

ORIGINAL ARTICLE / ARTÍCULO ORIGINAL**A BEHAVIORAL EXPERIMENTAL APPROACH ON THE TRANSMISSION OF
GYRODACTYLUS (MONOGENEA) OFF THE 3-SPINE STICKLEBACK
(*GASTEROSTEUS ACULEATUS* L.)****UN ENFOQUE EXPERIMENTAL SOBRE EL COMPORTAMIENTO EN LA
TRANSMISIÓN DE *GYRODACTYLUS* (MONOGENEA) FUERA DEL HOSPEDERO
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ABSTRACT

The aim of this study was to investigate some behavioral transmission factors of gyrodactylids and to ascertain how maturity and reproductive status may be employed in the colonisation of new hosts using the *Gyrodactylus gasterostei* Gläser, 1974 and *G. arcuatus* Bychowsky, 1933—*Gasterosteus aculeatus* L. transmission model from dead fish. Laboratory experiments included determining the maturity (presence of male copulate organ) and reproductive (presence of daughter) status of transmitting worms, in order to consider the behavioral factors that influence parasite choice to migrate to a new individual of the same host species. This is the first behavioral experimental study of the different developmental stages of two different species gyrodactylids using the stickleback model. Indeed, the results showed the comparison between behavioral frequencies and individuals. The analysis was elaborated among all individuals who survived the observational period, Behavioral differences related to maturity or some other characteristic were noted. The present work examines the initial ethological aspects of these behavioral strategies in detached and individual gyrodactylids. It is remarkably difficult to establish a behavioral measure of *Gyrodactylus* in the laboratory during most observations; data analysis showed no behavioral differences among these monogenean species.

Keywords: Behavior - *Gyrodactylus* - *Gasterosteus aculeatus* - Monogenea - Transmission

RESUMEN

El objetivo de este estudio fue investigar algunos factores en el comportamiento durante la transmisión del monogeneo *Gyrodactylus* sp. y determinar si la madurez y estado reproductivo del parásito pueden ser empleados en la colonización de nuevos hospederos. El modelo de transmisión empleado fue *Gyrodactylus gasterostei* Glaser, 1974 y *G. arcuatus* Bychowsky 1933 - *Gasterosteus aculeatus* L. que migraron de peces sacrificados. Los experimentos en el laboratorio incluyen la determinación de la madurez (presencia del órgano copulador masculino) y reproductiva (presencia de la hija en útero), y determinar los factores de comportamiento que influyen en la elección de los parásitos para migrar a un nuevo hospedero. Este estudio es el primer enfoque experimental en el comportamiento con relación a diferentes etapas de desarrollo en dos especies de *Gyrodactylus* utilizando el mismo modelo de hospedero. El presente trabajo examinó los aspectos iniciales del comportamiento de las estrategias de comportamiento en gyrodactilidos aislados de forma individual. Los resultados muestran que la comparación de las frecuencias de comportamiento entre los individuos que sobrevivieron al registro de observaciones y el análisis de los datos no mostró diferencias de comportamiento entre estas especies de monogeneos.

Palabras clave: Comportamiento – *Gyrodactylus* - *Gasterosteus aculeatus* – Monogenea - transmisión

INTRODUCTION

In nature, the interaction between parasite and host has a dual response; it is this interaction and diverse routes that makes the study of parasites and their infective stages a remarkable study. Gyrodactylids are monogenean flukes with a direct life-cycle, and are capable of rapid multiplication. Harris (1985) described this mode of reproduction as being fairly unique, allowing rapid population growth on their host and conferring an ability to transfer to a new host at all times during their life-cycle. As parasites, gyrodactylids are forced to employ a range of “successful” strategies in order to reach their target, a new fish. During this process of *transmission* or *colonisation*, gyrodactylids appear to display and adapt to the behaviour of the hosts using a wide range of behaviours in their transmission. In particular, it was suggested that individual

gyrodactylids moved to the surface film of the water and that therefore, as guppies are surface feeders; detached parasites were more likely to contact a new host (Cable & Harris, 2002), by swimming-like behaviour in the water column e.g. *G. rysavyi* (El-Naggar *et al.*, 2004), *Ieredactylus rivuli* (Schelkle *et al.*, 2011) and transmitting from dead hosts, suggesting that they might provide a significant reservoir of infection (Olstad *et al.*, 2006) during scavenging feeding (Grano-Maldonado, 2014b).

