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DISEASE IN WILDLIFE OR EXOTIC SPECIES

Rhabdomyosarcoma of Soft Tissues in an Adult Brook Trout (*Salvelinus fontinalis*)

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Summary

This report describes a spontaneously arising rhabdomyosarcoma of soft tissues in a brook trout (*Salvelinus fontinalis*). The lesion was examined by means of histology, immunohistochemistry (IHC) and transmission electron microscopy (TEM). The cross-reactivity of the primary antibodies used in the IHC was investigated *in silico* using the Protein Blast system. Microscopically, the lesion appeared as a 'small round cell' undifferentiated sarcoma with rare myotube formation. IHC identified expression of sarcomeric actin and vimentin and these molecules showed the highest protein sequence identity. Lower protein sequence identity coincided with negative immunolabelling for desmin, MyoD1, myogenin and CD3. TEM revealed myofibrils, but without a defined sarcomeric architecture. The diagnosis of solid alveolar rhabdomyosarcoma of soft tissues was achieved on the basis of histological and ultrastructural findings.

Keywords: immunohistochemistry; protein blast; rhabdomyosarcoma; salmonid

In human medicine, rhabdomyosarcoma (RMS) is a relatively rare cancer, but among children and young adults it is the most common soft tissue sarcoma. Common histological subclasses include embryonal, alveolar, botryoid and pleomorphic (Castleman *et al.*, 2011; Caserto, 2013; Tochitani *et al.*, 2013). The embryonal form (eRMS) occurs in children aged ≤10 years, accounts for ~67% of all RMS cases and has a favourable prognosis. In contrast, the alveolar form (aRMS) prototypically occurs in adolescents, comprises 30% of RMS cases and has a higher rate of metastasis following initial diagnosis (Keller and Guttridge, 2013). RMS is diagnosed infrequently in veterinary pathology (Turnbull, 2006) and reported cases of spontaneous RMS in fish are extremely rare (Turnbull, 2006). The zebrafish is considered to be a valid animal model for induced RMS (highly similar to human embryonal RMS) (Chen and Langenau, 2011).

The relative rarity of these tumours makes the diagnosis challenging and as a result they are sometimes simply included in the broad category of high-grade soft tissue sarcomas. The low prevalence of these neoplasms in the veterinary literature may be due to a failure to diagnose them because of their extreme variation in phenotype, age of onset and cellular morphology (Caserto, 2013). Histopathological findings of these primitive neoplasms typically consist of monomorphic sheets or packets of 'small round cells' with a lymphoid appearance or more mature stages characterized by obvious muscular differentiation (Caserto, 2013; D'cruze *et al.*, 2013). Immunohistochemistry (IHC) and transmission electron microscopy (TEM) are ancillary techniques that are often critical for the final diagnosis of RMS, because of the frequency of poorly differentiated tumours (Parham and Ellison, 2006).

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In this report we describe the application of recently proposed guidelines (Caserto, 2013) to the diagnosis of a rare spontaneously arising neoplasm, RMS, in a salmonid fish. An adult brook trout (*Salvelinus fontinalis*), from an Italian farm, was presented with an exophytic non-ulcerated mass (7.5 × 4.5 × 3.5 cm) in the area of the opercular openings. No therapeutic interventions were attempted and the fish was killed humanely. Samples of the mass were fixed by the local veterinary practitioner in 10% neutral buffered formalin and sent to our laboratory for histological investigations. After routine processing, sections (4 μm) were stained by haematoxylin and eosin (HE). Histochemistry was performed with phosphotungstic acid haematoxylin (PTAH) (Luna, 1968). IHC was performed as described in Table 1.

Prediction of the cross-reactivity of primary antisera and interpretation of immunolabelling took into account the sequence similarity of salmonid and human proteins. Protein identity was assessed *in silico*, by matching the primary amino acid sequence of salmonid and human proteins, against which the antibodies were directed. The matching was carried out using Protein BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome).

TEM was performed from the formalin-fixed samples, which were dehydrated in graded alcohols and embedded in Durcupan AcM resin (Sigma Aldrich, St. Louis, Missouri, USA). Semithin sections (1 μm) were stained with toluidine blue and examined by light microscopy. Selected ultrathin sections (90 nm) were stained with uranyl acetate and lead citrate. The ultrastructural observation was made with a Philips EM 208 transmission electron microscope (Philips, Eindhoven, The Netherlands) operating at 100 kV.

