15

Differentiating the *Gyrodactylus* Species an Ectoparasite Infection of Guppy *Poecilia reticulata*

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Abstract

Guppies (*Poecilia reticulata*) from natural populations are prone to infections from several species that potentially have a profound impact on their life history evolution. Ornamental fishes are very important source for the transmission of parasites to new sites and to different countries along the transcontinental movements of fish hosts.

Poecilia reticulata is a common ornamental fish representing tropical guppy all over the world. This serves as a host for a wide variety of parasites ranging from protozoans to metazoans. *Gyrodactylus turnbulli* and *Gyrodactylus bullatarudis* found to infect *Poecila reticulata*. *G. turnbulli* is reportedly a specialist which is found caudally on the host whereas *G. bullatarudis* is considered a generalist occurs more rostrally on the host.

Gyrodactylus sp causes mass mortalities to the ornamental fishing industry therefore the identification of the parasite is important. The identification of species lies mainly on the opisthaptoral armature specially the hamuli, marginals in association with the penis. Earlier the cirrus pouch was used for to discriminate the different species of *Gyrodactylus*. Flat preparations of the worms were made and fixed with

Malamberg's fixative and the measurement of hamuli and marginals were made under the light microscope. The measurements made were subjected to image analysis software. The immunopholerescent staining techinique with pholoiden used for study the different structures at different depth with the help of a confocal microscopy were used in the present study. The opisthaptor muscular arrangements with the distribution of flame cells together with the detailed structure of the pharynx have been carried out. The opisthaptor showed a ring like muscular arrangement and the muscular path is more intense as two bundles towards the median hamuli.

The musculature enhances firm attachment with the help of marginals and median hamuli, affecting the osmoregultion and causing the death of the fish host.

Key words: *Gyrodactylus* sp., *Poecilia reticulata,* Opisthaptoral armature, Hamuli, Marginals, Immunopholerescent, Confocal microscopy, Flame cells

Introduction

Guppies belong to the family Cyprinodontiformes which includes killifishes, rivulines, topminnows, mosquitofishes, splitfins and pupfishes. It has a cosmopolitan distribution specially distributed wildly in the tropical climates such as South America, Venezuela, Barbados, Trinidad, northern Brazil and the Guyanas [1]. It is widely introduced and established elsewhere, mainly for mosquito control, but had rare to non-existing effects on mosquitos and negative to perhaps neutral effects on native fishes [2].

Guppies are ideal community fish. They seem to be annoying to other species in the same tank, as they often follow other fish around incessantly, but they do no harm. Guppies range in colour from the drab green feeder guppy to bright orange, red, blue and yellow fancy guppies. They can breed very quickly. Males have longer, more colourful finnage and are smaller than females, which tend to be dull coloured. However, the most definitive feature in males is the gonopodium, a stick-like modified anal fin compared to the normal rounded anal fin in females.

Guppies are transported globally for its ornamental uses. During collection and on arrival at the recipient site the fish are kept in a big tank in large numbers. Presumably this enhances the transmission of disease causing agents to a new location. This study attempts to identify the *Gyrodactylus* spp monogenean ecto parasite of guppies. The genus *Gyrodactylus* von Normann, 1832 are parasitic helminths belonging to the class Monogenea [3]. There have been over 400 described species of *Gyrodactylus* [4]. They are a very diverse class with species found within brackish, fresh and sea water enviornments. The gyrodactylids have been found on teleost fish, although some species have been found to parasitize cephalopod molluscs and some amphibian species [5]. The main method of transmission of *Gyrodactulus* is by direct contact between fish, with fin contact being one of the simplest ways for parasites to move onto new host. The high stocking densities of fish within fish farms allow rapid spread and multiplication [6]. The spread of *Gyrodactylus salaris* and the impact of this species have resulted in mortality of juvenile salmon estimated to be US $ 500 million to the Norwegian fishing industry [7]. The very same parasite can cause similar economic loss to the ornamental fishing industry.

Gyrodactylus spp have a direct life cycle, with the absence of stages utilising an intermediate host, allowing the full life cycle to be studied both in the wild and laboratory[4]. The Gyrodactylids are viviparous containing embryo which on inside another within the uterus of parent [5]. The gyrodactylid produce live larvae which attach to the parental host directly after the birth or float in the water column for a short period of time before subsequently attaching to a new host.

The first born female is always produced asexually within the parent, still in the embryonic stage. The second born female and any successive females develop from an oocyte moves into the parental uterus after the birth of the first daughter [5].

