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Running title:

Spontaneous Neoplasia in Captive Syngnathids

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Data Availability Statement

All relevant data are within the paper and its associated files.

Conflict of Interest Statement

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Abstract

Syngnathidae (seahorses, pipefish and seadragons) are charismatic species commonly kept in commercial aquaria, but published literature on syngnathid diseases is limited and immunohistochemical techniques not routinely employed. A retrospective review of 2541 syngnathid submissions received between March 2003 and October 2016 identified 18 neoplasms including germ cell tumours, exocrine pancreatic and intestinal carcinomas, chromatophoromas, and single cases of lymphoma, thyroid and renal carcinoma, swim bladder and pituitary adenoma. Big bellied seahorses accounted for 19% of submissions but 50% of neoplasms were diagnosed in this species. This study includes the first reported cases of germ cell tumours, chromatophoroma, thyroid carcinoma and pituitary adenoma in *Syngnathidae* and the first reports of neoplasia in pipefish species. Out of nine commercial antibodies trialled for immunohistochemical characterisation of neoplastic tissue only pan-cytokeratin proved cross-reactive. Electron microscopy was performed on four cases. Tumours should be considered as differential diagnosis in cases with buoyancy issues, debilitated or emaciated animals, and may predispose to secondary infections. This study highlights the value of histopathological disease surveillance for commercial aquarium settings.

Key words

Syngnathid, seahorse, pipefish, neoplasia, immunohistochemistry, transmission electron microscopy

Introduction

Seahorses, pipefish and seadragons are bony marine fish found globally which belong to the family *Syngnathidae*. These charismatic fish have intrigued people for centuries and inspired many myths and legends. It is therefore unsurprising that they are very popular within public aquarium and hobbyist communities.

Although neoplasms have been previously described in syngnathids, reports of these lesions are sparse and the only large study (n=172) was restricted to seven neoplasms and two neoplastic-like lesions in three syngnathid species derived exclusively from one collection (LePage et al., 2012). Morphological diagnoses of entities involved in reported cases to date are based exclusively on histomorphological features, without additional ancillary testing.

The purpose of this study was to conduct a comprehensive review of archive material of the Pathology Department of the International Zoo Veterinary Group (IZVG) between 2003 and 2016 to establish the prevalence, tissue involvement and biological behaviour of neoplasms in *Syngnathidae*, compare findings to the previous study (LePage et al., 2012), and further characterise tumours by immunohistochemistry and electron microscopy where indicated.

Material and Methods

A retrospective review of submissions received between March 2003 and October 2016 (n=25936) was undertaken and 2541 syngnathid cases were identified. Eighteen neoplasms were included in the study, initially classified based on morphological diagnoses made by veterinary pathologists specialised in zoo and wildlife pathology. Immunohistochemistry was performed according to Standard Operating Procedures of the Veterinary Pathology Department of the University of Liverpool, UK, using an automatic immunostainer (Link 48, Dako). Nine commercially available antibodies routinely used for diagnostic purposes in domestic species were trialled, including pan-cytokeratin, vimentin, PNL-2, Melan-A, S100, CD20, CD3, IBA-1 and Lysozyme. Sections of a whole big bellied seahorse served as positive and negative controls for immunohistochemistry. Subsequently, electron microscopy was carried out on four cases (two dysgerminomas, one seminoma, one chromatophoroma) from paraffin embedded material following a previously described protocol (Finotello, Masserdotti, Baroni, & Ressel, 2017).

Results

The study population comprised 1969 seahorses, 416 pipefish and 156 sea dragons. An overview of species is available in Table 1. Most numerous were big bellied (n=495), slender (n=318), lined (n=302), and yellow (n=213) seahorses, broad nose pipefish (n=142) and weedy seadragons (n=114). Overall, syngnathid submissions were submitted by 64 different collections dominated by commercial aquarium attractions, including distribution and breeding centres. They were located in the UK (n=40), Germany (n=10), China (n=2), Denmark, Finland, France, Ireland, Italy, Portugal, South Korea, Spain, Thailand, The Netherlands, Turkey and the United Arab Emirates. Twenty-two collections submitted 1-9 cases, 20 collections 10-50 cases, 16 collections 51-99 cases and 6 collections 100 cases or more (up to 252). The 18 neoplastic cases derived from 13 individual collections located in China, Denmark, Germany, Spain, The Netherlands and the UK. A single case was submitted by 8 collections, two cases by 5 collections, respectively. The overall prevalence of neoplasms submitted was 0.7% (18/2541), however, prevalence per collection ranged as wide as 0.8% (2/252) to 13.3% (2/15). Cases were not seen from collections that submitted less than 10 cases over the duration of the study (equating to >1 case submission per year), and from only two collections submitting under 50 cases (averaging 3.8 submissions per year). The age of animals diagnosed with neoplasms was given as “adult” in 14 cases, 5 years in two cases, and >4 and 2 years in one case, respectively. Neoplasms were detected in 11 males and 7 females; species affected included big bellied seahorse (n=8), long snouted seahorse (n=4), broad nose pipefish (n=3) and lined, short snouted and thorny seahorse (each n=1). In comparison, big bellied seahorses comprised 19% of submissions but were affected by 50% of neoplasms (8/16). The prevalence of neoplasia in pipefish was equivalent to the overall prevalence in the study population (0.7%); no cases occurred in seadragons. Neoplasms were the primary cause of death in 7/18 cases (38%), whereas infectious branchitis unrelated to neoplasia was regarded the cause of death further 7/18 cases (38%). The cause of death was unclear in the remaining 4 case (22%). The annual distribution of syngnathid submissions, and neoplasms is depicted in Fig. 1; no neoplasms were detected prior to 2010. On average, 2.75 cases were seen per subsequent year (2010-2016). The highest number of cases was seen in 2016 (n=7, 38.8%).

Diagnosed neoplasms were classified as ovarian and testicular germ cell tumours (n=5), exocrine pancreatic and intestinal carcinomas (each n=3), chromatophoromas (n=2), lymphoma, thyroid and renal carcinoma, swim bladder and pituitary adenoma (each n=1). Table 2 provides details of species, clinical history, age, sex, and tumour types. Table 3 correlated tumour types with concurrent disease processes and the cause of death.