Behavioural flexibility in gyrodactylids may be important in the transmission process. Parasites that had not yet given birth for the first time were suggested to be less likely to transfer to a new host than worms that had already given birth at least once (Harris, 1985). Little is known regarding the biological basis of gyrodactylid behavioural during host selection and the maturity factors underlying

transmission, particularly with respect to the behavioural-mature status of gyrodactylids moving to new hosts. The use of the sensory and motor abilities to active and modify its behaviour patterns allowing transmission to new host promoting a parasite ability to survive and reproduce. Host behavioural changes due to parasitism have been studied (Levri, 1999) and are often assumed to be adaptations of the parasite. However, behavioural aspects of parasites as gyrodactylid may be a general response biological characteristic for parasite transmission. For this reason, alternatives behavioural studies which consider the stage development hypothesis should be tested. As *Gyrodactylus* has no specific transmission stage in its life-cycle, movement between hosts must be achieved by strategies employed by the adult. Species employ to interpret both their host and ambient environments employing structural features, such information may assist in the interpretation of transmission behaviours, like in this particular case. This transfer may be facilitated by the response of mechanoreceptors present on the tegument, detecting the aquatic turbulence (Grano-Maldonado, 2011a) or their responses to chemical stimuli (ie. fish) or the presence of a photoreceptor, which register the shadow produced by a passing fish could increase the chances of a successful transmission which gyrodactylids employ in host location during the transmission process (Grano-Maldonado 2014a).

The aim of this study is to assess the behavioural flexibility concerning maturity and reproductive stage employed the *Gyrodactylus gasterostei* Gläser, 1974 and *G. arcuatus* /*Gasterosteus aculeatus* L. infection model to examine these questions, concerning transmission process off the host. The present work examines the ethological initial aspects of these behavioural strategies in detached and individual gyrodactylids.

MATERIALS AND METHODS

Source of hosts and parasites

A *G. gasterostei* / stickleback model was used for the purposes of this study. Fish used for the study were collected along specimens were collected on previous methodology (Grano-Maldonado 2014a, b). This experiment was designed to examine the behaviour characteristics of worms (n=31) moving off dead hosts at 10°C. For this experiment we hypothesised that host transfer might be more favoured in those parasites having MCO (male copulatory organ) and absence of daughter present *in utero*. Individual sticklebacks were euthanised with an overdose (0.01·L⁻¹) of anaesthetic 2-phenoxyethanol (Merck-Germany) and were placed in individual Petri dishes containing clean water at 10 ± 1° C. Dead hosts were observed under an Olympus SZ30 stereomicroscope at different magnifications, with the time at which each gyrodactylid looped off the fish during 60 min being recorded for each individual gyrodactylid. Worms detaching naturally from the host tissue within 60 min were then used for analysis. Worms were carefully removed with a 200 µl pipette and were placed individually into 3 cm Petri dishes containing 5ml of filtered (0.45 µm Minisart Sartorius Stedim, Biotech) water taken from the same source as that used for fish maintenance and incubated at 10°C. There are several observation techniques that are used to record performance or behaviour including i.e frequency, rate, duration and interval recording. All of these techniques rely on precisely identifying the behaviours in observable and measurable performance terms and to make the results consequential and consistent. The target gyrodactylid behaviour may need to be defined in a way that it is observable and measurable, if exist. Clearly identifying specific behaviours being observed makes communicating and interpreting the results of the observation more accurate. We refer the term Frequency counts

(a record of the number of times a specific behaviour occurs within a specific period of time). Frequency counts are useful for recording behaviours which have; a clear beginning and ending, are of relatively short duration, and tend to occur a number of times during the specified time period (Martin & Bateson, 1993; Lehner, 1998).