The male reproductive organ (penis) only becomes mature when the second born female is in development which discounts the possibility of self fertilization as development takes place at different times. Sexual reproduction takes place very rarely in some species which only produces clonally *Gyrodactylus gasterosteus* which prasitizes

the three-spined stickleback *Gasterosteus aculeatus* [8]. This method of reproduction allows the gyrodactylids to increase in numbers causing an explosion of the parasite population. In some species of *Gyrodatylus*, the daughter embryo can be born within 24 hours of the birth of the parent [5].

The damage caused by the attachment of the gyrodactylids to the host epithelium and gills using the hamuli and marginal hooks is relatively low when the parasites are in low numbers. The wounds caused by feeding can be very severe an can cause ulceration of the epithelium as the eversible pharynx removes epithelial cells as it feeds [6]. The range of movement by the gyrodactylids once attached to the host is fairly small, with the parasite and the damage caused by each individual parasite being relatively localized, but the impact of a higher number of parasites causes serious damage to the fish [6]. The ulceration caused by the parasite suppresses the immune system and can allow the secondary infection of the wound by bacteria and fungus [9]. The secondary infections have a likely role in the high mortalities associated with *Gyrodactylus* infections [9].

The gyrodactylids have two main features which are important during host-parasite interactions; opisthaptor and the adhesive secretion of the cephalic papillae. The posterior opisthaptor is the principal region used in attachment to the host epithelium [6]. The opisthaptor contains two large hamuli. The hamuli are surrounded by 16 marginal hooks which are used to anchor the gyrodactylid to the host epithelium. The head papillae are situated on the cephalic lobes and produce a adhesive secretion which attaches the anterior end of the parasite to the host epithelium. The primary form of attachment of the gyrodactylids does not change during the life cycle of the parasite. This is in contrast t many monogenean parasites which use the marginal hooks as the primary form of attachment during the larvals stages and use the hamuli during the adult stages [10]. The marginal hooks are the primary form of attachment and the hamuli are secondary in their attachment role. The most secure form of attachment is obtained using the 16 marginal hooks which pierce the epithelium and holds on to the host. Different species are commonly found to inhabit specific areas on the fish host.

They are usually separated into skin and gill parasites; skin parasites are more likely to feed on the mucus layer coating the host where as the gill parasites usually feed on the rich supply of blood in the gills [10].

The attachment mechanisms of gyrodactylids can be very species specific due to the mode of life of the fish host. The opisthaptor is muscular due to its attachment function and consists of two centrally positioned large hamuli and sixteen peripheral marginal hooks [10]. The measurement of many different pats of the *Gyrodactylus* attachment allows the species to be identified. The difference between many species, especially those inhabit the same host can be relatively small [11]. The use of a high number of measurements reduces the variation present within members of the same species which may attributed to other factors such as those that are influenced by the environment [8]. The measurement of the marginal attachment hooks, the hamuli and the ventral bars at certain specific points has been successful in refining the systematic of *Gyrodactylus* spp and allow the separation of the parasites at a species level [12]. The measurement of the hard parts of the parasite has been found to be a greater taxonomic significance than measurement of the soft parts. One of the advantages of measurement of the hard parts that are prepared in a consistent fashion is that the variation within species is unlikely to be caused by the bad preparation of whle mounts and is more likely to be environmentally determined [8]. Shinn *et al* 1993 [13] used the sonication technique t remove the hamuli from the opisthaptor of the *Gyrodactylus* and allow them to look in more detail to identify the specific function of each part and the muscles associated with them. The differences in the function of these parts themselves contribute to the species identification. These variations are measurable and allow inter-species differences to be compared. The use of hard part measurements and the production of a formal set of methods for measurement have allowed the identification of separate species which may nevertheless be similar in one or more respects. Despite similarities in hamuli shape or over all size differences in the observed measurements of, for instance, the marginal hook sickles can still be taxonomically significant [12]. A range of molecular techniques have been used in order to differentiate *Gyrodactylus* species from one another, in separating pathogenic and

non pathogenic species and in the assessment of genetic variation within a species. Chaetotaxy is the compilation of a map of sensory receptors which can be found over the body of many different species. This technique has been used by Shinn *et al.* [14] to distinguish the pathogenic species in Europe and lead the way for to develop a soft ware discriminate harmful species from non harmful counterpart. Likewise the visualization and mapping of protonephridial system has been carried out in the past using a phase contrast microscope [12].

The objective of this study is to discriminate the different *Gyrodactylus* species with the use of special stains along with the confocal microscopy. Further the distribution of *Gyrodactylus* over the host species is also considered.

