably due to the outbreed nature of these animals.