Twelve basic behaviours were observed and evaluated; each specimen was observed every minute by the method denominated “scan sampling” according with Martin & Bateson (1993). The behavioural record elaborated for this study was based in gyrodactylid individual activities under the microscope every minute during 60 min. The data obtained of the occurrence of each behaviour was recorded: A. motionless - attached only by their haptor (worm static); B. Haptor attached and exploring surroundings; C. Looping “worm moving” across the dish; D. Haptor detached from dish (no twitching); E. Haptor attached from dish and twitching; F. Haptor detached and twitching; G. Erratic movements (moving in a way that is not regular twitching); H. Haptor detached but head attached to bottom; I. Keeping a “C” body shape and no twitching; J. Keeping a “C” body shape and twitching; K. Motionless (no moving but non dead); L. Mom Gives birth. Worms were considered dead when no movement were noticed after a gentle touch within a needle. Following observation, each worms were fixed in 10% neutral buffered formalin (NBF), each specimen was then mounted on a glass slide in a drop of distilled water ensuring that the haptoral hooks were flat, stained and fixed *in situ* by the addition of a drop (~3 µl) of Malmberg's fixative (ammonium picrate glycerine, APG; saturated picric acid and 100% glycerin) to the edge of the coverslip which was drawn under the coverslip by capillary action. The coverslip was then sealed with transparent nail vanish. The maturity and reproductive status of worms were recorded using a compound microscope (Olympus BX51) at 100× / oil immersion

magnification. Additionally, parasites were identified to species through morphological and morphometric analysis of the opisthaptor hard parts as described in Grano-Maldonado (2014a,b).

Data analysis

All data were analysed by comparing behavioural frequencies and developed status among all the individuals (*G. gasterostei* and *G. arcuatus*) which survived the observational record. Also, to determinate the behavioural differences between them not parametrical statistical tests, Kruskal-Wallis and Mann-Whitney, were employed. All statistical analyses were performed using the software SPSS version 20.

RESULTS

These experiments were carried out with a view to gaining some insight into the transmission strategies of gyrodactylids and individual behaviour during transmission off the fish host. During 31 h of observation of 31 individuals of recorded video, from 12 conducts originally described, just 8 conducts were used and identified these behaviours being described in this study in terms of possible transmission. The comparison of the behavioural frequencies between all individuals (*G. gasterostei* and *G. arcuatus*) showed there are not statistically significant differences (Kruskal-Wallis Test; $\chi^2=25.36$; $df=30$, $p=0.70$). The average of the twelve behaviours recorded in *G. gasterostei* and *G. arcuatus* during the observation period shown in Table 1. Comparative analysis showed that there were no statistically significant differences between the behavioural pattern of both species ($U = 62.0$, $p = 0.56$).

Table 1. Behavioural pattern displayed by *Gyrodactylus arcuatus* and *G. gasterostei* off the host. Values express the average frequencies \pm SD.

Behaviour	<i>G. arcuatus</i>		<i>G. gasterostei</i>	
	Average	SD	Average	SD
A	38.50	29.99	30.07	19.89
B	33.25	24.42	26.26	19.08
C	23.25	21.50	17.44	19.20
D	47.25	24.12	32.33	22.46
E	7.75	8.30	1.67	3.49
F	39.25	30.43	30.70	20.27
G	15.75	18.55	5.93	13.13
H	0.75	0.96	4.00	5.51
I	5.75	6.24	6.63	9.64
J	0.00	0.00	0.19	0.40
K	6.00	5.48	8.30	9.24
L	1.00	2.00	0.59	3.08

Because no significant differences between the behavioural pattern of individuals and species were observed, we compared using the Kruskal-Wallis test, behavioural patterns among individuals with presence of a daughter, without the presence of daughter and

who gave birth, regardless of the species. The analysis showed no statistically significant differences in the condition of presence or absence of a daughter ($\chi^2=0.37$, $df=2$, $p=0.85$). Behavioural averages frequencies are presented in Table 2.

Table 2. Behavioural pattern exhibited by individuals who gave birth, with a daughter without daughter. Values express the averages frequencies \pm SD.

Behaviour	Gave birth		With a daughter		Without daughter	
	Average	SD	Average	SD	Average	SD
A	22.25	6.85	32.69	20.63	32.29	24.21
B	19.50	10.41	27.15	20.48	29.36	21.06
C	14.25	13.74	17.85	21.00	19.64	19.89
D	36.00	20.72	32.31	23.59	35.57	24.15
E	0.50	1.00	1.85	4.41	3.57	5.39
F	29.50	9.85	28.46	20.32	35.57	24.96
G	1.00	2.00	7.31	15.80	8.86	14.27
H	4.25	2.22	2.38	4.74	4.50	6.28
I	6.75	7.89	6.46	12.23	6.50	6.53
J	1.00	0.00	0.08	0.28	0.00	0.00
K	13.50	7.77	3.85	5.37	10.29	10.32
L	0.00	0.00	1.54	4.48	0.00	0.00

We conducted a second analysis by Mann-Whitney test to compare the behavioural pattern with respect to the presence or absence of the male copulatory organ (MCO) in individuals regardless of species. This analysis

showed that the behaviour is not affected by the presence of a MCO ($U=66.0$, $p=0.73$). Behavioural averages frequencies are presented in Table 3.

