

Transfection of siRNA into *Brachionus plicatilis* (Rotifera)

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Abstract Transfection experiments have played a prominent role in functional genomics, being used to probe phenotypes with reporters and knockouts with techniques like RNAi, gene over-expression studies, and the creation of transgenic animals. Rotifers are not amenable to many classic genetic manipulations, hence have not been a model system in the genomics revolution. However, no one has explored whether rotifers are susceptible to gene expression knock-out via transfection, and with the establishment of the rotifer transcriptome sequencing project, an increasing amount of sequence data is available to define target genes for manipulation. As a first step towards transfection of rotifers, we describe our attempt to transfect double-stranded, fluorescently-labeled siRNA into resting eggs, hatched rotifers and amictic eggs with varying levels of success. Transfection was successful when hatched rotifers (rather than resting eggs or amictic eggs) were directly treated, and possibly successful when treated as resting eggs. Highest levels of transfection (as determined by significant fluorescence of the siRNA in the pseudo-

coelom) were observed in fed rotifers that were 2 h old at the time of treatment, which involved combining lipofection and electroporation methods of transfection. Successful transfection of siRNA into these animals now provides a possible mechanism for the exploration of rotifer gene function.

Keywords Transfection · siRNA · Resting eggs · Amictic eggs · RNAi · Gene expression

Introduction

Manipulation of gene expression in animals has led to a deeper understanding of gene function, regulation, metabolic pathways, and the production of phenotypes with desirable characteristics (van der Weyden et al., 2002). Rotifers have not played a prominent role in the genomic revolution, as they are not amenable to many classic genetic manipulations. However, to our knowledge, methods to assess functional genomics of rotifers using transfection protocols have not been described. Transfection is the process of introducing DNA or RNA into an organism to elicit changes in phenotype (Dean, 2005). Many transfection experiments are loss-of-function studies where transfected DNA strongly suppresses the expression of a target gene (Mehier-Humbert & Guy, 2005), allowing phenotypes to be probed with reporters and knockouts with techniques including RNAi (Fire, 1999). Gene over-expression studies

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have also advanced the field, as has the creation of transgenic animals of many species.

Transfection techniques have been successful in many invertebrates, including nematodes (*Caenorhabditis elegans*, May and Plasterk, 2005), which are morphologically similar to rotifers. Development of a transfection method for rotifers may lead to the ability to directly assess gene function, where other genetic manipulations have been less successful. With an increasing amount of sequence data available, as a result of the rotifer transcriptome sequencing project (D. Mark Welch, personal communication), many rotifer genes have been described that are candidates for knock-out experiments. Development of transfection tools for rotifers would open up a wide variety of fields that are currently closed to rotifer researchers. As a first step towards transfection of rotifers, we describe methods used in an attempt to transfect double-stranded, fluorescently-labeled siRNA into resting eggs, hatched rotifers, and amictic eggs with varying levels of success.

Materials and methods

Rotifers used for transfection experiments were from the *Brachionus plicatilis* (Muller) species complex, Manjavacas clade (Gomez et al., 2002). This population has previously been referred to as the Russian strain and has been maintained in the lab since 1983 and stored as resting eggs. All experiments were performed with rotifers hatched from a single batch of resting eggs produced on 14 September 1998 and stored dry at -20°C .

Treatment of resting eggs

Approximately 2,000 resting eggs were placed into wells of a 96-well plate and hatched in 50 μl of experimental solution according to the treatments described in Table 1A. Resting eggs were incubated at 25°C under constant fluorescent illumination of 2,000 lux for 3 h. For treatments requiring electroporation, eggs and solution were transferred to electroporation cuvettes and electroporated at the end of the incubation. After 3 h in the various treatments, resting eggs (electroporated and non-electroporated) were transferred to 60×15 mm petri

dishes with 15 ppt artificial seawater (ASW, Instant Ocean) and rotifers were hatched at 25°C under constant fluorescent illumination of approximately 2,000 lux. Hatchlings were transferred from the hatching dish into clean 15 ppt ASW and remained unfed. Hatched rotifers were photographed for quantitation of fluorescence at 2, 24, and 48 h after hatching.