Grossly, the mass was not encapsulated and was well demarcated, fleshy and white to grey/cream in colour and glistening, with multifocal haemorrhagic foci. Microscopically, the tumour was composed of densely cellular sheets of monomorphic round cells, separated by thin fibrous septa. Neoplastic cells were round, 15 μm in diameter, discrete, with scant eosinophilic and fibrillar cytoplasm, a high nucleus to cytoplasmic ratio, round central and frequently indented or convoluted nuclei, with finely stippled or irregularly distributed chromatin and a prominent, often bizarre shaped, magenta-coloured nucleolus. Scattered throughout these round cells were rare multinucleated elongate cells with tandem nuclei and eosinophilic fibrillar cytoplasmic inclusions

Table 1
Immunohistochemical methodology and sequence similarity data for primary antisera

Antibody specificity	*Antibody name and source*	*Antigen retrieval method*	*Dilution*	*Human protein*	*Salmonid protein*	*E (significance)*	*Protein sequence identity*	*Homology*	*Cross-reactivity*
Myogenin	Mouse monoclonal antibody F5D (Ventana, Tucson, Arizona, USA)	95°C with Tris–EDTA pH 8.0 for 20 min	Prediluted	P15173.2	CAA87010.1 *Oncorhynchus mykiss*	5^{-75}	53%	Yes	No
Myod1	Mouse monoclonal antibody 5.8A (Dako, Glostrup, Denmark)	95°C with Tris–EDTA pH 8.0 for 20 min	1 in 50	P15172	NP_001117109.1 *Salmo salar*	5^{-114}	60%	Yes	No
Desmin	Mouse monoclonal antibody DE-R-11 (Ventana)	95°C with Tris–EDTA pH 8.0 for 20 min	Prediluted	P17661.3	CAC83053.1 partial *Oncorhynchus mykiss*	0	69%	Yes	No
Vimentin	Mouse monoclonal antibody V9 (Ventana)	95°C with Tris–EDTA pH 8.0 for 20 min	Prediluted	P08670.4	CAA90601.1 *Oncorhynchus mykiss*	0	74%	Yes	Yes
α–sarcomeric actin	Monoclonal antibody IgM (Sigma Aldrich, St. Louis, Missouri, USA)	95°C with citrate buffer pH 6.0 for 20 min	1 in 1,000	P60709.1	AAF80342.1 *Oncorhynchus mykiss*	0	98%	Yes	Yes
CD3 ε	Mouse monoclonal antibody F7.2.38 (Dako)	750 Watt with Tris–EDTA pH 8.0 for 10 min	1 in 50	AAH49847.1	ACZ57825.1 *Salmo salar*	2^{-5}	52%	Yes	No

Appropriate positive and negative controls were included in each experiment.

('strap cells') reminiscent of myotubes and large round to polygonal cells with abundant eosinophilic cytoplasm containing the same fibrillar cytoplasmic structures (myofibrils) (Fig. 1). Anisocytosis and anisokaryosis were moderate, mitotic activity was high with up to 15 mitoses per high-power (×400) field, with frequent atypical mitoses. Numerous apoptotic figures were detected. There were multiple haemorrhagic areas and skeletal muscle was infiltrated multifocally.

IHC revealed diffuse cytoplasmic expression by the neoplastic cells of sarcomeric actin (tested in carp; Sigma datasheet) (Fig. 2) and multifocal expression of vimentin. Myogenin, MyoD1, desmin and CD3 IHC were negative. Internal control of skeletal muscle showed non-specific cytoplasmic expression of myogenin and MyoD1 as the only true nuclear expression of these markers should be considered positive (Sebire and Malone, 2003). There was no labelling for desmin and vimentin and a positive result for sarcomeric actin in the internal control skeletal muscle. The internal control of lymphoid renal tissue was negative for CD3 expression.

TEM confirmed the presence of a monomorphic population of discrete round cells, with markedly convoluted nuclei with irregularly clumped, electron-dense chromatin. No distinct Z discs were noted in any neoplastic cells; however, aggregates of myofilaments were observed in single cells. Occasionally, myofilaments clustered together in parallel bands; however, clear organization in sarcomeres was lacking (Fig. 3).

