STUDIES OF DESICCATION AND PARASITISM IN ANCIENTLY ASEXUAL BDELLOID ROTIFERS, WITH NOTES ON THE ROLE OF SEXUAL CONFLICT IN SOME HUMAN CULTURAL PRACTICES

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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January 2011

STUDIES OF DESICCATION AND PARASITISM IN ANCIENTLY ASEXUAL BDELLOID ROTIFERS, WITH NOTES ON THE ROLE OF SEXUAL CONFLICT IN SOME HUMAN CULTURAL PRACTICES

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Cornell University 2011

Asexuality has major theoretical advantages over sexual reproduction. Why, then, do exclusively asexual metazoan lineages rarely endure? The Red Queen hypothesis posits that asexuals are extinguished by relentlessly co-evolving parasites and pathogens. If so, any long-lasting asexual lineage must have unusual alternative mechanisms to deal with these biological enemies. In theory, asexual host organisms could persist by dispersing rapidly among habitat patches independently of parasites. Might this scenario apply to bdelloid rotifers, a class of tiny freshwater invertebrates that abandoned sex millions of years ago? Bdelloids thrive in ephemerally moist microhabitats, withstanding periods of desiccation by contracting into tiny anhydrobiotic propagules that are carried on the wind to colonize new patches. Chapters One and Two demonstrate that lab-cultured populations of three bdelloid species can rid themselves of five deadly fungal parasites during desiccation, especially when dried and allowed to disperse in an artificial wind chamber. Chapter Three shows that natural populations of bdelloids colonize elevated habitat patches more rapidly than other microfauna, and that these wind-founded populations have significantly lower parasite incidences than control populations at ground-level.

Results support the hypothesis that desiccation-tolerance and wind dispersal decouple bdelloids from coevolving enemies in space and time, allowing these asexuals to "outrun" the Red Queen.

Consequences of sex include conflicts over paternity. In Chapter Four, I argue that sexual conflict underpins a puzzling human cultural phenomenon. A quarter of preindustrial societies practice some form of male genital mutilation (MGM), despite its clear costs. Substantially altering the naturally selected genital morphology is likely to reduce a man's capacity to compete with other males for fertilizations, especially outside the pair-bond. MGM could therefore reduce the ability of young men to challenge the paternity of older, married men. In societies where paternity uncertainty and reproductive conflict are high, elders may be favored to enforce MGM via sanctions and incentives. As predicted by this sexual conflict hypothesis, MGM rituals are highly public, watched mainly by men, and facilitate access to social and material benefits. MGM is more common in polygynous societies, particularly when co-wives reside separately; it is also associated with lower-than-expected incidences of extramarital sex.

BIOGRAPHICAL SKETCH

Christopher Gordon Wilson was born in the United Kingdom in 1982, to Barbara and Richard Wilson of Broadstairs, Kent. He grew up in this quiet seaside town with his younger sister Stephanie, and they spent much of their childhood exploring tidal pools, fishing, crabbing, climbing trees and antagonizing one another. His affinity for rational inquiry expressed itself early and inopportunely during his time at St Joseph's Roman Catholic Convent School for Infants, and he found himself frequently at odds with the Holy Sisters of the Christian Retreat over matters both spiritual and temporal. Narrowly avoiding expulsion, though doubtless not perdition, he moved to Upton Junior School in 1988. Exposure to (and abuse of) chemistry sets from an early age helped him lead the school quiz team to victory in the 1992 Pfizer Chemistry Junior Blockbusters competition, which engendered a fondness for science. He began secondary education at Dane Court Grammar School in 1993, where he was first introduced to biology as an academic subject. A series of outstanding biology teachers and a longstanding interest in nature won him over from chemistry. He was sufficiently adept at memorizing biochemical, physiological and morphological trivia to succeed at high school biology. Indeed, he won a gold medal in the British Biology Olympiad in 2000, and was selected as one of four UK team members to compete in the International Biology Olympiad in Antalya, Turkey. The selection process entailed several visits to academic institutions, exposure to modern research facilities and direct contact with professional biologists, all of which helped to cement his enthusiasm for a career in this field.

Shortly after returning from Turkey in 2000, Christopher matriculated at the University of Oxford. He read Biological Sciences at St. Anne's College under the inspirational tutelage of Dr. Martin Speight, who implanted a lasting appreciation for

invertebrate ecology that would later bear fruit. He won a College Scholarship in 2001, with a first in Honour Moderations. During 2001-2002, Christopher served as President of the Oxford University Biological Society. He completed an Honours Research project in 2003 under the guidance of Dr. David Shotton, developing the foundations of a formal ontology to organize metadata associated with videos of animal behavior. He graduated from Oxford with a first-class degree in July 2003.

Christopher matriculated at Cornell University in August 2003, advised jointly by Paul W. Sherman and H. Kern Reeve. While at Cornell, his research interests included the nature of adaptation, costly signals of commitment, and the Red Queen hypothesis for the evolution of sexual reproduction. He and his research assistants studied the ecology of anciently asexual bdelloid rotifers and their fungal parasites in the laboratory and in the field. He also developed and taught several undergraduate courses focusing on behavior and evolution in human and nonhuman animals, with a particular focus on writing.

For my mother

With love and admiration

ACKNOWLEDGMENTS

I would like to express my heartfelt thanks to Paul Sherman for guiding me toward the rotifer project, for his unfailingly wise advice and infectious optimism throughout, and especially for his valued collaboration in the process of bringing the results to print. I am extremely grateful to my co-advisor H. Kern Reeve for his stimulating conversation on theoretical matters, and for his boundless enthusiasm, humor and insight. In addition to their time, both of my advisors gave generously and continuously of their resources and space, and I greatly appreciate this vital support.

My research was enhanced by the expert contributions of my committee members. Kathie Hodge gave generously of her time, resources and molecular expertise, and provided a much-needed crash-course in clavicipitaceous mycology. Nelson Hairston provided extensive feedback and advice that clarified my thinking and improved my writing, not just in the context of ecology, but with respect to scientific communication in general. Ron Booker was a constant source of creative experimental and technical ideas: many of the key empirical approaches described this thesis were inspired by his insight.

This thesis would not have been possible without the hard work and dedication of my undergraduate research assistants over the years: David Slottje, Grace Masters, Jennifer Hue, Olivia Love, Spencer Frost, Natalia Brito-Rivera and Angela Lau. I am also grateful for the expert advice of George Barron, Martin Daly, Diego Fontaneto, Sally Glockling and Claudia Ricci, and for technical support provided by ARSEF (Karen Hansen, Richard Humber), Colleen Kearns, Robert Johnson and Kent Loeffler.

I have had many enjoyable and productive interactions with members of the Department of Neurobiology and Behavior. Among the faculty, I wish particularly to thank Kraig Adler, Tom Eisner, Steve Emlen, Joe Fetcho, Rob Raguso, Tom Seeley

and Charlie Walcott. For their dedication, efficiency and professionalism, I am indebted to all the NBB staff, especially Stacey Coil, John Howell, Lori Miller, Brian Mlodzinski, Terri Natoli and Dawn Potter. It has been a privilege to interact with so many friendly and smart graduate students over the years. Their wide range of backgrounds, interests and talents enriched my social and intellectual experience in the department, and their advice and comments during Lunch Bunches helped to improve this thesis. In particular, I would like to thank Jessie Barker, Carlos Botero, Bernard Brennan, Patricia Brennan, Frank Castelli, Stephanie Correa, Becky Cramer, Sam Flaxman, Lynn Fletcher, Jason Gallant, Kristin Hook, Charlotte Jander, Julian Kapoor, Amina Kinkhabwala, Sandeep Kishore, Kristy Lawton, Jonathan Lee, Kevin Loope, Ann Marie McNamara, Jordan Moore, Jon Olthoff, Juliana Rangel, Kevin Rohmann, Dustin Rubenstein, C. Dustin Rubinstein, Judith Scarl, Sheng-Feng Shen, Caitlin Stern, Holly Summers, Mya Thompson, Biz Turnell and Maggie Wray. I am ever grateful to Pat Barclay, whose generosity with time, advice and resources contributed critically to my research and teaching in the field of human behavior.