Treatment of hatched rotifers

Rotifers were hatched from resting eggs in 60×15 mm petri dishes with 15 ppt ASW at 25°C under constant fluorescent illumination. The effect of rotifer age on transfection efficiency was tested by treating rotifers ranging in age from 2 to 48 h post-hatch. The mode of siRNA uptake by hatched rotifers is ingestion, with subsequent translocation through the gastrointestinal membranes into the pseudocoelom and reproductive tissues. Because feeding may alter permeability of the gastrointestinal membranes, the effect of feeding on transfection efficiency was tested by treating unfed and fed rotifers. Fed rotifers were hatched with the green alga, *Tetraselmis suecica* cultured in F medium (Guillard, 1983) at 25°C in 15 ppt ASW in 5 l bags under constant fluorescent illumination. Unfed rotifers were hatched in 15 ppt ASW. After hatching, rotifers were transferred into clean 15 ppt ASW either with (fed) or without (unfed) *Tetraselmis*. Prior to experimental treatments, fed rotifers were transferred into clean 15 ppt ASW for 1 h to clear the gastrointestinal tract of algae. Rotifers were exposed to experimental treatments 2–48 h after hatching and fluorescence was photographed 2–48 h after treatment.

Treatment of amictic eggs

Rotifers were hatched from resting eggs in 60×15 mm petri dishes with 15 ppt ASW and fed *T. suecica* at 25°C under constant fluorescent illumination. Oviparous rotifers 48 h old and carrying at least two amictic eggs were transferred into clean 15 ppt ASW for 1–2 h to clear ingested algae from their gastrointestinal tract. Fluorescence of amictic eggs was photographed, 2 h after treatment to quantify uptake of siRNA.

Table 1 Transfection treatments and optimized transfection conditions

<i>(A) Transfection treatments and for resting eggs, hatched rotifers and amictic eggs</i>						
Treatment	siRNA MRP4F	Transfection agent	Electroporation			
Control	No	No	No			
D1	Yes	Yes	No			
D1 + E	Yes	Yes	Yes			
E	Yes	No	Yes			
MRP4F only	Yes	No	No			
<i>(B) Optimized transfection conditions for resting eggs, hatched rotifers and amictic eggs</i>						
Life stage	Incubation period (h)	Electroporation parameters (Gene Pulser Xcell)	Electroporation media	siRNA ($\mu\text{g}/\mu\text{l}$)	Transfection agents	% Transfection agent
Resting eggs	3	Voltage: 50 V Pulse length: 100 ms Number of pulses: 5 Cuvette: 0.2 cm	PBS	0.0372	No transfection agent	NA
Hatched rotifers	3	Voltage: 25 V Pulse length: 100 ms Number of pulses: 5 Cuvette: 0.2 cm	PBS	0.0372	Dharmafect 1 (Dharmacon)	10%
Amictic eggs	3	Voltage: 25 V Pulse length: 100 ms Number of pulses: 5 Cuvette: 0.2 cm	PBS	0.0372	Dharmafect 1 (Dharmacon)	10%

D1, Dharmafect 1; E, electroporated

For D1 and D1 + E treatments, 6 μl MRP4F siRNA was combined with 19 μl PBS and 5 μl Dharmafect 1 was combined with 20 μl PBS in separate wells of a 96-well plate. Dharmafect 1 solution was added to the siRNA solution, mixed via pipette and incubated for 20 min. This transfection solution was added to a well containing resting eggs or 50 rotifers concentrated in a small volume of 15 ppt ASW (<20 μl). Control and E resting eggs and rotifers received 50 μl PBS. MRP4F-only resting eggs were incubated in 6 μl MRP4F siRNA for 5 min, then 44 μl PBS was added to the resting eggs. MRP4F-only rotifers received 6 μl MRP4F siRNA in 44 μl PBS. Resting eggs were incubated at 25°C under constant fluorescent illumination for 3 h. Rotifers were incubated at room temperature in the dark for 3 h. Treatments requiring electroporation were transferred to a 0.2 cm cuvette and electroporated (B). After treatment, resting eggs and rotifers were transferred to a petri dish with 15 ppt ASW. Rotifers were observed for fluorescence 2 h post-treatment, rotifers hatched from treated resting eggs were observed for fluorescence 2 h after hatching