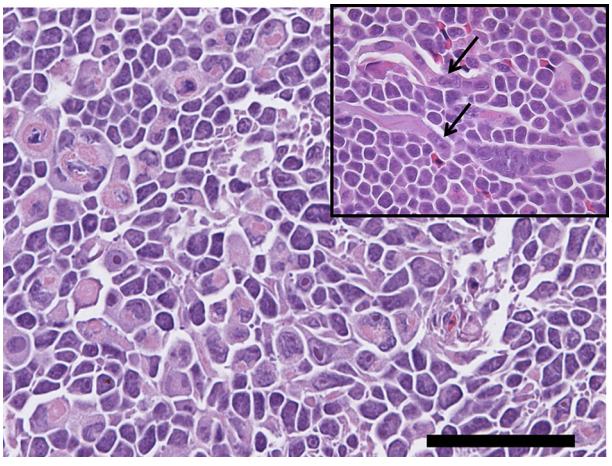

Fig. 1. Neoplastic cells showing bizarre shapes, anisocytosis and anisokaryosis, and a cytoplasm containing fibrillar eosinophilic material consistent with myofilaments. Occasional rare elongate multinucleated neoplastic cells with tandem nuclei and eosinophilic fibrillar cytoplasmic inclusions ('strap cells') reminiscent of myotubes are evident (Inset). HE. Bar, 50 µm.

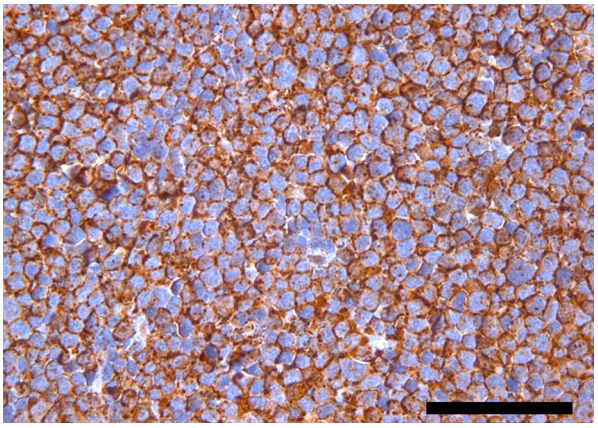

Fig. 2. IHC for sarcomeric actin. Neoplastic rhabdomyoblasts show diffuse coarse cytoplasmic labelling. Bar, 50 µm.

In fish, descriptions of spontaneous RMS are rare (Roberts, 2001). According to Iwanowicz *et al.* (2001) there are over 30 reports of rhabdomyogenic tumours in fish recorded from 1908, but these include rhabdomyomas and experimentally-induced cases. Recent reports of spontaneously-arising RMS include cutaneous neoplasia in four of 24 black plaice (*Pleuronectes obscurus*) diagnosed as dermal RMS (Syasina *et al.*, 1999), an embryonal RMS in a giant gourami (*Colisa fasciata*) (Iwanowicz *et al.*, 2001) and a cardiac RMS in a sea dragon (*Phyllopteryx taeniolatus*) (LePage *et al.*, 2012). In the first paper, tumours were characterized by striated muscular differentiation of neoplastic cells that morphologically resembled myoblasts and sometimes fused together to form myotubes containing cytoplasmic myofibrils (Syasina *et al.*, 1999). In the second paper, the neoplasm, located

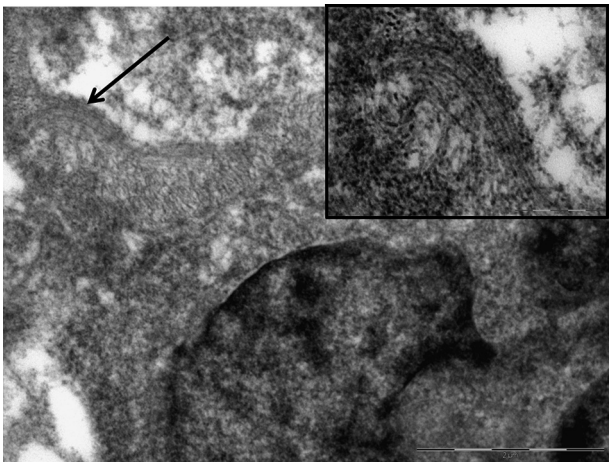

Fig. 3. TEM showing aggregates of myofilaments in the cytoplasm of single cells, clustered together in parallel or as haphazardly arranged bands (arrow, see Inset). Clear organization into sarcomeres was not evident. Bar, 2 µm.