Epithelial tumours labelled positive with pan-cytokeratin, whereas the other antibodies used showed no cross reactivity both in neoplastic lesions and in control tissues. Table 4 provides an overview of antibodies utilised and their reactivity in tumour cases and control material.

Electron microscopy was carried out on representative cases of two dysgerminomas, a chromatophoroma, and a seminoma, respectively. Relevant findings are included below.

Germ cell tumours (dysgerminomas, testicular germ cell tumour, seminoma):

Five big bellied seahorses (three females, two male) presented with gonadal neoplasms. Findings in the ovary were most consistent with dysgerminomas in female cases (Fig. 2 A/B). Tumours effaced normal ovarian architecture and entrapped or compressed remaining ovarian follicles and follicular debris. Neoplasms were poorly circumscribed, unencapsulated, densely cellular and composed of round cells arranged in sheets and occasional cord, separated by a scant fibrous connective tissue stroma. Neoplastic cells had distinct cell borders, moderate amounts of eosinophilic granular to vacuolated cytoplasm and large, centrally placed, irregularly round, vesicular nuclei with 1-2 prominent magenta nucleoli. Moderate anisokaryosis and anisocytosis were evident, with occasional multinucleation and large pleomorphic nuclei. Up to 3 mitoses per HPF (some atypical) and multifocal individual cell necrosis were seen. Immunohistochemistry was unrewarding. On electron microscopy, neoplastic round cells had fragmented nucleoli and dispersed chromatin. Further detailed examination was impaired by the fixation quality.

A morphologically similar neoplasm composed of poorly differentiated round cells was identified in one male and diagnosed as a testicular germ cell tumour (Fig. 2D). Another male presented with a metastasising seminoma (Fig. 2C), characterised by widespread infiltration of the coelom by a multilobular, densely cellular neoplasm composed of dense round cells with relatively distinct cellular borders, moderate amounts of stippled eosinophilic cytoplasm and peripheralised round anisokaryotic nuclei with pale basophilic finely stippled chromatin and one distinct basophilic nucleolus. There were moderate anisocytosis and anisokaryosis. Mitotic index ranged from 0-2 per HPF. Clusters of neoplastic cells were enclosed and embedded within large amounts of dense fibrous stroma (scirrhous reaction) which formed multifocal tubular spaces, often enclosing marginally palisading neoplastic cells. Areas of necrosis comprised colonies of Gram-negative bacteria.

Tumour metastases enclosed the distal oesophagus, effaced parts of the kidney, liver and integument are were seen within multiple vessels throughout the fish. Intraluminal testicular cells resembled disseminated neoplastic cell populations. On electron microscopy a more detailed examination of the seminoma was severely impaired by the fixation quality.

Carcinomas (intestinal, exocrine pancreatic, thyroid, renal) and adenomas (swim bladder and pituitary):

Tumours typical of carcinomas were detected in the intestines (n=3), exocrine pancreas (n=3), thyroid (n=1) and kidney (n=1) of 8 cases, and a swim bladder and pituitary adenoma, respectively, were seen in two animals (Figs. 3 and 4). No species predisposition was evident; carcinomas were seen in three broad nose pipefish, two long snouted seahorses and a big bellied, thorny, short snouted seahorse, respectively.

Growth pattern of intestinal and pancreatic carcinomas was aggressive, with infiltration into the coelomic cavity in all cases and variable compression of adjacent tissues. Intravascular metastases were detected in two cases of pancreatic carcinomas. Body condition was emaciated in three fish, moderate in two and good in one case, and debilitation was considered the underlying cause of concurrent fatal disease processes (amoebic and bacterial gill disease) in three cases. Intestinal parasites were not detected.

Metastases were not seen in thyroid or renal carcinomas. Due to its size and accompanying compression of the brain, the pituitary adenoma was considered clinically relevant. The swim bladder adenoma was likely responsible for buoyancy issues reported in the clinical history of the affected case.

Carcinomas stained positive with pan-cytokeratin, with cellular positivity ranging from <5 to 30% (intestinal carcinomas), 10-70% (pancreatic carcinomas), 40% in thyroid and 80% in renal carcinoma, respectively, with variable staining intensity (see Table 5).

Chromatophoromas:

Pigment cell tumours occurred on the tail of a long snouted and in the branchial arch of a big bellied seahorse, respectively (Fig. 5A). Both were most consistent with malignant melanophoromas, based on the presence of melanin (confirmed by Masson Fontana staining). Secondary amoebic gill disease was diagnosed in the big bellied seahorse. Vimentin, Melan-A and S100 employed for immunohistochemistry were not cross reactive. Electron microscopy successfully identified large electron-dense granules within the cytoplasm of the neoplastic cells (Fig 5B).

Lymphoma:

A single big bellied seahorse presented with a disseminated round cell tumour widely expanding vessels and infiltrating the parenchyma of most viscera. Tumour cells were arranged in sheets, approximately two times red cell size, uniformly round, with minimal cytoplasm and dense, basophilic chromatin. They resembled small lymphocytes, and a diagnosis of malignant lymphoma was made based on cellular morphology (Fig. 5 C/D). Immunohistochemical markers employed for further confirmation (CD-20, CD-3, Lysozyme) were not cross reactive.

Discussion

Morbidity and mortality of syngnathid species in captivity is primarily related to infectious diseases lead by bacterial infections, specifically mycobacteriosis and bacterial dermatitis, as well as parasitic infections, in particular scuticociliatosis and amoebic gill disease. Gas bubble disease and epaxial myopathy are important environmental diseases (LePage et al., 2015; Stidworthy, 2017). To date, studies into and reports of neoplasms are rare (LePage et al., 2012; Stilwell, Boylan, Howard, & Camus, 2018; Willens, Dunn, & Frasca, 2004). 2541 syngnathid cases could be reviewed for this study, compared to 172 animals included in previous research (LePage et al., 2012), and 41 different species from 64 collections with a worldwide distribution were included. The prevalence of neoplasms in our study equates to 0.7%, low compared to previous results in captive yellow seahorses (4.1%) housed at a single collection. Prevalence data per collection ranged as wide as 0.8% (2/252) to 13.3% (2/15) in our study, however, these results are based on submitted cases only, constituting a markedly differing representation of cases per seahorse population housed in respective aquaria. Therefore, the prevalence of 0.7% is considered more accurately reflective of the evaluated captive population.