RNA/DNA

Fluorescently-labeled, double-stranded RNA oligonucleotide (19-mer siRNA) was synthesized (Qiagen) based on the target rotifer mate recognition pheromone (MRP) DNA sequence (Snell, T. W. & D. Mark Welch, unpublished). The sequence started at nucleotide 4, hence it is called MRP4: sense r(CGACGGCCAGU-GAAUUGUA)dTdT and antisense r(UACAAUUCACUGGCCGUCG)dTdT. The 3' end of the sense strand was fluorescein (F) labeled. Transfection treatments included concentrations of MRP4F siRNA ranging from 0.0124–0.0744 $\mu\text{g}/\mu\text{l}$.

Transfection agents

Lipofection agents interact with exogenous DNA to form a lipid-DNA complex that can bind and fuse with cell membranes (Felgner et al., 1987). DNA delivered into cells in this manner has resulted in both transient and stable transfections of various cell lines. Numerous transfection agents Dharmafect 1–4 (Dharmacon), ESCORT IV (Sigma) and RNAiVect (Qiagen) were tested with MRP4F siRNA at volumes of 2–7 μl per 50 μl transfection solution (4–14%).

Other transfection agents, including jetPEI (Qbiogene) are cationic polymers that condense with

DNA to form positively charged particles that interact with anionic proteoglycans on the cell surface. DNA is protected by the agent through its buffering capacity as the complex enters the cell through endocytosis (Qbiogene). jetPEI was tested with MRP4F siRNA at volumes of 0.7–2.8 μl per 50 μl transfection solution (1.4–5.6%).

Electroporation

Electroporation is a common method of transiently and stably introducing foreign macromolecules into eukaryotic cells, where cells are exposed to a high-voltage electric field that temporarily rearranges the cell membrane (Chang et al., 1992). The increase in membrane permeability enhances transfer of exogenous macromolecules from the surrounding environment into the cells (Mehier-Humbert & Guy, 2005). Standard methods of electroporation allow for optimization of variables (e.g., wave type, voltage, and pulse length) to optimize transfection efficiency. Another approach to electroporation is nucleofection (Amaxa Biosystems, Cologne, Germany), which uses electrical parameters in combination with specific nucleofector solutions to efficiently transport DNA into the nucleus of target cells. Both electroporation methods were tested to maximize transfection efficiency with acceptable rotifer mortality.

A range of siRNA exposure times (0–22 h), electroporation variables (Gene Pulser Xcell Bio-Rad); voltage: 10–1,750 V; pulse length: 1–100 ms; number of square wave pulses: 1–5; cuvette: 0.1–0.4 cm), nucleofection programs (Amaxa Biosystems; live rotifers A-001, A-002, resting eggs X-001), electroporation media (2 ppt ASW, 15 ppt ASW, PBS (van den Hoff et al., 1992), EC buffer (Qiagen), siPORT (Ambion), Cell Line Nucleofector Solutions L and V (Amaxa), Human T-cell and Smooth Muscle Nucleofector Solutions (Amaxa Biosystems) and Cytomix (van den Hoff et al., 1992) and siRNA concentrations (0.0124–0.0744 $\mu\text{g}/\mu\text{l}$) were tested to determine conditions which maximize rotifer survival with maximum transfection efficiency for resting eggs and hatched rotifers. Electroporation methods were also tested in combination with transfection agents (Dharmafect 1–4 (Dharmacon), ESCORT IV (Sigma), jetPEI (Qbiogene), and RNAiVect (Qiagen) with volumes ranging from 0.7 to 7 μl per 50 μl transfection

solution (1.4–14%) in an effort to maximize transfection efficiency with acceptable mortality.