in the region of the head, was comprised histologically of pleomorphic cells showing occasional clear cytoplasmic cross-striations, numerous multinucleated giant cells and rare mitotic figures (Iwanowicz *et al.*, 2001). In the third paper, the neoplasm was located in the ventricular lumen and was formed of multinucleated neoplastic cells (strap cells) with cytoplasmic striations, immersed in a myxoid matrix (LePage *et al.*, 2012). The histological features of these three cases are different to the case described herein because muscular differentiation was obvious in all three. In the case described in a sea dragon, other typical histological features included the presence of a myxoid basophilic matrix, moderate oedema and rare mitoses, while in the present case, tumour cells appeared densely packed and mitoses were numerous and atypical. Similar to the case report in giant gourami, some multinucleated giant cells were also noted in the present case.

In human and veterinary medicine, the histological classification of RMS consists of two main histotypes, eRMS and alveolar aRMS. The eRMS histotype includes three variants that are named according to their predominant cellular morphology. The two most common are represented by the myotubular variant of eRMS, which is dominated by multinucleated strap cells forming myotubes with frequent cross-striations (often only identified by histochemical staining with PTAH, which highlights striations as dark blue/purple lines among pale blue/purple myofibres) and the rhabdomyoblastic variant, which is dominated by round to polygonal cells with abundant eosinophilic cytoplasm with only rare PTAH-positive cross-striations (Van Vleet and Valentine, 2007; Caserto, 2013). The case described here shared some morphological features of both types of eRMS: predominant round to polygonal cells reminiscent of myoblasts and rare multinucleated cells reminiscent of myotubes (strap cells) containing cytoplasmic cross-striations; however, the scant amount of cytoplasm in the neoplastic cells was not typical of eRMS and could have erroneously led to the diagnosis of a lymphoid neoplasm.

The aRMS is characterized by small, poorly differentiated, round cells with scant cytoplasm, arranged into packets of cells separated from each other by fibrous connective tissue, forming alveolar-like patterns. aRMS is further subdivided into the classic alveolar pattern, which is characterized by extensive sloughing of neoplastic cells into a central open space with additional neoplastic cells lining the fibrous septa, and the solid variant, which is characterized by sheets of small round cells closely packed together with lack of rhabdomyoblastic differentiation (Van Vleet and Valentine, 2007; Caserto, 2013). In the present case, the cellular pattern was a poorly differentiated 'small round cell category' sarcoma, more similar to aRMS and in particular to the solid variant. Moreover, the absence in the majority of neoplastic cells of a clear muscular differentiation was closer to aRMS. The final diagnosis was a solid variant of aRMS.

Human aRMS and eRMS are thought to arise by a multistep process leading to loss of tumour suppressor genes and genes affecting the regulation of apoptosis and cellular senescence. Recent molecular studies have suggested that aRMS represents an arrested stage of development of undifferentiated myoblasts. The cause of the arrested stage of development could be related to the *PAX-FKHR* mutation and fusion, which leads to increased levels of MyoD1 and myogenin, resulting in cell proliferation and myogenic differentiation, respectively (Bentzinger *et al.*, 2012; Caserto, 2013).

In addition to routine histopathology, the panel of immunohistochemical markers proposed by Caserto (2013) was performed. IHC is useful in differentiating RMSs from other mesenchymal tumours or embryonal tumours, but the expression of muscle-specific markers depends on the degree of differentiation of the neoplastic cells along the myogenic lineage. In this case, there was diffuse marked positivity of the majority of the cells for sarcomeric actin and multifocal expression of vimentin, which are markers of striated muscle (Costa *et al.*, 2002) and employed in human and veterinary medicine in the diagnosis of RMS (Hill and Laskin, 2001; Caserto, 2013; Parham and Barr, 2013).

In contrast, MyoD1, myogenin and desmin, which are elective markers for the diagnosis of RMS (Caserto, 2013), were negative. In this case the protein sequence identity between human and salmonids for these two markers was low, so the negative result was likely due to a lack of cross-reactivity, as the internal control of fish striated muscle was also negative (Table 1).