In the wild, the average life span for seahorse ranges from 4 to 6 years, captive populations average 4.25 years, though this varies significantly between syngnathid species and is significantly influenced by stress, poor conditions, and the high risk of disease in captive scenarios. The youngest animal affected in the present study was 2 years of age, all animals were adult on submission. No sex predilection could be determined in the study population. Big bellied seahorses, which accounted for 19% of the submission material, showed the highest prevalence of neoplasms (50%, 8/16). This may be related to the comparatively longer life span of this species (7 years). In our study, neoplasms were not detected prior to 2010, with the highest number of cases per year seen in 2016 (see Fig. 1). A steady increase in case numbers over the years (see Fig. 1) is a likely contributing factor. Cases submitted in

2016 were derived from five different collections, occurred in 3 different species and comprised five different tumour entities. No common predisposing factor was recognised.

Tumours previously reported in syngnathids include a cardiac rhabdomyosarcoma (n=1), renal adenoma and adenocarcinoma (n=1, respectively), renal round cell tumours (n=2), exocrine pancreatic carcinoma (n=1), intestinal carcinoma (n=1), swim bladder adenoma (n=1), brood pouch fibrosarcoma (n=1) and a dermal tumour of vascular origin, most closely resembling a lymphangioma (n=1) (Boylan et al., 2015; LePage et al., 2012; Stilwell et al., 2018; Willens et al., 2004). Taken together with the data in our study, most commonly reported tumours in syngnathids to date are gonadal germ cell tumours (n=5), intestinal and exocrine pancreatic carcinomas (n=4, respectively), lymphoma/round cell tumours (n=3), renal adenocarcinoma/adenoma (n=3) as well as chromatophoromas and swim bladder adenomas (n=2, respectively). Our study includes the first reports of thyroid carcinoma and pituitary adenoma in syngnathids.

Germ cell tumours (GCTs) arise from primordial germ cells and can occur in the testis, the ovary or extragonadal sites. They are broadly divided in two classes; germinomatous or seminomatous germ-cell tumours include dysgerminoma and seminoma. The nongerminomatous or nonseminomatous germ-cell tumours include all other germ-cell tumours, pure and mixed. Gonadal neoplasms are rare in fish; individual reports of seminomas in African lungfish and sea bass are recognised (Masahito, Ishikawa, & Takayama, 1984; Nigrelli & Jankowska, 1953; Weisse, Weber, Matzkin, & Klide, 2002) and germ cell tumours are reported in experimental zebra fish models (Sanchez & Amatruda, 2016). This is the first report of germ cell tumours in a syngnathid species, the big bellied seahorse. Previously, a connection has been made between ovarian neoplasms and exposure to chemical agents in laboratory fish (Grizzle & Goodwin, 1998), and in recent years, increasing amounts of research suggest that emerging oestrogenic pollutants in the aquatic environment may be associated with reproductive neoplasms (Adeel, Song, Wang, Francis, & Yang, 2017) and increasing number of intersex animals (Brighty, 2002). Hormone levels are to date not routinely measured in aquarium settings, but it is likely that fish are exposed to increasing levels of a range of environmental pollutants, including endocrine disrupters.

Odontomas of freshwater angelfish associated with retroviral infections are the most significant alimentary neoplasms of fish, other tumours of the alimentary tract are rare in all fish and are therefore not regarded as significant in ornamental or display fish (Groff, 2004). In syngnathids, intestinal and pancreatic carcinomas are aggressive tumours that can metastasise, are associated with emaciation, and resulting debilitation may predispose affected individuals to concurrent fatal infections. In zebrafish, carcinomas and mixed malignant intestinal neoplasms have been associated with the nematode parasite *Pseudocapillaria tomentosa* (Kent, Bishop-Stewart, Matthews, & Spitsbergen, 2002). Intestinal parasites were not evident in any of the affected individuals in our study or the previously reported case (LePage et al., 2012). Intestinal adenocarcinomas have also been reported in fish models for environmental carcinogenesis (Bailey, Williams, & Hendricks, 1996) after exposure to chemical agents. Environmental carcinogens may play a role and a link has also been established in humans between dietary factors and alimentary cancer (Ryan-Harshman & Aldoori, 2007). A large proportion of syngnathids in our study received a predominantly live diet of enriched artemia, copepods and mysis shrimp, very similar to their diet in the wild. Though a degree of accumulation of toxins through the food chain cannot be excluded, this has a greater impact on predators at the end of the chain. Diets rich in highly processed food or deviating markedly from natural diets, factors proposed in other species including humans, are unlikely to play a role.

Spontaneous neoplasms of the exocrine pancreas (adenomas, adenocarcinomas and cystadenomas) are rare in fish. They have, however, been induced in teleosts exposed to diethylnitrosamine, methylazoxymethanol, and polycyclic aromatic hydrocarbons (Grizzle & Goodwin, 1998).

Round cell tumours presumptively diagnosed as lymphomas were evident in one case in this study, and two yellow seahorses previously described (LePage et al., 2012). Vascular metastasis and systemic disease were a feature in all cases. Infection with retrovirus has been associated with lymphosarcoma in some fish species such as Japanese medaka, northern pike and muskellunge (Groff, 2004; Harada, Hatanaka, & Kubota, 1990; Papas, Dahlberg, & Sonstegard, 1976), however, virus associated round cell tumours appear to occur with a higher prevalence in these species. Due to active haematopoiesis in the kidney, this is a likely site of origin for lymphohaematopoietic neoplasms.