While at Cornell, I benefited from the generosity of several funding sources. I wish particularly to thank the Knight Institute for Writing in the Disciplines, which supported me for five semesters, and the College of Agriculture and Life Sciences. Research funding was provided by Sigma Xi and the Department of Neurobiology and Behavior; I am grateful to everyone involved in providing these vital grants.

Finally, this journey would not have been possible without a great deal of support, companionship and love. I wish to thank Zack Bassman, Sofia, Robert and Jared Bassman, Audrey DiFelice, Randall Hance, all my friends associated with Kappa Delta Rho, Karatedo Doshinkan, Gateway Theatre and the Cornell Shakespeare Troupe, Dean Lillard, Evan and Amy Marshak, Alice Moore, Drew Mudge, Kevin Nagel, James Selwyn, and especially Richard, Stephanie and Tessa Wilson.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ARSEF	USDA Agriculture Research Service Collection of Entomopathogenic Fungal Cultures
CI	Confidence interval
eHRAF	Human Relations Area Files (electronic version)
EPC	Extra-pair copulation
FGM	Female genital mutilation
MGM	Male genital mutilation
PRF	Polygyny risk factor
PSF	Probability sample file
RH	Relative humidity
rPSF	Probability sample file (reduced)
SCCS	Standard cross-cultural sample
SD	Standard deviation
SE	Standard error

CHAPTER 1

ANCIENTLY ASEXUAL BDELLOID ROTIFERS ESCAPE LETHAL FUNGAL PARASITES BY DRYING UP AND BLOWING AWAY[1]

Abstract

Asexuality has major theoretical advantages over sexual reproduction. An important evolutionary puzzle, therefore, is why exclusively asexual metazoan lineages rarely endure. The Red Queen hypothesis posits that asexuality is rapidly extinguished by relentlessly co-evolving parasites and pathogens. If so, any long-lasting asexual lineage must have unusual alternative mechanisms to deal with these biotic enemies. Bdelloid rotifers are freshwater invertebrates that abandoned sexual reproduction millions of years ago. We show that cultured populations of bdelloids can rid themselves of a deadly fungal parasite through complete desiccation (anhydrobiosis), and disperse by wind to establish new populations in its absence. Under Red Queen models, spatiotemporal escape can decouple and protect asexuals from co-evolving enemies. Our results may therefore help to explain the persistence of the anciently asexual Bdelloidea.

Introduction

Sexual reproduction reduces the efficiency of gene transmission by up to 50%, disrupts favorable gene combinations, spreads disease and is energetically expensive (*1*). Yet, paradoxically, sex is nearly ubiquitous: obligate asexuality occurs in less than 1% of animal species (*1, 2*), and its scattered distribution at the tips of

[1] Reprinted from Science, Vol. 327, Wilson, C. G. and Sherman, P.W. Anciently asexual bdelloid rotifers escape lethal fungal parasites by drying up and blowing away, pp. 574-576, © 2010 American Association for the Advancement of Science (AAAS). Reprinted by permission of the AAAS.

phylogenetic trees implies that abandoning sex condemns a clade to extinction before it can radiate sufficiently to achieve high taxonomic rank.

The Class Bdelloidea (Phylum Rotifera) is a famous exception. During three centuries of observation, over 450 species of these tiny freshwater invertebrates have been described, but neither males nor meiotic eggs have ever been recorded (*3*). Molecular evidence supports the inference that bdelloid rotifers have been obligately asexual for tens of millions of years (*2-5*).

Hypotheses for sexual reproduction suggest that it is maintained because it removes deleterious mutations, facilitates co-evolution with parasites and pathogens, or both (*2, 5-8)*. However, mutational hypotheses have been challenged empirically (*1*, *4*, *5*, 9), and mutational load may be less problematic than predicted since the bdelloids have persisted despite accumulating mutations faster than related sexual clades (*4*). By contrast, the co-evolution (Red Queen) hypothesis has received considerable empirical and theoretical support (*1, 5-11*).

Under Red Queen models, biotic interactions favor sex by relentlessly imposing fluctuating, time-lagged frequency-dependent selection (*6-10*). However, asexuality can be maintained in one special case: when vulnerable hosts can temporarily shed locally co-adapted parasites and pathogens, and disperse without them to uninfected habitats (*11*). Migration and clonal diversity at the population level can then substitute for recombination and genetic diversity at the individual level, allowing mobile hosts to avoid the costs of sex while continuing to "outrun" their enemies (*11-12*). Dispersal further favors asexuality by reducing intergenerational transmission of infections (*13*).

Three unusual characteristics of bdelloid rotifers suggest that this scenario may apply to them (*5, 11, 12*). First, bdelloids can survive extended (up to 9 years) and repeated bouts of complete desiccation (anhydrobiosis) at any life stage (*14*, *15*). Second, anhydrobiotic bdelloids have an extraordinary potential for wind dispersal as

tiny (usually <300μm) ovoid propagules (called "tuns") (*14-17*), resulting in circumglobal distribution of some taxa (*18*). Third, bdelloids can thrive in almost any moist habitat, rapidly colonizing even the most ephemeral patches of moss or rainwater on every continent (*14-18*).

All identified parasites of bdelloid rotifers are oomycetes or hyphomycete fungi. Most belong to *Rotiferophthora*, a genus of obligate, lethal fungal endoparasites that are exclusive to bdelloids (*19-20*). Infections spread when rotifers ingest spores (conidia), which lodge in their pharynx and produce assimilative hyphae. As the rotifer is killed and digested, hyphae puncture its integument and, at the air-water interface, produce conidiophores carrying hundreds of new conidia.

Results and Discussion

We investigated whether populations of the bdelloid rotifer *Habrotrocha elusa elusa* can escape the fungal parasite *Rotiferophthora angustispora* (*19*) in space and time, through anhydrobiosis and subsequent wind dispersal. We transferred rotifers from a monoclonal population singly to Petri dishes (*20*). A control group was allowed to proliferate without parasites, whereas 6 experimental groups were inoculated after 9 days with approximately 712 (± 210 SD) conidia of *R. angustispora*. The first experimental group remained hydrated throughout the experiment, but the remaining five were desiccated 72 hours after inoculation, maintained at 39.8% R.H. (± 1.8% SD) for 7, 14, 21, 28, and 35 days respectively, and then rehydrated.