Materials and Methods

Poecilia reticulata were obtained from the pet shop regularly on arrival to Scotland from tropical countries mostly Singapore. The fish were anesthetized in benzocane and killed instantly to check for ecto and endo parasites. Initial examination was carried out under the dissecting microscope and the same fish was moistened with the same aquarium water throughout.

First the fish were decerebrated after anesthetic treatment used for the study. The total length and weight of the fish were measured protocol used for examination and quantification of parasites in a fish host was followed for skin, fin, gills [15]. The parasites were observed in wet mounts and identified up to the generic level. This study included 151 fish specimens from the pet shop.

Flat preparations of the worms were made for the light microscopic identification. A low concentration saline solution (0.01M) added in preparing the flat worms to enhance the functions of gyrodactylus excretory system and to make the system more clearly visible via light microscope. The measurements of the hard sclerotised parts of the opithaptor were made as described by Shinn *et al*. [10]. The measurements were subjected to image analysis software.

A modified fluorescence staining technique was used to stain the excretory system and musculature of gyrodactylids [16]. The slides for light microscopy were viewed under x 1000 magnification and for the confocal microscopy laser scanning was used.

The fluorescent stain resorufin (Sigma-Aldrich R 3257) was prepared by using 1 mg of resorufin dissolved in 1 ml of distilled water. 100µL of the solution was diluted with 900 µL distilled water to form a solution with concentration of 10µgml^{-1}. A fresh solution was made for each batch of specimens since the resorufin solution is relatively unstable and degrades over short period of time.50µL of the resorufin solution was added into the embryo dish containing worms, covered with foil and incubated for different length of time to identify the optimal length of staining time. The fluorescently labeled gyrodactylids were washed 5 times by water and mounted on to the slides.

Phalloidin stain was made using 1ml triton-X (Sigma Aldrich T9284) dissolved in distilled water. 4µL of resulting solution was added to the 200µL of neutral buffered formalin. 5µL of the phallodin was then added to methanol to produce phallodin stain and was added to the embryo dishes with worms. The dishes were covered with foil and left for different times to find out the optimal time for staining the worm.

Results

Gyrodactylus sp is having two cephalic lobes with an opithaptor with a pair of hamuli and sixteen marginal hooks [13].

The *Gyrodactylus* sp recovered from the gills and skin of fish of 35 -165 mm length. Of the total of 151 *P. reticulata* specimens examined 100 (66%) were found to be parasitized by *Gyrodactylus* sp. Mixed infection was found to be common in the ornamental fish as they are stocked in transportation tanks before and after shipment.

The data on the parasite infection levels were recorded in terms of Prevalence (percentage), Range (intensity range) and Mean intensity (Table 1).

Table 1: The list of *Gyrodactylus* spp and corresponding prevalence (%), mean intensity, range values of *Poecilia reticulata.*

Parasite species	Organ infested	Prevalence	Mean intensity	Range
Gyrodactylus turnbulli	Skin, gills and fins	75.3	66.5±35.4	0-650
Gyrodactylus bullatarudis	Skin, gills and fins	13.5	50.2±1.35	0-375

The prevalence mean intensity and range varied drastically for the two species. The possibility of selectivity of the sites by the parasite species depends on the water flow and the temperature of the water in which the fish are kept (Table 2).

Table 2: The distribution of the *Gyrodactylus turnbulli* and *Gyrodactylus bullatarudis* from different areas of the body of *Poecilia reticulata* .

Parasite species	Head	Skin	Pectoral fin	Pelvic fin	Caudal fin	Anal fin
Gyrodactylus turnbulli	125	714	168	345	34	898
Gyrodactylus bullatarudis	950	500	175	234	45	105

There are two species of *Gyrodactylus* which parasitize the *Poecilia reticulata.* The two species can be readily identified on the basis of the large differences in the shape of their ventral bar and the shape of the hamulus (Fig1, 2, 3). The morphometirc measurements of the opisthaptoral scelerite show variation and led to the species specific identification (Table 3). The total measurements of the *Gyrodactylus* sp put into the spreadsheet of image analysis software. Then the grouping made individually to identify the differences among the species.