Though different types of non-haematopoietic renal neoplasms including nephroblastoma, adenocarcinoma, renal cell carcinoma, cystadenoma and renal tubular adenoma have been reported in fish, spontaneous renal neoplasms are considered rare in fishes (Groff, 2004; LePage et al., 2012; Lombardini, Hard, & Harshbarger, 2014). Taken together with findings in the previous study, three cases of renal carcinoma/adenoma were identified, arising in two yellow seahorses and a broad nose pipefish, constituting the first report in the latter species. In rare instances there is suggestion of a genetic predisposition of certain populations to specific renal neoplasms, environmental carcinogenesis, or speculation of an unknown infectious aetiology acting as a promoter. A genetic basis is not considered to play a role in syngnathids.

Chromatophoromas are tumours of pigment-producing cells of the skin, subclassified based on the type of pigment and include erythrophoromas, melanophoroma, xanthophoromas, guanophoromas, and iridophoromas. Pigment cell neoplasms are common in fish and are thought to be the primary type of neoplastic disease in certain fish species, possibly linked to a genetic basis or exposure to chemicals (Groff, 2004; Hayes & Ferguson, 1989). This is the first report of pigment cell tumours in seahorses, and the first report of a pigment cell tumour occurring in the opercular cavity.

The swim bladder of fish is an out-pocketing of the digestive tube that contains gas and functions as a hydrostatic organ. It is therefore unsurprising that swim bladder lesions result in buoyancy issues. Retrovirus infections have previously been associated with aggressively behaving swim bladder sarcomas in salmon (Paul et al., 2006).

Specific conditions of endocrine glands are rarely recognised in fish, and the endocrine tumours thyroid carcinoma and pituitary adenoma have not previously been reported in syngnathid species or more generally. Thyroid tumours, which can be grossly evident as expansile mass lesions in the ventral opercular cavity, need to differentiated histology from thyroid hyperplasia or goitre, common conditions in various wild and captive teleosts associated with poor water quality and abnormal nutrition (Hoover, 1984). On immunohistochemistry, TTF-1 signal could not be seen in either thyroid carcinoma or control thyroid, suggesting that the antibody was not cross-reacting with syngnathid tissue.

In teleosts, the pituitary gland is composed of two embryologically distinct components, the neurohypophysis and the adenohypophysis. Neoplasms of the pituitary could not be identified in a previous large scale review of neoplasms in fish (Groff, 2004). Specific chemical toxicity has been reported to cause enlargement, pseudocystic fluid-filled spaces, congestion and changes in tinctorial properties (Couch, 1984) but, to the best of the authors' knowledge, this is the first report of a pituitary tumour in a teleost.

Immunohistochemistry (IHC) is not commonly employed for routine diagnostics in fish species and limited information is available on the cross reactivity of antibodies used commonly in veterinary diagnostic settings. As part of this study, the authors aimed to try and further investigate the potential usability of nine commercially available antibodies. Though previous publications could demonstrate cross-reactivity of a range of antibodies including cytokeratin, chromogranin A, S100, desmin and α-smooth muscle actin in some fish species (Coffee, Casey, & Bowser, 2013; Paquette et al., 2015), a case report utilising endothelial markers (CD31, Factor VIII, VEGF, VEGF-R) to try and characterise a dermal mass from a lined seahorse showed no antibody cross reactivity (Boylan et al., 2015). In our syngnathid case material, only pan-cytokeratin was cross reactive. This could be due to several factors. Fixation is an important cause of variation in the reproducibility of IHC. For most tissues submitted for routine histology, fixation for 24 hours in room temperature is recommended. The submission process of fish cases often includes prolonged fixation and decalcification times, fixation in formalin and alternative fixatives (for example ROTI®Histofix ECO Plus) and the tissue to fixative ratio can vary significantly. Duration of fixation, fixative formula, and tissue to fixative ratio can affect the extent and intensity of IHC (Engel & Moore, 2011), and target antigens may be distorted by the process.

Several of the antibodies utilised in our study are mouse monoclonal antibodies, and it is feasible that the targeted epitope is not expressed in syngnathid tissues. Utilisation of polyclonal antibodies, which recognise multiple epitopes, might prove more sensitive.

Antibody binding epitopes can be masked in formaldehyde-based fixation due to cross-linking of amino groups on adjacent molecules, in addition to the formation of methylene bridges. For this reason, antigen retrieval, an additional step to unmask the epitope, is sometimes required. In our study, all antibodies underwent heat induced antigen retrieval in low or high pH.

Transmission electron microscopy (TEM) is an established technique to characterise fine subcellular details in neoplasms. In the present case series, TEM was useful to conclusively characterise one tumour type (chromatophoroma). In the other two cases (germ cell tumours), the ultrastructural features were suboptimal and failed to identify robust cellular details in order to support a more definitive diagnosis. This is a common pitfall in the processing of TEM material from formalin fixed and paraffin embedded samples, frequently the only material available for the investigation of archive cases.

It is noteworthy that no cases were seen from collections that submitted less than 10 cases over the duration of the study (equating to >1 case submission per year), and from only two collections submitting under 50 cases (averaging 3.8 submissions per year). No data is available to highlight the respective percentages of stock submitted; however, this demonstrates the value of routine surveillance of aquarium stock.

In summary, as surveillance of diseases of captive syngnathids by histological screening continues to improve, neoplasia may become a more frequently diagnosed cause of morbidity and mortality. This study includes the first reported cases of germ cell tumours, chromatophoroma, thyroid carcinoma and pituitary adenoma in *Syngnathidae* and the first reports of neoplasia in pipefish species. Tumours should be considered as differential diagnosis in debilitated or emaciated animals, buoyancy issues and may predispose to secondary infections.

Exposure to environmental pollutants, including endocrine disrupters may play a role. At present, routine water testing does not include screening for a range of potentially harmful chemicals. Going forward, evaluation of hormone levels could prove to be a useful surveillance tool.

Pan-cytokeratin can be used successfully to characterise syngnathid tissues. Further studies into the utilisation of additional polyclonal antibodies may be beneficial.

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Table 1: Overview of sygnathid species reviewed including scientific names.