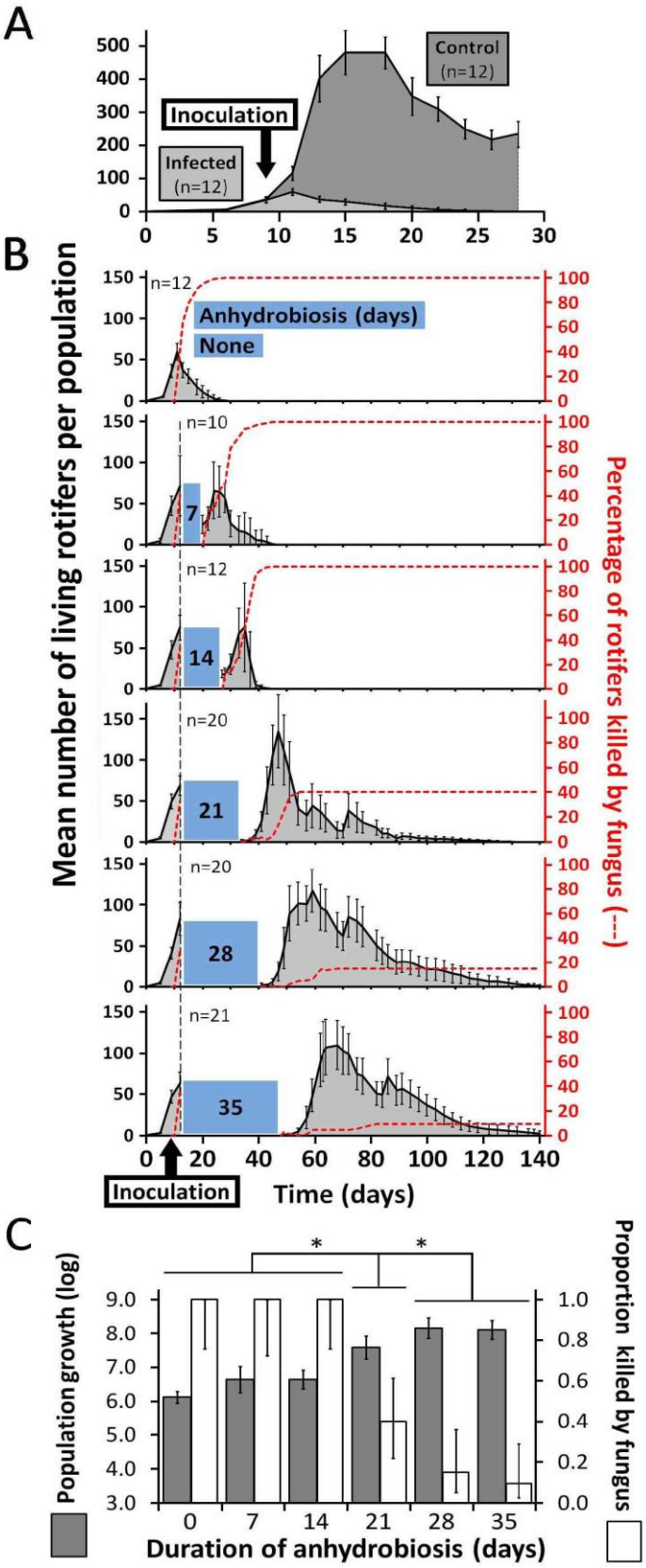
Without anhydrobiosis, *R. angustispora* exterminated *H. elusa* populations in 13.8 ± 2.6 days (mean ± SD, $n = 12$), during which time the uninfected control populations were peaking (Fig. 1.1A). In the dishes that were rehydrated after 7 days of desiccation, the parasite initially seemed absent. Recovered rotifers were healthy, desiccation had fractured and scattered the fungal conidiophores, and water samples

were not infectious to fresh rotifer cultures, suggesting absence of viable conidia (*20*). However, within 48 hours hyphae began to re-emerge from the remains of dead rotifers, generating new conidiophores and exterminating the populations in 18.6 ± 4.0 days (Fig. 1.1B). Excluding a four day lag while the fungus regenerated, the time course of the resurgent infection in the rehydrated populations did not differ from that observed in the hydrated, inoculated group (i.e., complete extermination in 14.6 ± 4.0 days vs. 13.8 ± 2.6 days; unpaired *t*-test, $n = 22$, $t = 0.544$, $P = 0.59$). Identical fungal regeneration was observed after 14 days of desiccation (with extermination in 14.7 ± 2.3 days).

However, dramatically different results occurred in the other three experimental groups. Following 21 days of anhydrobiosis, 60% of rotifer populations remained free of fungal infections for the duration of the experiment (20 weeks). Longer periods of desiccation were even more effective: after 28 and 35 days, 85% and 90.5% of rehydrated populations remained fungus-free, and so grew significantly more (Fig. 1.1C).

In nature, desiccated bdelloids are dispersed by wind, adding a second dimension in which they might evade parasites (*16*). To simulate wind dispersal, we established a heavily infected population of *H. elusa* in a dish with a natural, friable substrate (sterilized moss and silt), and placed it in a wind chamber with a turbulent flow equivalent to a light breeze (*20*). Desiccated substrate particles were blown toward empty target dishes 30-40 cm away. After 7 days in the chamber, these 'wind dispersal' dishes had accumulated 5.01mg (± 2.52 SD) of material; they were then hydrated and monitored for 6 weeks.

Figure 1.1 (A) Growth curve (±95% CI) for rotifers (*H. elusa*) inoculated with *R. angustispora*, versus uninfected controls. **(B)** Growth curves for inoculated populations subjected to extended desiccation. The proportion of infected rotifers in each dish was used to construct a mean mortality curve. As all parasitized populations were eventually exterminated, this curve finally stabilizes at a value equal to the proportion of dishes in which infections returned after desiccation. **(C)** Statistical summary of treatment differences from (B). Population growth was quantified for each dish as the natural logarithm of the total area under its growth curve; shaded bars show the mean for each treatment group (±95% CI). Open bars show the proportion of populations that were exterminated by the fungus (±95% CI). $*P < 0.05$ by one-way ANOVA, Tukey post-hoc (for growth), and by χ^2 (for mortality), corrected post-hoc.


A
Control
(n=12)
Inoculation
Infected
(n=12)
B
Mean number of living rotifers per population
Percentage of rotifers killed by fungus (---)
n=12
Anhydrobiosis (days)
None
n=10
7
n=12
14
n=20
21
n=20
28
n=21
35
Inoculation
Time (days)
C
Population growth (log)
Proportion killed by fungus
Duration of anhydrobiosis (days)

Enough rotifers survived to establish populations in 17 of 24 target dishes. Fungal infections appeared in 7 of the newly established populations, and exterminated them within 16.4 ± 5.0 days (Fig. 1.2A). However, the 10 remaining wind-dispersed populations (58.8%) never exhibited infections.