Transfection protocols (detailed in Table 1 legend) and conditions were manipulated for each life stage (Table 1B) to determine parameters producing maximum fluorescence intensity (mean body/egg fluorescence) either within the rotifer pseudocoelom or amictic eggs. For each life stage, fluorescence intensity and transfection efficiency (% whole body fluorescence) of MRP4F was compared across five treatments (Table 1A). Transfection into rotifers was considered successful if fluorescence was observed within rotifer tissues and pseudocoelom, and overall body fluorescence was significantly greater than control auto-fluorescence. Successful transfection of amictic eggs was indicated by significantly higher fluorescence intensity relative to control auto-fluorescence.

Quantification of transfection

Treated rotifers 2–48 h after transfection were examined for fluorescence by epifluorescent microscopy (Olympus BH-2; excitation at 490 nm; 25 \times magnification). Digital images were captured using an Olympus E-300 camera with 1.6 s exposure controlled by Olympus Studio (version 1.2) on an Apple G5 iMAC.

Fluorescence intensity (mean gray value in optical density units of a single integrated image after 1.6 s exposure) within the outline of the rotifer body or amictic egg was quantified from digital photos using ImageJ (version 1.34s; National Institutes of Health). High mean gray values corresponded to high levels of fluorescence and saturation due to high levels of fluorescence was not encountered. Background fluorescence intensity (measured using the same outline of the rotifer body or amictic egg over background) was subtracted from rotifer or egg fluorescence to yield net fluorescence.

Net fluorescence intensity was analyzed using the statistics program JMP (version 1.1.2; SAS Institute Inc.). Analysis of variance (ANOVA) was performed to determine significant differences among treatments for each life stage. Chi-square and *t*-tests assessed significance between treatments and controls. In order to assess which treatment(s) maximized fluorescence intensity, pairwise comparisons across all

treatments were performed with the Tukey–Kramer HSD test, adjusting significance levels for multiple comparisons.

Results

Rotifers from treated resting eggs

Although significant differences in fluorescence intensity in rotifers were observed over all treatments (Table 2A), none of the rotifers treated as resting eggs demonstrated whole body fluorescence, indicating transfection into the pseudocoelom was not successful. Fluorescence from the MRP4F, when present, was confined to the gastrointestinal tract and mastax (Fig. 2). It is unclear whether this fluorescence was indicative of successful transfection since alternative mechanisms can explain this pattern of fluorescence: (1) post-hatch ingestion of residual MRP4F transferred from the electroporation cuvette into hatching dishes (not successful transfection); (2) successful transfection with subsequent localization to gut tissues resulting from metabolic activity, or (3) successful transfection with subsequent degradation in regions other than gut tissues. Highest levels of fluorescence were observed in MRP4F-only (at 24 and 48 h post-treatment) and D1 + E treatments (at 48 h post-treatment) (Fig. 1A, Table 2B). At 2 h after transfection, MRP4F-only was not different from auto-fluorescence in the control, but by 24 h MRP4F-only had significantly more fluorescence than controls, and this continued through 48 h (Fig. 1A, Table 2B). Fluorescence for all other treatments was not significantly greater than control auto-fluorescence at 2 h or at 24 h (Table 2B).

Treated hatched rotifers

In general, fluorescence was confined to the gastrointestinal tract and mastax due to ingestion of MRP4F (Fig. 2), but whole body fluorescence was observed in 50% of treatments (Fig. 1B), with the highest proportion observed in D1 + E at 2 h (41%) and D1 at 2 h (29%) (Fig. 1). The frequency of whole body fluorescence in both of these treatments was higher than controls by chi square analysis (D1 + E: $\chi^2 = 6.730$, $P = 0.0095$, D1: $\chi^2 = 8.510$, $P = 0.0035$). ANOVA revealed significant differences in fluores-

cence intensity for transfection treatments with hatched rotifers (Table 2A); however, Tukey–Kramer HSD analysis indicated that only D1 at 2 h was significantly greater than controls (Fig. 1B, Table 2B).