Seahorse species	**Scientific name**	**No**	**Pipefish species**	**Scientific name**	**No**	**Sea dragon species**	**Scientific name**	**No**
Big bellied seahorse	Hippocampus abdominalis	495	Broad nose pipefish	Syngnathus typhle	142	Weedy sea dragon	Phyllopteryx taeniolatus	114
Slender seahorse	Hippocampus reidi	318	Snake pipefish	Entelurus aequoreus	81	Ribboned sea dragon	Haliichthys taeniopherus	25
Lined seahorse	Hippocampus erectus	302	Greater pipefish	Sygnathus acus	54	Leafy sea dragon	Phycodurus eques	17
Yellow seahorse	Hippocampus kuda	213	Alligator pipefish	Syngnathoides biaculeatus	47			
Barbour's seahorse	Hippocampus barbouri	136	Banded pipefish	Doryrhamphus dactyliophorus	23			
Long snouted seahorse	Hippocampus guttulatus	116	Blue striped pipefish	Doryrhamphus excisus	14			
Short snouted seahorse	Hippocampus hippocampus	81	Janss pipefish	Doryrhamphus janssi	6			
Tiger tail seahorse	Hippocampus comes	61	Scribbled pipefish	Corythoichthys intestinalis	6			
Dwarf seahorse	Hippocampus zosterae	29	Black striped pipefish	Syngnathus abaster	5			
White's seahorse	Hippocampus whiteii	29	Ghost pipefish	Solenostomus cyanopterus	4			
Narrow bellied seahorse	Hippocampus angustus	21	Many banded pipefish	Dunckerocampus multiannulatus	4			
Sea pony	Hippocampus fuscus	21	Lesser pipefish	Syngnathus rostellatus	3			
Pacific seahorse	Hippocampus ingens	13	Ribboned pipefish	Haliichthys taeniophorus	3			
Japanese seahorse	Hippocampus japonicus	12	Long snouted pipefish	Doryichthys boaja	2			
Tiger snout seahorse	Hippocampus subelongatus	7	Short snouted pipefish	Pugnaso curtirostris	2			
Kelloggi seahorse	Hippocampus kelloggi	7	Worm pipefish	Nerophis lumbriciformis	2			
Thorny seahorse	Hippocampus histrix	6	Spotted pipefish	Nerophis maculatus	1			
Cape seahorse	Hippocampus capensis	3	Darkflank pipefish	Sygnathus taenionotus	1			
Species unknown		99	Yellow banded pipefish	Dunckerocampus pessuliferus	1			
			Species unknown		15			

Table 2: Details of species, clinical history, age, sex, and tumour types.

Species	**Clinical history**	**Age**	**Sex**	**Tumour type**	**Cause of death**
Long snouted seahorse	Abnormal position in tank (at bottom)	Adult	Female	Chromatophoroma	**Neoplasm**
Thorny seahorse	No presenting signs	Adult	Female	Intestinal carcinoma	Branchitis
Big bellied seahorse	Suspected mycobacteriosis	5 years	Male	Lymphoma	Branchitis
Short snouted seahorse	Lethargy	Adult	Female	Thyroid carcinoma	Branchitis
Big bellied seahorse	Several deaths in group over 12 months, suspected age related	Adult	Female	Ovarian dysgerminoma	Hepatic lipidosis
Big bellied seahorse	Wound caudal tail	Adult	Male	Seminoma	**Neoplasm**
Big bellied seahorse	No presenting signs	Adult	Female	Ovarian dysgerminoma	Unclear
Big bellied seahorse	Parasites seen, copepods and Uronema	Adult	Female	Ovarian dysgerminoma	Unclear
Lined seahorse	Abnormal position in tank (at bottom)	Adult	Male	Swim bladder adenoma	Branchitis
Long snouted seahorse	Nodules found in intestines	Adult	Female	Pancreatic carcinoma	Neoplasm
Big bellied seahorse	Swollen head and body	> 4 years	Male	Chromatophoroma	Branchitis secondary to neoplasm
Long snouted seahorse	Abdominal distension by fluid	Adult	Male	Pancreatic carcinoma	**Neoplasm**
Big bellied seahorse	Bilateral discolouration below gills	5 years	Male	Intestinal carcinoma	**Neoplasm**
Broad nose pipefish	Gas bubbles	Adult	Male	Pancreatic carcinoma	Branchitis
Big bellied seahorse	Euthanised, unknown cause	Adult	Male	Testicular germ cell tumour	Hepatic lipidosis
Long snouted seahorse	Skinny and pale	Adult	Male	Intestinal carcinoma	Branchitis
Broad nose pipefish	Sudden death, no clinical signs	Adult	Male	Renal carcinoma	Branchitis
Broad nose pipefish	Sudden death	2 years	Male	Pituitary adenoma	**Neoplasm**

Table 3: Correlation of observed tumour types with concurrent disease processes and the causes of death.

Tumour type	**Concurrent diseases**	**Cause of death**
Ovarian dysgerminoma	Chronic hepatopathy, adenomatous intestinal hyperplasia	Hepatopathy, unclear
Pancreatic carcinoma	Nematodiasis, branchitis (AGD[‡]+Uronema), emaciation	Tumour metastases, branchitis
Intestinal carcinoma	Branchitis (Ichthyobodo and BGD[§], AGD), cestodiasis, MB[**], emaciation, epaxial myopathy	Branchitis / debilitation, carcinoma
Chromatophoroma	AGD, ovarian degeneration, hepatic lipidosis	Branchial melanoma +/- AGD
Lymphoma	AGD	AGD and neoplasm
Seminoma	NA	Seminoma metastases
Testicular germ cell tumour	Hepatic lipidosis, hyperplasia brood pouch	Hepatic lipidosis, buoyancy
Thyroid carcinoma	AGD	Branchitis
Renal carcinoma	Branchitis with AGD and Uronema, MB	Branchitis
Pituitary adenoma	Renal myxozoans	Pituitary adenoma
Swim bladder adenoma	MB	Branchitis

[‡] AGD = amoebic gill disease
§ BGD = bacterial gill disease
** MB= mycobacteriosis

Table 4: Overview of antibodies utilised and their reactivity in tumour cases and control material.