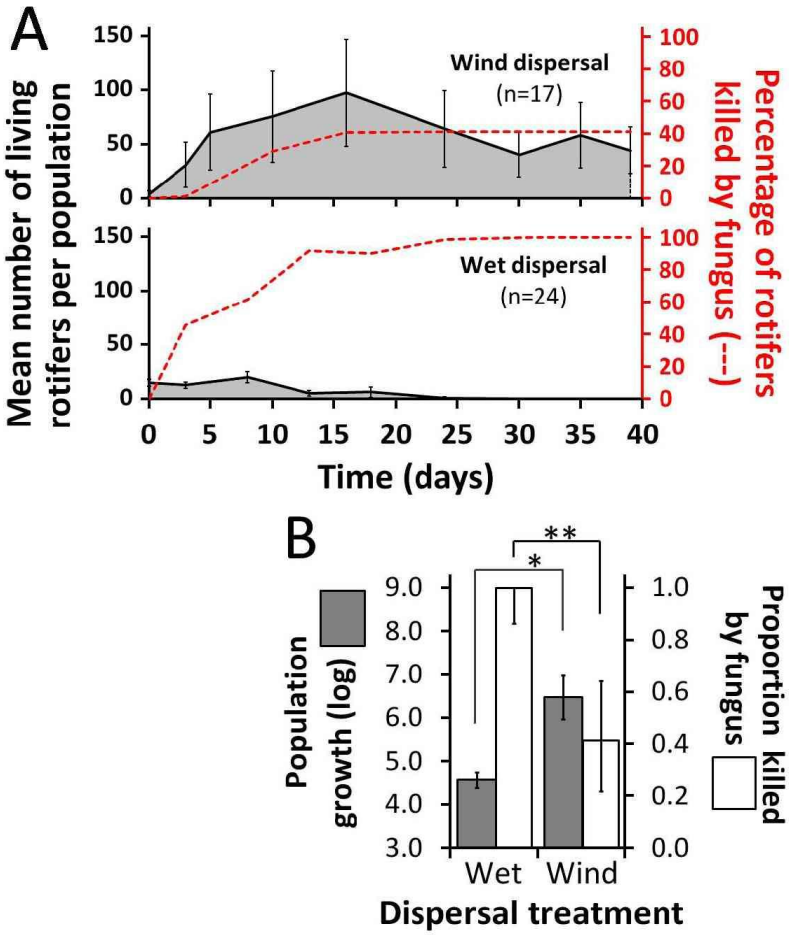


Figure 1.2 (A) Growth (±95% CI) and mortality curves for 17 *H. elusa* populations founded by artificial wind dispersal of substrate (5.01mg ± 2.52 SD) from an infected source culture desiccated for 7 days. Parasites exterminated 7 populations, but 10 remained uninfected (58.8%), compared with complete mortality of all 24 control populations founded concurrently by wet transfer of 5mg of the same infected source material. **(B)** Statistical summary of differences in (A) [see caption to Fig. 1.1(C)]; * $P < 0.0001$, unpaired t-test; ** $P < 0.0001$, Fisher's exact test.

We replicated this experiment, with similar results (i.e., 63.6% of wind-dispersed populations remained fungus-free: fig. 1.4). For comparison, 'wet dispersal' dishes (n=24) were created by pipetting 5.0 mg of well-mixed, suspended substrate from an identical infected source dish that was not desiccated. Rotifers established populations in all dishes, but fungal conidia also were readily transmitted, and all populations were exterminated in 22.3 ± 5.7 days (Fig. 1.2B).

Our first experiment indicates that anhydrobiosis allows *H. elusa* to shed *R. angustispora*, apparently because the fungus is less resistant to extended desiccation than its host (Fig. 1.1C; also fig. 1.3). Our second experiment demonstrates that wind dispersal acts in concert with anhydrobiosis to facilitate escape from *Rotiferophthora*, which is primarily waterborne. Three weeks of in situ desiccation (Fig. 1.1B) were required to achieve the same rate of parasite elimination (60%) that was seen after only 7 days in the wind chamber (Fig. 1.2; fig. 1.4). In nature, dispersal distances and maximal durations of anhydrobiosis are certainly far greater (*14-18, 20*). Together, our results demonstrate that anhydrobiosis, even for relatively short periods, enables this bdelloid rotifer to disperse without accompaniment by a lethally co-adapted fungal parasite.

Although facultative or recently evolved asexuality is common among organisms resistant to physical extremes, and those with passively dispersed dormant propagules (*1*), the extraordinary capabilities of the bdelloids in both regards sets them apart. Few animals or plants can withstand complete loss of cellular water, and usually just at specific life-stages (e.g. seeds, larvae, eggs) (*15*). Only bdelloid rotifers and certain tardigrades and nematodes can tolerate repeated bouts of desiccation at any life stage (*14, 15*) and, of these, only the rotifers occur frequently in samples of rain and wind (*16*). With their smaller, more aerodynamic tuns bdelloids can colonize tiny, isolated microhabitats more rapidly than tardigrades and nematodes (*17*).

The Bdelloidea have been called an 'evolutionary scandal' (*2*), because their ancient asexuality seemed to challenge all hypotheses for the long-term maintenance of sex. However, if anhydrobiotic dispersal enables bdelloid species to escape temporally and spatially from some or many natural enemies, their co-evolutionary burden would be substantially reduced. Our results are therefore consistent with a scenario in which bdelloids have evaded parasites and pathogens over evolutionary time without incurring the costs of sexuality, by playing a never-ending game of "hide-and-seek" (*11*, *12*). Decoupling from co-evolving enemies could help to explain the ancient asexuality of the Bdelloidea under the broad array of models that derive from or incorporate Red Queen dynamics.

Materials and Methods

Isolation of Rotifer Clone and Culture Conditions

All rotifer populations descended clonally from a single individual isolated from a sample of moss collected in June 2006 from the Mundy Wildflower Garden in Ithaca, NY, USA (42°27'1.50"N; 76°28'2.49"W). This clone was later identified as *Habrotrocha elusa elusa* (*21*), based on morphology of the head and eggs (*22*).

Stock populations of rotifers were propagated in non-axenic culture using a serial batch transfer method (*23*). Every 2 weeks, individual rotifers were transferred singly by pipette from a source population into 35mm plastic Petri dishes lined with 0.9ml of modified Czapek-Dox agar, prepared to the following recipe, which omits sucrose:

$NaNO_3$	3.0g
K_2PO_4	1.0g
$MgSO_4.7H_2O$	0.5g

KCl	0.5g
$FeSO_4.7H_2O$	0.01g
Agar	15.0g
Distilled water	1000 mL

To each dish was added 1.0mL of sterile rainwater, which we used in preference to distilled water since it better approximates the ionic composition of water in the rotifers' natural habitats. This was collected regularly in flasks on the roof of Mudd Hall, Cornell University, Ithaca, NY (42°26'50.35"N; 76°28'44.80"W), filtered and then sterilized by autoclave. Newly subcultured dishes were stored in sealed boxes of transparent plastic under a 12L:12D regime. Evaporation of water from the stored cultures was minimized by keeping the relative humidity (R.H.) in the boxes ≥ 97% using standing dishes of distilled water. Rotifer populations grow rapidly and reliably under these conditions, apparently sustained solely by naturally occurring bacteria that slowly metabolize the agar (*23*). Population dynamics typify the standard microbiological growth curve, with lag, log, stationary and decline phases (Fig. 1.1A). Although we typically sub-cultured several individuals to fresh dishes every 2 weeks, dishes can sustain populations of >100 individuals for many more weeks without further addition of food or fresh medium.

The 95 populations in our first experiment were initially established by the same method, using individuals transferred singly from a source culture which had originated with a single founding rotifer 2 weeks earlier. Originally, 100 new cultures were created; however, 5 failed to thrive. This is the source of the slight imbalance in final sample sizes. Experimental populations were not subcultured further or transferred to new dishes at any point.