Treated amictic eggs and transfer of fluorescence from amictic egg to hatchling

Fluorescence intensities of amictic eggs were significantly different across treatments (Table 2A). Amictic eggs treated with D1 exhibited highest levels of fluorescence intensity, which were significantly different from E, MRP4F-only and control treatments (Fig. 1C, Table 2B).

To determine if fluorescence, due to successful MRP4F transfection, observed in amictic eggs was transferred into the hatchling, amictic eggs treated with D1 were photographed and transferred individually into wells of a 96-well plate. Rotifers hatched from these eggs were photographed, 2 h after hatching. None of the hatchlings had fluorescence within their gastrointestinal tract or distributed throughout their body. Fluorescence intensity in hatchlings from D1 transfected amictic eggs was not significantly different from that of hatchlings from control amictic eggs (t -test $P = 0.210$; Fig. 3), indicating that transfection of MRP4F into rotifers via treatment of amictic eggs was not successful.

Fed versus unfed rotifers

Initially all treatments were conducted with unfed rotifers in order to minimize the interference between siRNA fluorescence and algal auto-fluorescence when quantifying fluorescence intensity. Comparisons of fluorescence intensity and transfection efficiency were made between unfed and fed rotifers. In order to quantify fluorescence from MRP4F rather than from consumed algae, rotifers were placed in clean 15 ppt ASW for at least 1 h to clear their guts (i.e. no red algal fluorescence remained within the rotifers). The effect of feeding on siRNA transfection into hatched rotifers treated with D1 at 2, 24, and 48 h after hatching revealed significant differences (ANOVA, Table 3A). Fed rotifers transfected at 24 h post-hatch exhibited 1.4 times more fluorescence than unfed rotifers (Fig. 3B). More importantly, 77% of fed rotifers at 24 h had whole body fluorescence

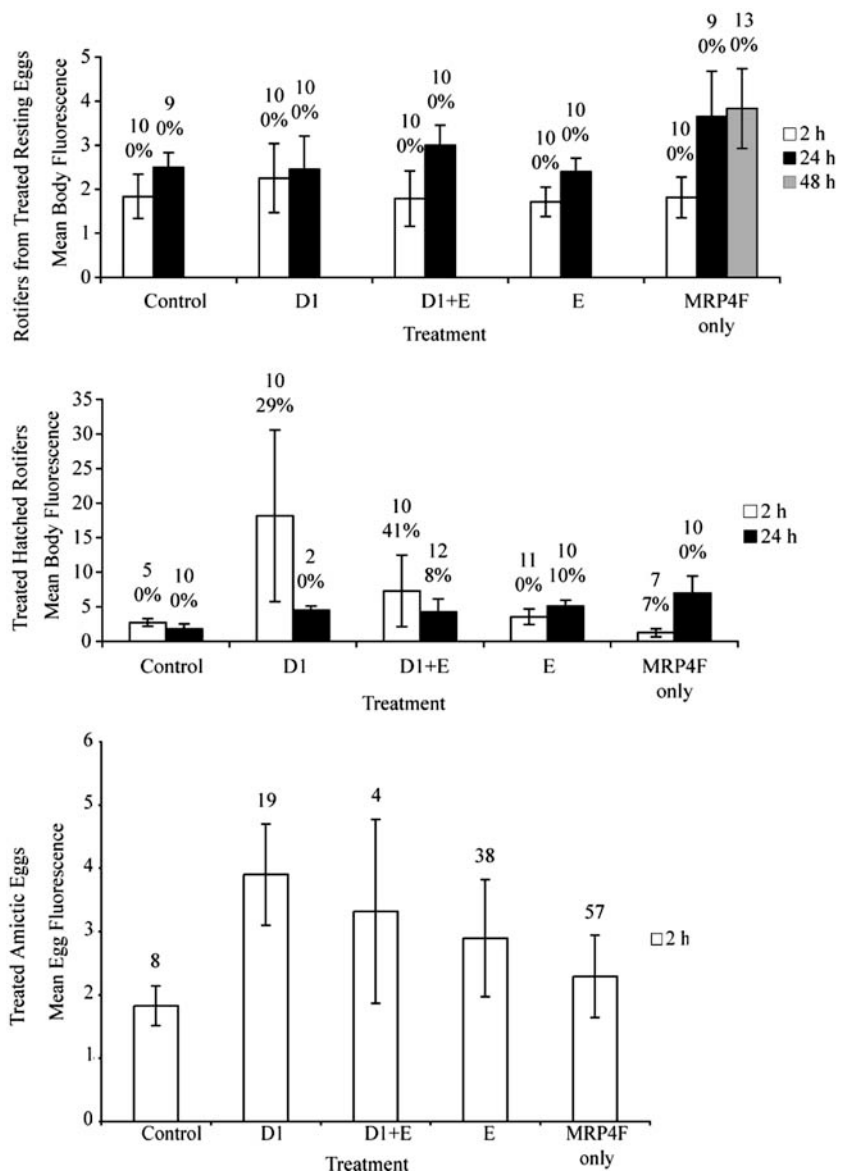
Table 2 Statistical analysis of mean fluorescence intensity of rotifers from treated resting eggs, hatched rotifers, and amictic eggs