Antibody	Species	Clone	Manufacturer	Reactivity	Comments
Pan-Cytokeratin	Mouse monoclonal	Clones AE1/AE2	Dako M3515	Yes	50% of neoplasms epithelial (pancreatic, intestinal, renal, thyroid, swim bladder)
Vimentin	Mouse monoclonal	Clone V9	Dako M0725	No	
PNL-2	Mouse monoclonal		Santa Cruz sc59306	No	
Melan-A	Mouse monoclonal	A103	Dako M7196	No	Stained mucus in goblet cells
S100			Dako Z0311	No	Non-specific staining of renal tubules
CD-20	Epitope specific rabbit antibody		ThermoScientific RB-9013-P	No	
CD-3	Rabbit polyclonal		Dako A0452	No	
Iba-1	Goat polyclonal		LifeSpan Biosciences Inc LS-B2402 AIF1	No	Non-specific diffuse staining
Lysozyme	Rabbit polyclonal E.C. 3.2.1.17		Dako A0099	No	

Table 5: Variable staining intensities of carcinomas with pan-cytokeratin.

Morphological diagnosis	**Positivity to pan-cytokeratin (%)**	**Intensity of stain**
Intestinal carcinoma	30	++
Intestinal carcinoma	15-20	++
Intestinal carcinoma	<5	+/-
Pancreatic carcinoma	70	+
Pancreatic carcinoma	50	+/-
Pancreatic carcinoma	60	++
Thyroid carcinoma	40	++
Renal carcinoma	80	+++

Legend:	
Annotation	**Intensity of stain**
-	Negative
+/-	Very weak
+	Weak
++	Moderate
+++	Marked

Figure legends

Figure 1: Annual distribution of sygnathid cases submitted to IZVG Pathology between 2003-2016 (blue) and correlation with neoplasms occurring over the same period (orange). No neoplasms were diagnosed prior to 2010.

Figure 2: Germ cell tumours, H&E.

A and B: Adult big bellied seahorse, ovary, A x2, B x20. Dysgerminoma: Effacement of ovarian architecture by densely packed sheets of poorly differentiated germ cells. Remnants of follicular debris (arrowhead) secondary to follicular collapse are intermingled with neoplastic populations.

C: Adult big bellied seahorse, testis, x2, inset x10. Seminoma: Densely packed round cells (germ cells) with distinct cell borders, scant amounts of cytoplasm and large, round, vesiculate nuclei with coarse chromatin widely expand and efface seminiferous tubuli.

D: Adult big bellied seahorse, testis, x10. Testicular germ cell tumour: Effacement of testicular architecture by densely packed sheets of poorly differentiated germ cells. Findings are similar to those seen in the ovary (A, dysgerminoma).

Figure 3: Pancreatic and intestinal carcinoma, H&E and immunohistology.

A and C: Adult male long snouted seahorse. A: Pancreatic carcinoma: Packets of metastasising neoplastic epithelial cells markedly expand gills, x10. C: A pancreatic carcinoma was evident in the coelomic cavity and labelled positive with pan-cytokeratin, x40.

B and D: Adult male long snouted seahorse. B: Intestinal carcinoma: The mucosa is expanded by nests and tubuli of neoplastic epithelial cells, x2. C: Neoplastic cells label positive with pan-cytokeratin, x40.

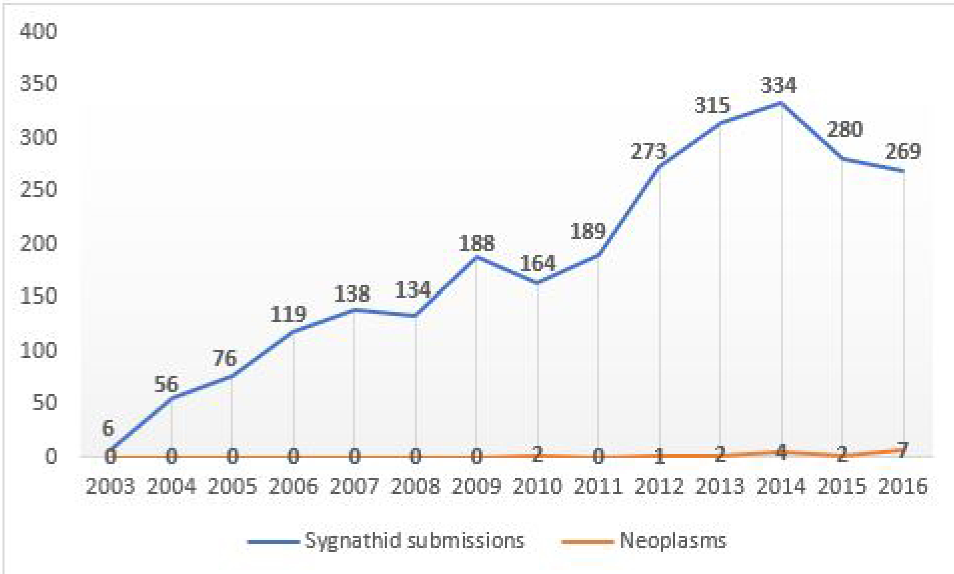
Figure 4: Carcinomas and adenomas, H&E.

A: Adult male broad nose pipefish, kidney, x2, inset x20. Renal carcinoma: Approximately 40% of renal parenchyma is effaced by a well-differentiated renal carcinoma with central necrosis.

B: Adult female short snouted seahorse, thyroid gland, x2, inset x10. Thyroid carcinoma: Marked expansion of the thyroid gland by neoplastic epithelial populations arranged in nests and tubuli, with colloid remnants. Inset: Neoplastic cell label positive with pan-cytokeratin.

C: 2 years old male broad nose pipefish, pituitary gland, x2, inset x20. Pituitary adenoma: An expansile pituitary adenoma widely fills the cranial vault and compresses the brain.

D: Adult male lined seahorse, swim bladder, x2. Swim bladder adenoma: The swim bladder lumen is extensively occluded by a well-differentiated epithelial tumour.



Annual distribution of sygnathid cases submitted to IZVG Pathology between 2003-2016 (blue) and correlation with neoplasms occurring over the same period (orange). No neoplasms were diagnosed prior to 2010.