Parasite Isolate and Culture

Rotiferophthora is a genus of hyphomycete fungi with 27 described species (*24*). These fungi were chosen for our experiments both because they "are the most frequently recorded and have the greatest number of species of any parasite or predator of bdelloid rotifers" (*19*), and because they are "devastating parasites…capable of wiping out entire populations of rotifers in Petri dishes in a few days" (*19*). Our isolate of *Rotiferophthora angustispora* (*23, 19*) was provided by the Agriculture Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF), located at Cornell University. The ARSEF accession number for this isolate is 5610, and it was originally recovered in 1997, from decaying straw in the United Kingdom. The fungus was provided in pure culture on potato dextrose agar; it was transferred into our rotifer cultures and sustained using a serial transfer method described by Barron (1985) (*23*). We are grateful to the U.S. Department of Agriculture ARS Biological Integrated Pest Management Research Unit for providing the ARSEF isolate.

R. angustispora is "recovered frequently" from various soil habitats in Ontario, Canada, where it was originally described (*23*). We also have recovered this species from soil in the vicinity of Ithaca, NY on at least 4 separate occasions and at 4 different sites: more frequently than any other *Rotiferophthora* species. One of these Ithaca isolates was used to replicate the results of our dispersal experiment (see below), and this strain was subsequently deposited with ARSEF (accession #8991).

Infection Protocol

A suspension of *R. angustispora* conidia was prepared by individually transferring 100 infected rotifers with fully developed conidiophores into 1mL of distilled water and vortexing. Conidial concentration (79.1 μL^{-1} ± 23.4 SD) was

assessed by taking a 10µL sample of the suspension, staining the conidia with 5µL of lactophenol cotton blue, and counting by hemocytometer. After the experimental rotifer populations had been growing for 9 days, each was inoculated with 9µl of the suspension, which was vortexed between transfers to maintain a uniform distribution of conidia. We calculate that each dish received approximately 712 conidia (± 210 SD).

Population Counts

Living and infected rotifers in each population were counted at intervals of 2-3 days for the duration of the experiment (20 weeks), using a 20x20mm counting grid and a compound microscope. Rotifers were considered alive if they showed any locomotion, feeding behavior or internal organ movements; they were considered infected if any fungal hyphae had punctured the integument. These categories are mutually exclusive, since host movements invariably cease at such an advanced stage of infection by *Rotiferophthora*. We did not count rotifers that had died of causes not obviously related to fungal infection.

Dishes could not be counted while desiccated, since anhydrobiotic rotifers contract into an opaque "tun" and enter a state of complete physiological dormancy (*25*), in which neither survival nor infection can be determined reliably. Immediately prior to the onset of dehydration, the mean number of living rotifers was 72 (± 35 SD) in the dishes that were to be dried. Therefore, the fungal conidia from the initial inoculum (712 ± 210 SD) outnumbered the rotifers by an order of magnitude when desiccation began.

Desiccation and Rehydration

Drying of the infected *H. elusa* populations was initiated 72 hours after inoculation, by spacing the 35mm Petri dishes 3cm apart on shallow rectangular plastic trays (33 x 25 x 8cm) and removing the lids to expose them to the air in a sealed room. Relative humidity in the room was kept at 39.8% (±1.8% SD), measured using electronic thermo-hygrometers (Fisher Scientific, cat. #11-661-14), and maintained by two commercial humidifiers (Holmes, models #HM630; #HM1865) and a high-capacity dehumidifier (Whirlpool, model #AD70USS) with built-in hygrostats. The temperature (23.0^{o}C ± 1.45 SD) was also measured daily.

Dishes exposed to these conditions appeared to have lost their water layer after 12 hrs. However, a strip of $CoCl_2$ test paper could still be inserted easily into the moist agar, and its color change indicated the presence of free water. Moreover, rotifers could still be seen moving slightly on the surface of the agar, trapped in a glossy residual film of water. After a further 6 hours, the agar itself had reached the fully desiccated appearance that it would retain until rehydration, becoming a tough, plastic-like sheet of minimal thickness adhering so tightly to the bottom of the dish that it seemed to disappear entirely. $CoCl_2$ test paper could no longer be inserted into this material; there was no color change after scraping the paper across the surface; no movement was visible on the dish, and the rotifer tuns were distinguishable only as faintly raised topographical features, which crumbled into fine particles if scraped with a minuten needle.

During the extended desiccation, care was taken to limit mechanical disturbance to the dishes and fluctuations in temperature or relative humidity, since these are known to be harmful to anhydrobiotic rotifers in culture (*26*). In previous laboratory studies, bdelloids have recovered after more than 50 days of anhydrobiosis (*26*), considerably longer than the maximum duration in our experiment (35 days).

Some individuals that dried in nature have even recovered after 9 years without water (*27*). Exposure to increased humidity just prior to rehydration is known to improve recovery rates (*27*), and so desiccated dishes were placed in a pre-hydration "priming chamber" at 55% R.H. for 90 minutes before rehydration, after which 1.0mL of sterile rainwater was added. Following rehydration, the agar partially recovered its gelatinous state, and recovered rotifers easily detached themselves from this substrate. The number of recovering rotifers was lower than the population size prior to desiccation; recovery rate varied according to the duration of anhydrobiosis (Fig. 1.3).

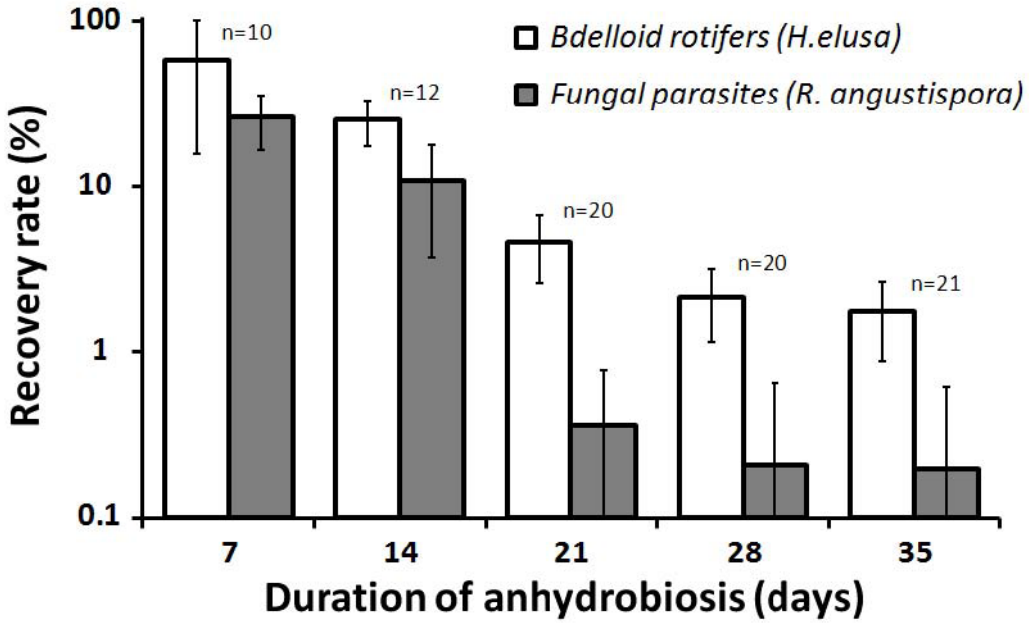


Figure 1.3 Mean proportions (±95% CI) of individual bdelloid rotifers and germinating parasitic fungi that survived anhydrobiosis. Since both organisms take at least 48 hours to produce a secondary generation, active individuals observed during the 48 hours immediately following rehydration must have been desiccation survivors. These counts were divided by the number of individuals present initially at desiccation, and the resulting recovery rate is displayed on a logarithmic scale relative to the duration of anhydrobiosis. Bdelloid recovery rates were more than twice as high as those of parasites at 7-14 days, and became over ten times greater at 21-35 days, by which time most dishes had no recovering parasites at all (c.f. Fig. 1.1B, C).