Fig 1 The photomicrograph of the hamuli of *G. bullatarudis*

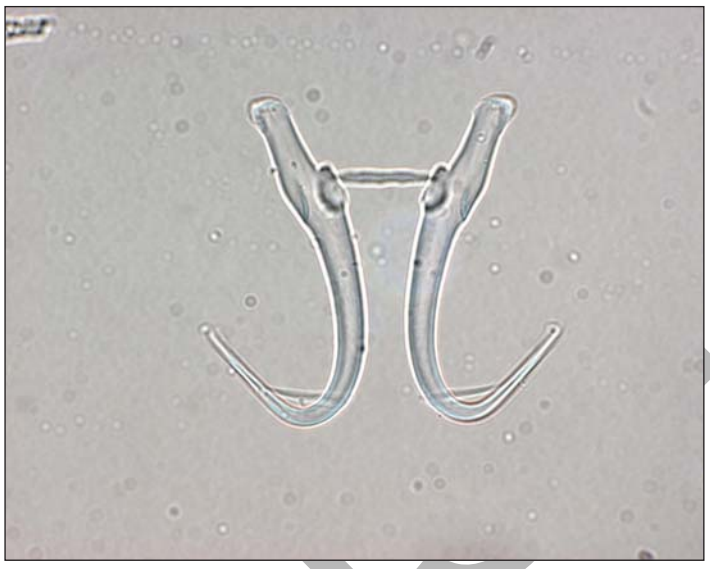

Fig 2 The photomicrograph of the hamuli of *G. turnbulli*

Fig 3: The individual hamulus showing the curvature in *G. bullatarudis*

Table 3: Measurements of the sclerotised parts of the opisthaptor of *Gyrodactylus* spp from *Poecilia reticulata.*

Measurement	***Gyrodactylus turnbulli*** **(n=20)**	***Gyrodactylus bullatarudis*** **(n=25)**
Hamuli total length	46-60 µm	59-66 µm
Ventral bar width	20.1-26.9 µm	20-22 µm
Marginal hook length	24.3-30.3 µm	23-39 µm

There are also distinct striations on the ventral bar membrane of *G. turnbulli. G. bullatarudis* has a pair of excretory bladders situated within the trunk region next to the pharynx and below the cephalic lobes which are not present in *G. turnbulli.*

A visual comparison of the success of the two fluorescent stains resorufin and phallodin in staining the excretory systems of *Gyrodactylus* found that phalloidin is the superior of the two stains. The optimal time for staining with phalloidin was found to be 30 minutes, which produced detailed fluorescent images of the flame cells present within the excretory system by staining the actin surrounding them. The majority of the fluorescent visualizations were therefore using palloidin and all provided similar quality using optimized method. Phalloidin did not stain the canals of the excretory system. The canals within the excretory system are differentiated into three types. The main canal is the largest of the three types and is the site of collection of excretory products before they are passed out of the excretory pore into the surrounding environment. The secondary canal connects directly to the main canal in the posterior and anterior parts of the excretory system. The secondary branch is the smallest of the canals and is always associated with the secondary canals; the flame cells are located at the terminal point of the secondary branches.

Discussion

The fluorescent phalloidin was found to be more effective and successful of the two tested stains to stain the excretory flame bulbs. The fluorescent marker has been used for successfully visualize the excretory system of *Gyrodactylus* spp. Pahlloidin is a phallotoxin which actively binds and stabilizes actin (F actin). According to previous work by Sato *et al* (2002) [16] , the excretory system of *Schistosoma mansoni* is approximately 10 times larger than the *Gyrodactylus* spp. The method used has been changed in order to cope with the extreme differences in size of the specimen in this study. The success of staining canals on one or two occasions of the experiment using resorufin suggests that there are no serious problems with the uptake of the stain and that further optimization is required. *S. mansoni* inhabits the human body. Staining by the method of Sato *et al*. [16] using resorufin and physiological saline was used as the staining medium for *S. mansoni* to take up the stain. However, for *Gyrodatylus* spp on the other hand fresh water was used, it is therefore possible that the staining could be better with small amount of saline water as this helped in the visualization of the excretory canals during phase microscopy.

A double staining technique could be performed using both resorufin and phalloidin, in order to get a clear picture of the whole excretory system which would hopefully highlight the canals as well as the flame cells. The phalloidin stain is rapid, although it kills the parasite hence they would used to stain first with resorufin which requires active uptake inorder to visualize the canals as well as the flame bulbs.

Conclusion

This study made an attempt to identify and differentiate the genera *Gyrodactylus* into two different species and found out the site of attachment by means of the microspoic study and further the staining to stain the excretory cells and canals some of which were described by Malammberg [11,12]. The time consuming nature of phase contrast microscopy which was very frequently used by researchers needs a revision with some quick methods. The most efficient method for to determine the positions of the flame bulbs are with phallodin fluorescence microscopy method. The phase contrast microscopy will be furnishing the diversified needs of the researchers instead of using electronmicroscopy.

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