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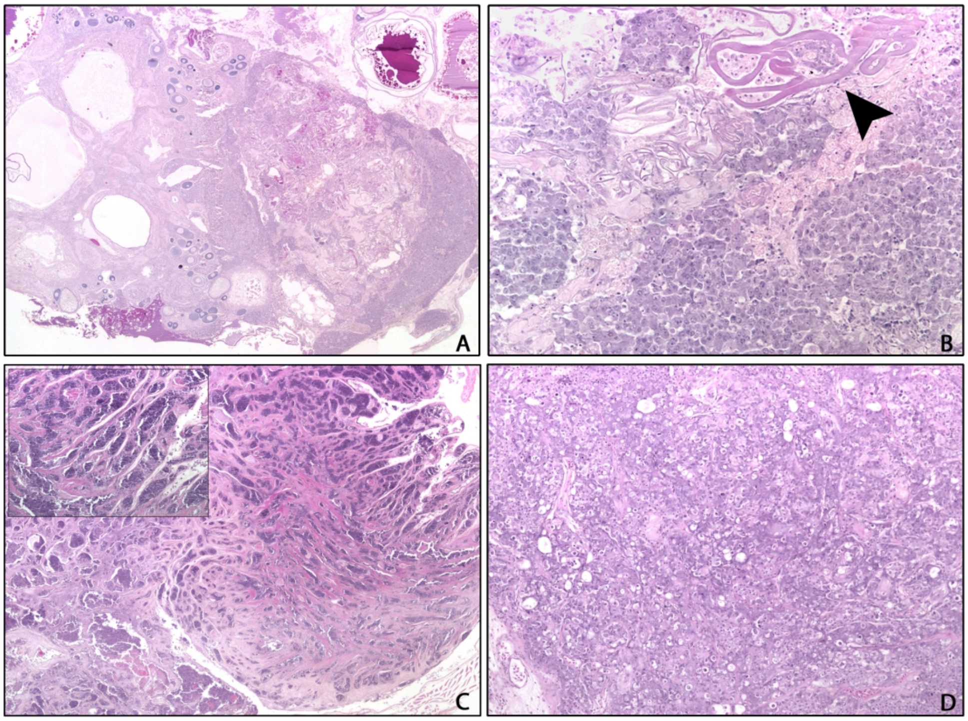

Germ cell tumours, H&E.
A and B: Adult big bellied seahorse, ovary, A x2, B x20. Dysgerminoma: Effacement of ovarian architecture by densely packed sheets of poorly differentiated germ cells. Remnants of follicular debris (arrowhead) secondary to follicular collapse are intermingled with neoplastic populations.
C: Adult big bellied seahorse, testis, x2, inset x10. Seminoma: Densely packed round cells (germ cells) with distinct cell borders, scant amounts of cytoplasm and large, round, vesiculate nuclei with coarse chromatin widely expand and efface seminiferous tubuli.
D: Adult big bellied seahorse, testis, x10. Testicular germ cell tumour: Effacement of testicular architecture by densely packed sheets of poorly differentiated germ cells. Findings are similar to those seen in the ovary (A, dysgerminoma).

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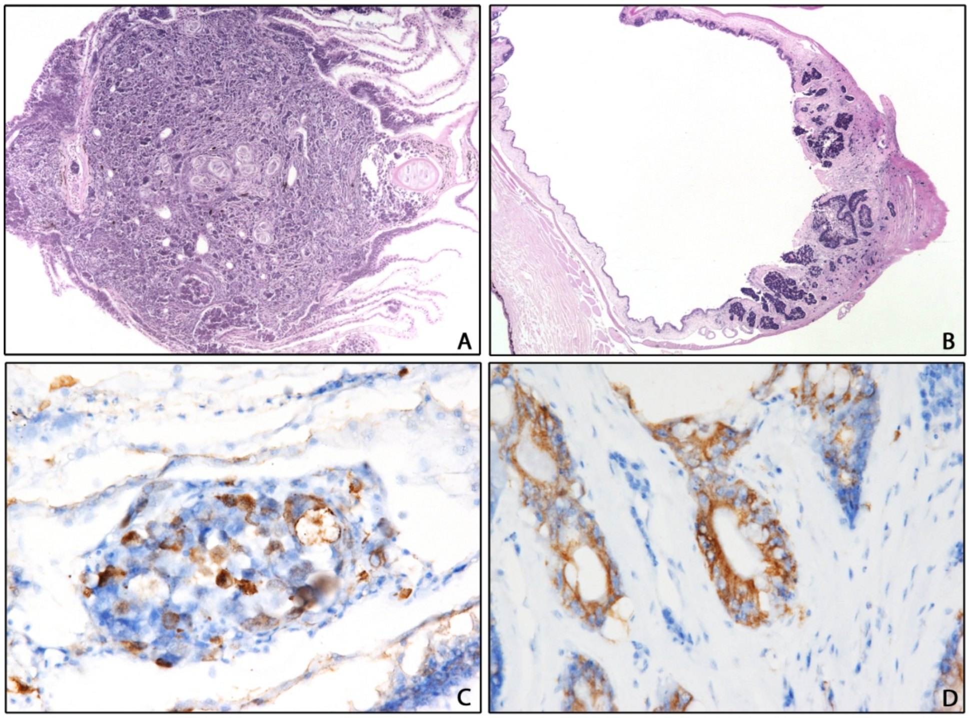

Pancreatic and intestinal carcinoma, H&E and immunohistology.
A and C: Adult male long snouted seahorse. A: Pancreatic carcinoma: Packets of metastasising neoplastic epithelial cells markedly expand gills, x10. C: A pancreatic carcinoma was evident in the coelomic cavity and labelled positive with pan-cytokeratin, x40.
B and D: Adult male long snouted seahorse. B: Intestinal carcinoma: The mucosa is expanded by nests and tubuli of neoplastic epithelial cells, x2. C: Neoplastic cells label positive with pan-cytokeratin, x40.