(A) ANOVA transfection treatments						
Life stage	Source	DF	Sum of squares	Mean square	F ratio	Prob > F
Resting eggs	Treatment	10	58.337	5.834	14.121	<.0001
	Error	100	41.313	0.413		
	Total	110	99.650			
Hatched rotifers	Treatment	9	2014.982	223.881	9.829	<.0001
	Error	77	1753.806	22.777		
	Total	86	3768.738			
Amictic eggs	Treatment	4	49.301	12.325	20.161	<.0001
	Error	121	73.973	0.611		
	Total	125	123.274			

(B) Tukey–Kramer HSD									
Resting eggs	Treatment	Mean	Hatched rotifers			Amictic eggs			
			Treatment	Mean	Mean	Treatment	Mean		
Resting eggs	MRP4F only 48 h	3.830	D1 2 h	A	18.179	A	D1 2 h	A	3.901
	MRP4F only 24 h	3.647	D1E 2 h		7.300	A	D1E 2 h	B	3.317
	D1E 24 h	3.000	MRP4F only 24 h		6.968	B	E 2 h	B	2.895
Resting eggs	Control 24 h	2.494	E 24 h		5.127	C	MRP4F only 2 h	C	2.225
	D1 24 h	2.458	D1 24 h		4.483	B	Control 2 h	D	1.829
	E 24 h	2.394	D1E 24 h		4.237	B			
Resting eggs	D1 2 h	2.254	E 2 h		3.552	B			
	Control 2 h	1.839	Control 2 h		2.738	B			
	MRP4F only 2 h	1.811	Control 24 h		1.773	B			
Resting eggs	D1E 2 h	1.793	MRP4F only 2 h		1.239	B			

(A) One-way analysis of variance to determine if there was a significant difference in fluorescence intensity among treatments. (B) Tukey–Kramer HSD with adjusted significance levels for multiple comparisons to determine which treatment maximized fluorescence intensity

Fig. 1 Mean fluorescence intensity of (A) rotifers from treated resting eggs, (B) treated hatched rotifers and (C) treated amictic eggs (Table 1A). Number above bars indicates sample size. Percentage above bar indicates percent of rotifers exhibiting whole body fluorescence. Vertical lines on bars indicate standard deviation



compared to 20% of unfed rotifers ($\chi^2 = 7.79$, $P = 0.0053$) indicating that feeding significantly enhances fluorescence intensity and transfection efficiency in hatched rotifers.