However, the initial survivors rapidly resumed reproduction, and the population entered a new growth phase (Fig. 1.1B). The rehydrated dishes were stored in sealed, humid boxes as described above, and removed only briefly for counting. In this way, loss of water by evaporation was kept to a minimum, and population dynamics could be studied for months without addition or removal of any medium to or from the dishes.

During desiccation, the dried dishes had been exposed to the ambient environment and it was therefore surprising that there was no obvious contamination of the rehydrated dishes by any undesired bacteria or fungi. The recovered *H. elusa* in each dish were able to subsist on the bacterial inoculum they happened to have received. Naturally, the peak of the secondary, post-rehydration growth curves in the rehydrated dishes did not equal the peak of the control growth curve observed on fresh agar, but the shape was unchanged. Moreover, although there was considerable variation in the degree of growth achieved by each population after rehydration, a highly significant portion was due to the treatment (Fig. 1.1C). The ultimate fate of each population was primarily determined by the duration of anhydrobiosis and the consequent probability of parasite removal, rather than the unpredictable composition of the adventitious bacterial inoculum.

Assessing Fungal Regeneration

To establish whether fungal conidia had survived the shortest periods of desiccation (7 and 14 days), we briefly agitated each plate in these treatment groups by vortex approximately 24 hours after rehydration. This process had no adverse effects on the recovered rotifers, but was intended to re-suspend any viable fungal conidia. A 100 µL sample containing no dead or living rotifers was then transferred from each experimental dish into a matched, 9-day old fresh population of *H. elusa* (the lost

volume was restored to the original plates by addition of 100 µL of sterile rainwater). The 10 dishes matched to the 7-day treatment group were observed for the duration of the main experiment (20 weeks), but none developed fungal infections.. The same test was conducted for the 12 plates rehydrated after 14 days, with the same negative outcome. We therefore did not test any rehydrated plates in subsequent weeks. However, we did establish a control by taking 100 µL samples from the plates in the first experimental group, which was never dehydrated. When these were transferred to fresh dishes, the matched populations were all exterminated by *R. angustispora*. We concluded that free conidia are susceptible to inactivation by even short periods of desiccation, and they probably require water for successful transmission.

When the fungus survived desiccation at all in our experiments, it did so as hyphae within dead rotifers. A substantial percentage of rotifers (34.0 ± 11.9 SD) had been infected with a germinating conidium before desiccation began, and had begun to develop protruding hyphae or conidiophores. Following rehydration, these fragile fungal structures were entirely absent, having apparently been destroyed by the treatment. However, within approximately 48 hours, fresh hyphae began to emerge from a fraction of the rehydrated corpses. These hyphae went on to produce conidiophores and initiate a new infection cycle. The number of rehydrated corpses in each plate that generated new external hyphae in this manner was lower than the number of rotifers that had been infected prior to desiccation. If we consider each visibly infected rotifer present at desiccation to represent a single fungal 'individual', and each rehydrated corpse with regenerating hyphae to represent a fungal 'survivor' of desiccation, we can directly compare the desiccation tolerance of individual rotifers and parasites (Fig. 1.3). After 7 days and 14 days of desiccation, the rotifers recovered more than twice as effectively as the parasites, and this rose to a tenfold advantage when desiccation lasted for 21 days or longer. These asymmetries in desiccation

tolerance between individuals of the two species underpin the eventual outcomes at the population level (Fig. 1.1 B, C).

Dispersal Treatments

To create a heavily-infected source dish for the spatial dispersal experiment, a single rotifer was transferred to a 100mm Petri dish with modified Czapek-Dox agar and sterile rainwater (6mL), according to the methods described above. After clonal proliferation for 30 days, the original agar substrate was cut into wedges and removed from beneath the rotifer population. A more natural and friable substrate was added, consisting of 0.8g of sterilized, milled sphagnum moss (Mosser Lee Co.) and 0.4g of sterilized soil from the location where *H. elusa* was originally collected. This culture was inoculated with 0.5ml of *R. angustispora* conidial suspension, prepared as described above (and therefore containing approximately 40,000 conidia). After 24 hours, the dish was placed in the center of the wind chamber: a rectangular, sealed Plexiglas box (1.2 x 0.4 x 0.76 m), containing five 48V fans (ETRI Co.). These generated a turbulent flow with a mean velocity of 0.97 $m \cdot s^{-1}$ (± 0.43 SD) and maximum gusts of 2.24 $m \cdot s^{-1}$ (a 'light breeze' on the Beaufort Scale), measured using a hot-wire anemometer (Testo 425). The chamber was kept in a room whose relative humidity was maintained at approximately 40% by the methods described above. Desiccation of the moss and soil was assessed with $CoCl_2$ test paper, which no longer changed color after 12 hours. A sterile spatula was used to initiate crumbling of the dried substrate, which then proceeded passively through the action of the artificial wind.

Twenty-four target Petri dishes (35mm) of known mass were embedded in the Styrofoam floor of the chamber in order to accumulate blown material. They contained neither rotifers nor water, only a layer of agar which had been allowed to

dehydrate according to the method described above. The distance between the source dish and these target dishes was 30-40cm; however, it is likely that most particles circulated for some time in the chamber before accreting in the target dishes, so that the total distance travelled could have been somewhat greater. In nature, bdelloids readily disperse at least 60m from a source population (*28*), and have been collected in a windsock sample at an elevation of 16m and a distance of 1km from the nearest upwind body of standing water (*16*). Iterative long-distance dispersal by wind may also be responsible for the distribution of closely-related clones of some bdelloid species at sites as far apart as Europe, Africa and New Zealand (*29*).

After one week, the 'wind dispersal' target dishes were removed from the wind chamber and re-weighed to calculate the mass of deposited substrate (5.01mg ± 2.52 SD). They were placed in a pre-hydration priming chamber at 55% R.H. for 90 minutes, after which 1.0mL of sterile rainwater was added to each dish.

For the 'wet dispersal' control condition, a heavily-infected source dish was created as described above. However, immediately after addition of the moss and soil substrate, and inoculation with *R. angustispora*, the dish was shaken to suspend this mixture uniformly, and ten samples of 0.1mL were pipetted to weighing boats of known mass. These were allowed to dry over 12 hours at 40% R.H. and then weighed, so that we could calculate the mean dry mass of material in a given volume of infected suspension (47.4mg mL^{-1} ± 25.2 SD), and therefore match the mass deposited on the wind dispersal dishes. Accordingly, after a further 12 hours, 108µL of infected suspension was dispensed by pipette from the wet source dish into 24 Petri dishes (35mm) lined with desiccated agar as in the wind dispersal condition. By adding 892µL of sterile rainwater, the final volume was made up to 1mL, to match the water volume of the rehydrated dishes in the wind dispersal condition. The rehydrated wind dispersal dishes and their wet-dispersal controls were maintained for several

weeks in a sealed, humid box, and living and infected rotifers were counted regularly as described above.

Replication and Robustness of Dispersal Results

After completing the first dispersal experiment described above, we obtained a second strain of *R. angustispora* from nature, isolated from a sample of moss and soil collected in October 2008 near the shore of Cayuga Lake in Ithaca, NY (42°27'32.46"N 76°29'37.51"W). We replicated the dispersal experiment using this isolate, to demonstrate the robustness of our results with a fungal strain whose geographic provenance matched that of our rotifers. We have made this second strain available via ARSEF (accession #8991).