Table 3. Behavioural pattern exhibited by individuals with MCO and without MCO. Values express the averages frequencies \pm SD.

Behaviour	With MCO		Without MCO	
	Average	SD	Average	SD
A	29.44	19.19	33.54	23.88
B	25.61	19.35	29.31	20.33
C	15.39	16.37	22.08	22.72
D	30.44	22.33	39.54	23.32
E	0.78	1.77	4.77	6.31
F	30.06	18.77	34.23	25.16
G	5.11	11.17	10.08	17.18
H	3.78	4.31	3.31	6.54
I	6.06	7.21	7.15	11.70
J	0.28	0.46	0.00	0.00
K	9.61	10.44	5.77	5.51
L	1.11	3.83	0.00	0.00

DISCUSSION

This study suggests that there are no significant differences between the behavioural pattern in *G. gasterostei* and *G. arcuatus* concerning the presence of a daughter *in utero* or the presence of MCO. These factors did not affect the host-seeking behaviour, and potentially the transmission success may affect in a similar manner. The observed host-seeking behaviour of gyrodactylids during this study may increase transmission success of gyrodactylids species. Decreased host-seeking behaviour during this time may also result in reduced movements towards the end of the observation; this may be for a possible temperature raise, thus also affecting the fitness of the worm parasite. However, every 5 min 5ml of cold water were add to keep the outside Petri dish cool; additional effects of infection by species remain to be elucidated and further studies could include measures of fitness, such as longevity under the same experimental circumstances.

One of the first descriptions of a specific migratory behaviour that facilitates

transmission of a gyrodactylid from dead hosts was detailed by Cable & Harris (2002). These latter authors described the method by which *Gyrodactylus turnbulli* Harris, 1986 and its hosts, guppies *Poecilia reticulata* (Peters), come into close contact. After death, guppies float at the water's surface, its burden of *G. turnbulli* parasites move off these fish into the water film, hanging motionless with the haptor held by surface tension. Since guppies are surface feeders, detached parasites in the water film are using this host's behaviour to increase the likelihood of contacting a new host. However, the majority of gyrodactylid species, if they are dislodged from the host's skin host, they sink until they reach a solid surface (Cable & Harris, 2002). Another interesting case of gyrodactylid behaviour was reported by El-Naggar *et al.* (2004). These authors suggested that *Gyrodactylus rysavyi* Ergens, 1973 was capable of directional swimming by flexing its body, this involving ~4-8 per sec looping contractions in any direction with a speed of ~1.7-5mm/sec and a range of ~15cm distance. This motility was suggested to be an exceptionally efficient infection mechanism with respect to the Nile catfish *Clarias gariepinus* (Burchell) host. By comparison, these authors reported that the closely related

Macrogyrodactylus congolensis (Prudhoe, 1957) Yamaguti 1963 and *M. clarii* Gusev 1961 does not possess the ability to swim, even though they parasitized the same Nile catfish host. Another clear example, Schelkle *et al.* (2011) suggested a swimming-like behaviour for the gyrodactylid *Ieredactylus rivuli* from *Rivulus hartii* (Boulenger, 1890) in Trinidad. Gyrodactylids use a variety of different strategies to infect new hosts (Bakke *et al.*, 2002), but robust experimentation to test these possible strategies is lacking.

One observation suggests gyrodactylids can transmit from dead hosts when live fish cannibalise the carcass of an infected one (Olstad *et al.*, 2006). This behaviour would increase the chances of parasites contacting a new fish host, particularly if potential hosts are either scavengers or benthophagous feeders. *Gyrodactylus* species usually have preferred sites on their host which, depending on the transmission strategy to facilitate their transmission to new host (Grano-Maldonado, 2011; 2014b). Gyrodactylids disperse effectively using a variety of mechanisms, but the most common is most likely through contact between living hosts. The transmission could be by direct host contact, via dead fish (scavenging or food items), transmission also occurs by contact with dead hosts, parasites attached to the substratum and worms drifting in the water column (Bakke *et al.*, 1992; Soleng *et al.*, 1999, Cable & Harris, 2002; Grano-Maldonado, 2011, 2014b). In benthic species, such as the 3-spine stickleback, transmission via the substrate is noticeable and can be one of the most important routes of transmission. Some gyrodactylids are capable of reproducing on several hosts, whilst on others they are unable to reproduce. Nevertheless, the colonisation of a host on which reproduction cannot occur may still play a role in the transmission of the parasite towards its final host (Bakke *et al.*, 1992).