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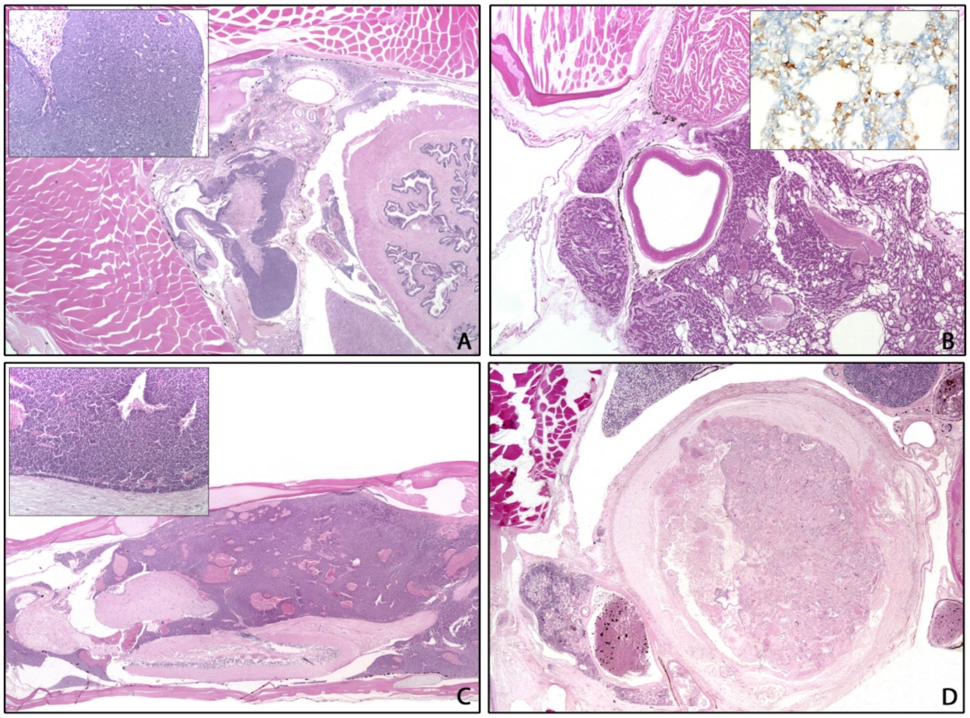

Carcinomas and adenomas, H&E.
A: Adult male broad nose pipefish, kidney, x2, inset x20. Renal carcinoma: Approximately 40% of renal parenchyma is effaced by a well-differentiated renal carcinoma with central necrosis.
B: Adult female short snouted seahorse, thyroid gland, x2, inset x10. Thyroid carcinoma: Marked expansion of the thyroid gland by neoplastic epithelial populations arranged in nests and tubuli, with colloid remnants. Inset: Neoplastic cell label positive with pan-cytokeratin.
C: 2 years old male broad nose pipefish, pituitary gland, x2, inset x20. Pituitary adenoma: An expansile pituitary adenoma widely fills the cranial vault and compresses the brain.
D: Adult male lined seahorse, swim bladder, x2. Swim bladder adenoma: The swim bladder lumen is extensively occluded by a well-differentiated epithelial tumour.

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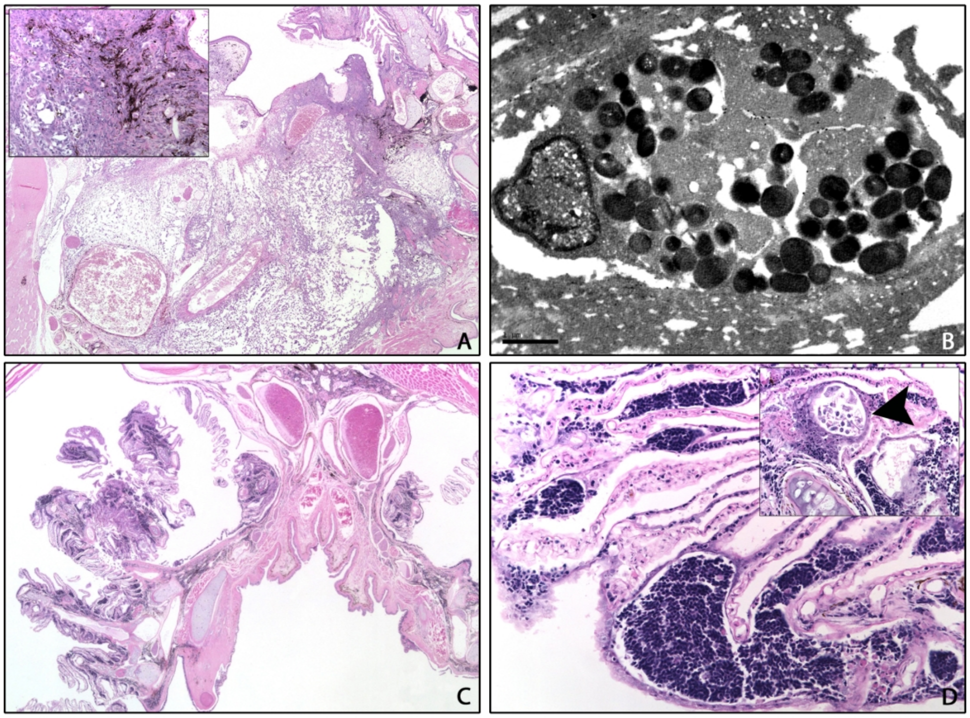

Chromatophoroma and lymphoma, H&E and TEM.
A and B: Adult male big bellied seahorse, branchial arch. Chromatophoroma: A: Branchial arch is expanded and effaced by a malignant melanophoroma, x2, inset x20. B: Large electron-dense granules are confirmed within the cytoplasm of neoplastic cells by TEM.
C and D: 5 years old male big bellied seahorse, gills. Lymphoma: C: Branchial vessels are packed with deeply basophilic cellular populations, x2. D: Neoplastic round cells fill vessels and infiltrate into branchial parenchyma. Concurrent amoebic gill disease with interlamellar cyst formation and intralesional amoeba (inset, arrowhead), x20.

176x130mm (300 x 300 DPI)