In mammalian and avian muscle, myoblasts contain vimentin. When myoblasts fuse together to form myotubes, desmin integrates into the myotube's pre-existing vimentin filament system, acting as a scaffold for the organization of desmin during the early phase of myogenesis. Desmin is first expressed around the nucleus and later occupies the rest of the cell. Within mature skeletal muscle, intermediate filaments express desmin along the transverse bands surrounding the Z discs and connecting myofibrils (Cary and Klymkowsky, 1994).

MyoD1 and myogenin are myogenic nuclear regulatory molecules, which act as transcription factors and stimulate myogenesis. MyoD1 induces

differentiation by activating muscle-specific genes and is important in the switch from cellular proliferation to differentiation. Nuclear expression of MyoD1 is restricted to skeletal muscle and has been demonstrated to be a sensitive marker of myogenic differentiation. The antibody strongly labels the nuclei of myoblasts in developing skeletal muscle tissue, while the majority of adult skeletal muscle is negative. Like MyoD1, myogenin is expressed earlier in skeletal muscle differentiation than desmin. The antibody labels the nuclei of myoblasts in developing muscle tissue (Cessna *et al*., 2001; Sebire and Malone, 2003).

More specific to skeletal muscle is expression of proteins involved in sarcomere construction, such as the α-actin isoform found in sarcomeres, identified by the antibody sarcomeric actin. As skeletal myocytes differentiate further, they begin to accumulate sarcomeric actin (Caserto, 2013). This marker has been shown to be specific for identification of tumours with rhabdomyoblastic differentiation, even when they fail to mark with desmin (Carter and Hall, 1992).

Sequence comparison using BLAST is generally used in proteomics to infer homology on the basis of shared protein sequences (Pearson, 2013). In the present investigation, protein sequence matching was carried out to predict the potential immunohistochemical cross-reactivity of different antibodies. To the best knowledge of the authors, guidelines for the prediction of interspecies cross-reactivity are lacking, but sequence comparison is used as a preliminary step for peptide synthesis for monoclonal antibody production, to check for the intraspecies consistency of sequence and to avoid intraspecies epitope cross-reaction (Lindskog *et al*., 2005). In the present study, all of the assessed identities were over the cutoff value of 30% with significant E (expectation value), which can be interpreted as a protein homology, relevant from the evolutionary point of view (Pearson, 2013). Among all markers, only sarcomeric actin and vimentin were characterized respectively by 98% and 74% identity, a highly significant E value and a strong and specific cytoplasmic immunolabelling (Table 1). However considering other intrinsic variables such as sample quality, protein cross-linking and antigen retrieval, which may play a significant role in the success of immunolabelling, in-silico prediction cannot be used alone and the inclusion of positive and negative internal controls is always essential. The lack of cross-reactivity of the majority of the specific muscular markers tested with salmonid tissues made TEM a very helpful technique in identifying the myofilament framework of muscle cells and in confirming the diagnosis of RMS.

In conclusion, a spontaneously arising solid aRMS in a brook trout was described for the first time. The tumour was diagnosed by histology and confirmed by TEM, while IHC showed poor efficacy due to lack of antibody cross-reactivity. Considering the scant immunohistochemical investigations conducted on salmonids, in-silico sequence similarity screening could be proposed as a useful ancillary technique for predicting and corroborating the interpretation of IHC. Finally, solid aRMS should be considered as a differential diagnosis for 'small round cell tumours' in fishes.

Conflict of Interest Statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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References

Bentzinger CF, Wang YX, Rudnicki MA (2012) Building muscle: molecular regulation of myogenesis. *Cold Spring Harbor Perspectives in Biology*, **4**. pii: a008342.

Cary RB, Klymkowsky MW (1994) Differential organization of desmin and vimentin in muscle is due to differences in their head domains. *Journal of Cell Biology*, **126**, 445–456.

Carter RL, Hall JM (1992) A note on immunohistochemical staining for sarcomeric actin in rhabdomyosarcomas and other round cell tumours. *Histopathology*, **21**, 575–577.

Caserto BG (2013) A comparative review of canine and human rhabdomyosarcoma with emphasis on classification and pathogenesis. *Veterinary Pathology*, **50**, 806–826.

Castleman WL, Toplon DE, Clark CK, Heskett TW, Farina LL *et al*. (2011) Rhabdomyosarcoma in 8 horses. *Veterinary Pathology*, **48**, 1144–1150.