This study is the first behavioural experimental

approach concerning different developmental stages on two different species gyrodactylids using the stickleback model. These results showed that the behaviour is not affected by the presence of daughter o penis (MCO) and behavioural sequences are very similar between species (they perform singular movements in the same way). However, Grano-Maldonado (2011, 2014b) showed that the presence of the MCO favoured migration to a new fish host of the same species. Thus, those individuals with MCO present will colonise sooner and consequently have more time available to find a new host once detached (Grano-Maldonado, 2011, 2014b). The present study was a part of an experimental section from an extensive previous research (Grano-Maldonado, 2011a; Grano-Maldonado *et al.*, 2011; Grano-Maldonado 2014a,b; Grano-Maldonado & Palaiokostas, 2015). The maturity and reproductive status of parasites that transferred to live hosts was recorded and it was found that 65.6% of the parasites had a MCO present whilst 54.2% had a daughter *in utero* (Grano-Maldonado 2014b) during this research resulting that parasites with MCO presence ($p < 0.05$) showed a significantly higher probability of transmission. However, there are no significant differences between *Gyrodactylus* species and behavioural conduct.

We attempted to link the previous research which developed from the same fish model/parasite and similar experimentation set up. In previous research, it was identified the presence of structures: ciliated photoreceptor located sub-surface, localised in close proximity to the spike sensilla in monogeneans as reported by Watson & Rohde (1994) and sense organs were conducted on the works of Lyons (1969, 1973). It is possible that parasites may initially detect water movements associated with an approaching host and then prepare themselves for attachment employing mechano-chemical and photo receptors (Grano-Maldonado, 2014a). To date, few

studies have explored the effect of some chemical compounds on the behaviour transmission of *Gyrodactylus* species on both their vertebrate hosts (Brooker *et al.*, 2011). This study provides the first example of a measurable behavioural approach to *Gyrodactylus* species during migration off the host.

A significant difference was not observed between numbers of parasites with daughter or MCO; however, it is likely that results for *Gyrodactylus* species were confounded by temperature, which may inhibit host-seeking behaviour. Gyrodactylids become less active as physiological age increases or experience environmental responses of receptors that may signal the presence of a fish close by, and they may be more susceptible to infection (Grano-Maldonado, 2014a). Indeed, the results show the comparison in behavioural frequencies between individuals; this analysis was elaborated among all individuals who survived the record, and if whether there behavioral differences between them life status (i.e. presence of daughter or MCO), or some other characteristic.

Regarding the data analysis no behavioural differences are among species. Taking into consideration, other possible factors affecting transmission in gyrodactylids, we may consider that skin and gills are in contact with the external environment and may be subject to morphological, histopathological and behavioural changes when exposed to substances which may 'damage' tissues or 'affect' gyrodactylid transmission, i.e. anaesthetic (Grano-Maldonado & Palaokostas, 2015) did not have an effect on gyrodactylid transmission. However, formaldehyde (Pahor-Filho *et al.*, 2014) caused mild to severe hyperplasia and detachment of respiratory epithelium in the gills of juvenile *Mugil liza* (Valenciennes, 1836). The tolerance to formaldehyde may be fish species-specific; also may affect

swimming performance at every developmental fish stage i.e. larvae or adult. Unfortunately, in our study, we did not consider the possible effect of formaldehyde in the sticklebacks-host, when it was employed to reduce in some cases the parasite load. In the best of our knowledge, all fish used in the experiments did not present any erratic behaviour or skin disorder. The *G. gasterostei*, *G. arcuatus* / *Gasterosteus* model is a simple and successful system to examine aspects of transmission of parasites from fish. Future studies to evaluate the tolerance to formaldehyde including possible gill and skin epithelium damage which may affect gyrodactylid transmission in sticklebacks warrant further investigation. Thus, further studies, such as power analysis to determine if any effect would emerge with a higher sample size, would likely reveal useful information; also, considering that gyrodactylids may not be comparable with other behavioural patterns from more evolved organisms.

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