Discussion

The ultimate purpose of transfecting rotifers with siRNAs is to achieve RNAi knockdown of gene expression (Fire, 1999). This technique allows gene expression to be selectively suppressed so that gene

function can be determined (Khvorova et al., 2003). RNAi would provide rotifer researchers with an extraordinarily powerful tool to investigate the function of the new genes being discovered in the rotifer transcriptome sequencing project currently underway (D. Mark Welch, personal communication). RNAi will be especially valuable to rotifer researchers as they perform gene mapping, dissect metabolic pathways and manipulate rotifer phenotypes.

Successful transfection of siRNA into rotifers requires that it be taken up intact by the tissues. In resting eggs, the mechanism of uptake is hydration,

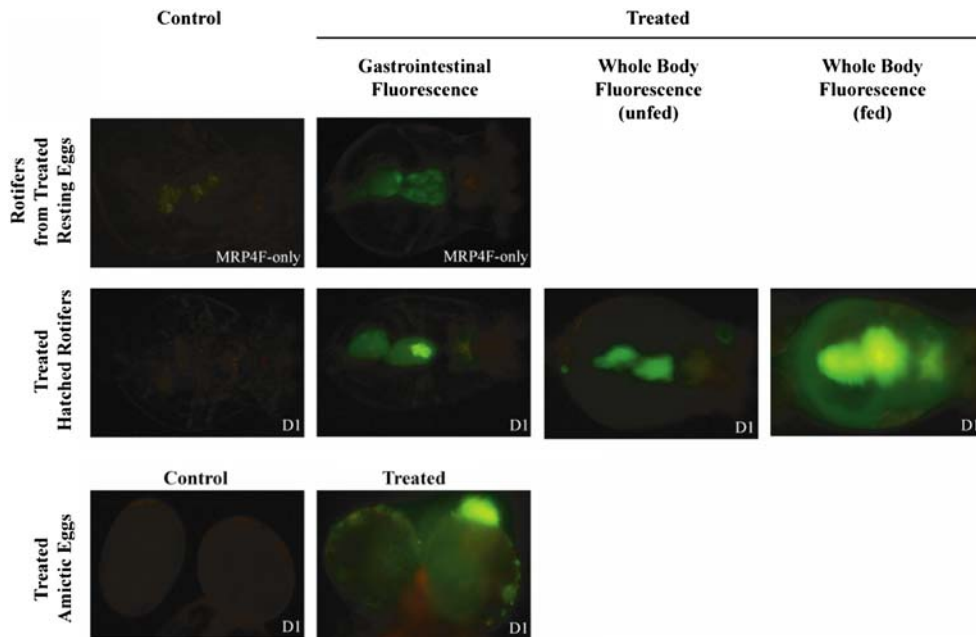


Fig. 2 Fluorescence patterns of rotifers from treated resting eggs, hatched rotifers, and amictic eggs. Transfection treatments are MRP4F-only for resting eggs, and D1 for hatched rotifers and amictic eggs

so when the dried resting eggs are re-hydrated during hatching, the siRNA is taken up along with water. This water uptake during the hatching process makes it unnecessary to treat resting eggs with transfection agents or electroporation to get siRNA incorporation into the hatchlings. Secondary exposure of the hatchlings to residual siRNA is possible; however, the effect of this minimal exposure was not quantified.

In hatched rotifers, the mechanism of siRNA uptake is through ingestion. Rotifer hatchlings ingest media within a few hours of hatching and accumulate molecules, such as siRNA, throughout the digestive tract. It appears that a large proportion of siRNAs accumulated in the lumen of the gastrointestinal tract is defecated without entering rotifer tissues. We assume that siRNAs must first penetrate tissues of the digestive tract in order to have an impact on rotifer metabolism. If siRNAs are to elicit an RNAi reaction in rotifers, penetration of the gut wall through to the pseudocoelom is necessary in order to enter reproductive tissues (i.e. ovaries and vitellarium) and maximize the possibility of an RNAi knockdown of gene expression that has been such a powerful genetic tool in other organisms (Reynolds et al., 2004).