Cessna MH, Zhou H, Perkins SL, Tripp SR, Layfield L *et al*. (2001) Are myogenin and myoD1 expression specific for rhabdomyosarcoma? A study of 150 cases, with emphasis on spindle cell mimics. *American Journal of Surgical Pathology*, **25**, 1150–1157.

Chen EY, Langenau DM (2011) Zebrafish models of rhabdomyosarcoma. *Methods in Cell Biology*, **105**, 383–402.

Costa ML, Escaleira RC, Rodrigues VB, Manasfi M, Mermelstein CS (2002) Some distinctive features of zebrafish myogenesis based on unexpected distributions of the muscle cytoskeletal proteins actin, myosin, desmin, α-actinin, troponin and titin. *Mechanisms of Development*, **116**, 95–104.

D'cruze L, Dutta R, Rao S, Anuradha R, Varadarajan S *et al.* (2013) The role of immunohistochemistry in the analysis of the spectrum of small round cell tumours at a tertiary care centre. *Journal of Clinical and Diagnostic Research*, **7**, 1377–1382.

Hill KA, Laskin WB (2001) Fibroblastic lesions. In: *Clinical Pathology of Soft Tissue Tumors*, E Montgomery, AD Aaron, Eds., Taylor & Francis, London, pp. 105–106.

Iwanowicz LR, Goodwin AE, Harshbarger JC (2001) Embryonal rhabdomyosarcoma of the giant gourami, *Colisa fasciata* (Bloch & Schneider). *Journal of Fish Diseases*, **24**, 177–179.

Keller C, Guttridge DC (2013) Mechanisms of impaired differentiation in rhabdomyosarcoma. *FEBS Journal*, **280**, 4323–4334.

LePage V, Dutton CJ, Kummrow M, McLelland DJ, Young K *et al.* (2012) Neoplasia of captive yellow sea horses (*Hippocampus kuda*) and weedy sea dragons (*Phyllopteryx taeniolatus*). *Journal of Zoo and Wildlife Medicine*, **43**, 50–58.

Lindskog M, Rockberg J, Uhlén M, Sterky F (2005) Selection of protein epitopes for antibody production. *Biotechniques*, **38**, 723–727.

Luna LG (1968) *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*. McGraw-Hill, New York, pp. 85–86.

Parham DM, Ellison DA (2006) Rhabdomyosarcomas in adults and children. *Archives of Pathology and Laboratory Medicine*, **130**, 1454–1465.

Parham DM, Barr FG (2013) Classification of rhabdomyosarcoma and its molecular basis. *Advances in Anatomic Pathology*, **20**, 387–397.

Pearson WR (2013) An introduction to sequence similarity ('homology') searching. *Current Protocols in Bioinformatics*, **3**. Unit 3.1.

Roberts RJ (2001) Neoplasia in teleosts. In: *Fish Pathology*, 2nd Edit., RJ Roberts, Ed., Baillière Tindall, London, pp. 151–168.

Sebire NJ, Malone M (2003) Myogenin and MyoD1 expression in paediatric rhabdomyosarcomas. *Journal of Clinical Pathology*, **56**, 412–416.

Syasina IG, Sokolovsky AS, Phedorova M (1999) Skin tumours in *Pleuronectes obscurus* (Pleuronectidae) represent a complex combination of epidermal papilloma and rhabdomyosarcoma. *Diseases of Aquatic Organisms*, **39**, 49–57.

Tochitani T, Matsumoto I, Hoshino K, Toyosawa K, Kouchi M *et al.* (2013) Spontaneous rhabdomyosarcoma in a common marmoset (*Callithrix jacchus*). *Journal of Toxicologic Pathology*, **26**, 187–191.

Turnbull J (2006) Musculoskeletal system. In: *Systemic Pathology of Fish. A Text and Atlas of Normal Tissues in Teleosts and their Responses in Disease*, 2nd Edit., HW Ferguson, Ed., Scotian Press, London, pp. 289–311.

Van Vleet JF, Valentine BA (2007) Muscle and tendon. In: *Jubb, Kennedy, and Palmer's Pathology of Domestic Animals*, 5th Edit., Vol. 1, MG Maxie, Ed., Elsevier Saunders, Philadelphia, pp. 185–280.

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