Wind and wet dispersal treatments were repeated using methods described above, with conidia of the new fungal isolate. The amount of material that accumulated in the wind dispersal target dishes after 7 days was 4.3mg (± 2.43 SD), and the volume of suspension used in the wet dispersal condition was adjusted to match this mass, following a mass-to-volume calibration procedure as described above. Fig. 1.4 displays the outcome of this second trial in the same format as Fig. 1.2. The results are in close agreement with the earlier experiment: 63.6% of wind-dispersed populations shed the parasite, whereas 100% mortality was seen in the wet dispersal condition ($P = 0.0004$, Fisher's exact test, n=27). Although the wet-dispersed populations in this trial experienced a particularly rapid period of growth shortly after transfer, the parasites quickly "caught up", and so the wind-dispersed rotifers enjoyed a longer period of growth overall, by an extremely significant margin ($P = 0.0003$, unpaired t-test, n=27, $t = 4.16$). The minor differences in growth patterns between this trial and that shown in Fig. 1.2 are probably due to fluctuations in bacterial species composition in the dishes and in the wind chamber at the times of the two trials.

Despite this variation, our initial finding that bdelloids evade parasites spatially appears robust for both strains of *R. angustispora*.

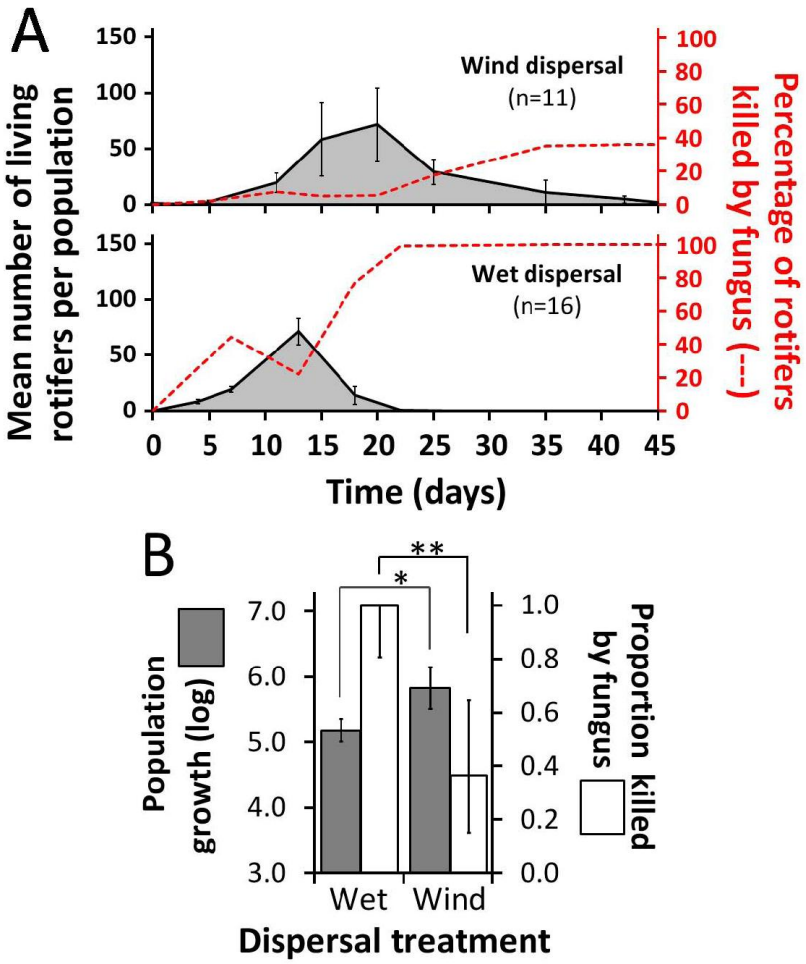


Figure 1.4 In a replicate experiment (see Fig. 1.2), wind-dispersing *H. elusa* escaped infection by a second (sympatric) strain of *R. angustispora* collected from nature. **(A)** Growth and mortality curves (±95% CI) for 11 rotifer populations founded by artificial dispersal (7 days in a wind chamber) of desiccated substrate (4.3mg ± 2.43 SD) from an infected source culture. Infections developed in 4 populations (36.4%), compared with 100% (n=16) of control populations founded concurrently by wet transfer of approximately 4.3mg of the same infected source material. **(B)** Statistical summary of differences in (A) [see caption to Fig. 1.1(C)]; * $P = 0.0003$, unpaired t-test; ** $P = 0.0004$, Fisher's exact test.

Statistical Analysis

The most important outcome in each trial was considered to be the extent and duration of growth experienced by bdelloid populations. In a sense, population growth is a direct measure of reproductive fitness; more specifically, a bdelloid clone can increase its chance of surviving extended desiccation by maintaining a large number of individuals in a given microhabitat. With more individuals, the clone also has a higher probability of colonizing new habitats by wind dispersal. At the end of each experiment, we evaluated the growth outcome for each population of bdelloids individually, by calculating the total area underneath its growth curve (one unit might be considered to represent one bdelloid present on one day). The resulting data were not normally distributed, due to the devastating effect of the parasite on a discrete portion of the populations; normality was restored by natural log transformation. For our first experiment, the mean values of these transformed growth data are shown in Fig. 1.1B. These were analyzed by one-way ANOVA with treatment group as a categorical factor, and Tukey's post-hoc test was used to detect significant differences among treatments while maintaining an overall error rate of 0.05. Statistical analyses were conducted using MINITAB, v.15. The desiccation treatments were found to cluster into three groups (0, 7, and 14 days; 21 days; and 28 and 35 days): growth differed significantly among, but not within these three groups.

As a secondary outcome variable, we examined the proportion of populations in each group that had succumbed to the parasite by the end of the experiment. This analysis used a test of multiple proportions based on chi-square, incorporating a Tukey-type correction for multiple comparisons as described by Zar (1999) (*30*). Significant differences ($P \leq 0.05$) were found among the same three groups of treatments (Fig. 1.1C). We provide 95% confidence intervals for the surviving proportions of populations in Fig. 1.1C, Fig. 1.2B, and Fig. 1.4B; these were

calculated using Wilson's 'score' method, which avoids many common problems with interval estimate methods for single proportions (*31*).

As in Fig. 1.1B, the dispersal data displayed in Fig. 1.2A and Fig. 1.4A represent the mean of many individual growth curves, and we calculated and log transformed the area for each curve separately as described above. The mean growth outcomes for wet and wind dispersal treatments could then be compared by t-test. We used Fisher's Exact Test to compare the proportion of dishes in which the parasite eventually returned, and once again, Wilson's 'score' method was used to construct the 95% confidence intervals of these proportions.