The mechanism of uptake by amictic eggs is probably through lipofection facilitated by the D1 transfection solution. The shell of the developing amictic egg allows certain materials to enter (Gilbert, 1988) and the presence of fluorescence in treated eggs demonstrates that siRNAs are able to penetrate. The location of the siRNA within the embryo is not possible to discern with our current resolution. Determining localization is especially problematic since no fluorescence was detectable in the hatchlings.

Our demonstration that siRNAs can be transfected into rotifers is the first step in being able to manipulate gene expression. The next step is to demonstrate RNAi knockdown in a gene whose function is understood, such as the gene coding for the mate recognition pheromone (MRP). We have a reliable mating bioassay to test for a reduction in the ability of females treated with MRP-siRNA to elicit the male mating response. In addition to using transfection methods to knock-out gene expression, we are also attempting to transfect plasmids containing a GFP reporter gene and/or a selectable marker to determine if plasmids can be stably transfected into rotifers, which will facilitate gene over-expression studies. Development of transfection protocols for

Fig. 3 Mean fluorescence intensity of D1-treated rotifers under fed and unfed conditions. Rotifers were treated at 2, 24, or 48 h after hatching. Number above bars indicates sample size. Percentage above bar indicates percent of rotifers exhibiting whole body fluorescence. Vertical lines on bars indicate standard deviation

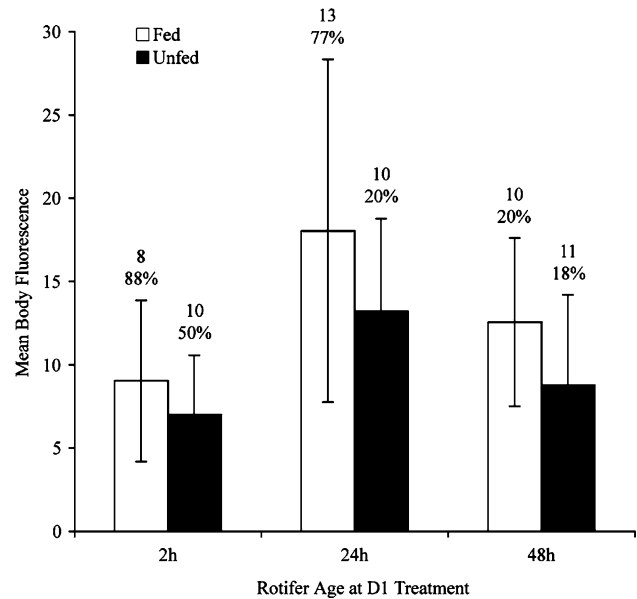


Table 3 Statistical analysis of mean fluorescence intensity of D1-treated hatched rotifers under fed and unfed conditions

Rotifers were treated at 2, 24, or 48 h after hatching. (A) One-way analysis of variance to determine if there was a significant difference in fluorescence intensity among treatments. (B) Tukey-Kramer HSD with adjusted significance levels for multiple comparisons to determine which treatment maximized fluorescence intensity

(A) ANOVA fed versus unfed

Source	DF	Sum of squares	Mean square	F ratio	Prob > F
Treatment	5	902.22	180.444	4.234	0.0025
Error	55	2342.971	42.6		
Total	60	3245.192			

(B) Tukey-Kramer HSD

Treatment	Mean		
Fed 24 h	A	18.04	
Unfed 24 h	A	B	13.21
Fed 48 h	A	B	12.56
Fed 2 h	B	9.031	
Unfed 48 h	B	8.765	
Unfed 2 h	B	6.997	

rotifers now provides a mechanism for the exploration of rotifer gene function.

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