REFERENCES

1. G. Bell, *The Masterpiece of Nature* (Univ. California Press, Berkeley, 1982).
2. O. P. Judson, B. B. Normark, *Trends Ecol. Evol.* **11**, 41 (1996).
3. D. B. Mark Welch, J. L. Mark Welch, M. Meselson, *Proc. Natl. Acad. Sci. U.S.A.* **105**, 5145 (2008).
4. T. B. Barraclough, D. Fontaneto, C. Ricci, E. H. Herniou, *Mol. Biol. Evol*. **24**, 1952 (2007).
5. B. B. Normark, O.P. Judson, N. A. Moran, *Biol. J. Linn. Soc.* **79**, 69 (2003).
6. S. A. West, C. M. Lively, A. F. Read, *J. Evol. Biol*. **12**, 1003 (1999).
7. W. D. Hamilton, *Oikos* **35**, 282 (1980).
8. W. D. Hamilton, R. Axelrod, R. Tanese, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3566 (1990).
9. M. Salathé, R. D. Kouyos, S. Bonhoeffer, *TREE* **23**, 439 (2008).
10. J. Jokela, M. F. Dybdahl, C. M. Lively, *Am. Nat*. **174**, S43 (2009).
11. R. J. Ladle, R. A. Johnstone, O. P. Judson, *Proc. R. Soc. Lond. B* **253**, 155 (1993).
12. O. P. Judson, *J. Theor. Biol.* **186**, 33-40 (1997).
13. A. F. Agrawal, *PLoS Biol*. **4**, 1364 (2006).
14. C. N. Ricci, *Hydrobiologia* **446**, 1-11 (2001).
15. A. Tunnacliffe, J. Lapinski, *Phil. Trans. R. Soc. Lond. B* **358**, 1755 (2003).
16. D. G. Jenkins, M. O. Underwood, *Hydrobiologia* **388**, 15 (1998).
17. C. E. Cáceres, D. A. Soluk, *Oecologica* **131**, 402 (2002).
18. D. Fontaneto, T. G. Barraclough, K. Chen, C. Ricci, E. A. Herniou *Mol. Ecol.* **17**, 3146 (2007).
19. G. L. Barron, *Can. J. Bot.* **69**, 494 (1991).
20. See Materials and Methods.

21. W. Milne, *J. Quek. Micr. Club* **13**, 47 (1916).
22. J. Donner, *Bestimmungsbücher zur Bodenfauna Europas*, **6**, 1-297 (1965).
23. G. L. Barron, *Can. J. Bot.* **63**, 211 (1985).
24. S. L. Glockling, *Mycol. Res*. **102**, 1142 (1998).
25. C. Ricci, D. Fontaneto, *Ital. J. Zool*. **76**, 240 (2009).
26. M. Caprioli, C. Ricci, *Hydrobiologia* **446/447**, 13 (2001).
27. R. Guidetti, K. I. Jönsson, *J. Zool*. **257**, 181 (2002).
28. G. M. Cohen, J. B. Shurin, *Oikos* **103**, 603 (2003).
29. D. Fontaneto, T. G. Barraclough, K. Chen, C. Ricci, E. A. Herniou *Mol. Ecol*. **17**, 3146 (2007).
30. J. H. Zar, *Biostatistical Analysis, 4th ed*. (Prentice Hall, London, 1999), p.564.
31. R. G. Newcombe, *Statist. Med*. **17**, 857 (1998).

CHAPTER 2

SPATIOTEMPORAL ESCAPE FROM FUNGAL PARASITES BY ANHYDROBIOTIC BDELLOID ROTIFERS: EXTENDED TRIALS WITH MULTIPLE SPECIES

Abstract

Despite the theoretical efficiency of asexual reproduction, obligately parthenogenetic metazoan lineages seldom persist over evolutionary time. Evidently, sexual reproduction provides critical compensatory benefits, although the nature of these is not fully understood. The prominent Red Queen hypothesis argues that sex facilitates adaptation to rapidly evolving parasites and pathogens, whereas clonal lineages are extinguished by infections. However, this view is challenged by rotifers of the class Bdelloidea, which abandoned sex millions of years ago, but have endured and diversified despite their parasites. Red Queen models predict such an outcome only under special ecological conditions; namely, asexual hosts must disperse rapidly among discrete habitat patches in a metapopulation during a parasite-free phase of their life history. Intriguingly, these dynamics seem to characterize the ecology of bdelloid rotifers, which tolerate complete desiccation of their ephemeral freshwater microhabitats by forming tiny anhydrobiotic propagules that can disperse by wind to colonize new patches. Previous laboratory experiments showed that desiccation and dispersal in a wind chamber facilitate the exclusion of a common fungal parasite from newly founded populations of one bdelloid species. Here, these results are extended to two more species of bdelloids and five more fungal parasites. Populations were inoculated with up to four parasites simultaneously, then desiccated and dispersed in the wind chamber. Even short durations of anhydrobiotic dispersal significantly

reduced infection by all parasite species, thereby significantly enhancing rotifer population growth. Results are therefore consistent with a prediction derived from the Red Queen hypothesis: stable asexuality can prevail if unusual mechanisms decouple organisms from coevolving enemies in space and time.

Introduction

In principle, animal populations could substantially increase their per capita growth rate by dispensing with the production of males, or ceasing to invest in male function. Up to twice as much effort could then be directed into eggs, which would give rise to independently reproductive females (Maynard Smith 1978, Lively & Lloyd 1990). However, despite the apparent efficiency of asexual reproduction, a substantial number of genetic and ecological hypotheses argue that strict adherence to this strategy is not evolutionarily sustainable (Kondrashov 1993). Theoretical disadvantages include an irreversible accumulation of deleterious mutations, the inability to combine beneficial mutations from different genetic backgrounds, and the vulnerability of a static genotype to rapidly co-evolving parasites and pathogens (Kondrashov 1993; West et al. 1999; Agrawal 2006). In nature, obligately asexual lineages are indeed rare (<1% of animal species; Burt 2000). Moreover, they generally are restricted to the recent tips of phylogenetic trees, which implies that abandoning sex leads to rapid extinction (Rice 2002). However, there is little consensus as to which of the proposed disadvantages of asexuality actually extinguishes such lineages, and drives the widespread maintenance of sexual reproduction.

Rotifers of the class Bdelloidea are of great relevance to this puzzle, since they appear to have persisted and diversified for millions of years in the absence of males and sexual reproduction (Mark Welch et al. 2009). Their evolutionary longevity

challenges all hypotheses predicting the demise of asexuals, and prompted John Maynard Smith (1986) to call the bdelloid rotifers "something of an evolutionary scandal." This designation led to considerably increased interest in a group that was once overlooked due to its relatively minor role in ecosystem function (Judson & Normark 1996). In particular, bdelloids have been examined for unusual genetic or ecological features that may have contributed to their exceptional success. Any of the competing selective hypotheses for sex would be strengthened if the "anciently asexual" bdelloid rotifers were shown to possess unusual mechanisms that circumvent the particular disadvantage it postulates (Butlin 2002).

In this regard, the extreme desiccation-tolerance of bdelloid rotifers has emerged as a focus of interest (Ricci & Fontaneto 2009). Many animals and plants have drought-resistant propagules or dormant developmental phases, but bdelloids can tolerate complete loss of water at any life stage, sometimes more than once during the lifetime of an individual (Ricci & Caprioli 2005). Desiccating bdelloids contract into a miniscule 'tun', and metabolism ceases until water returns. Living bdelloids have been revived from material kept dry for more than nine years (Guidetti & Jöhnson 2002), but periods of drought in their limno-terrestrial habitats more typically last several days or weeks.

Anhydrobiosis in bdelloids has several intriguing physiological, genetic and ecological cosnequences that assume special importance in light of the major hypotheses for the maintenance of sex (Ricci & Fontaneto 2009; Mark Welch et al. 2008; Gladyshev et al. 2009; Wilson & Sherman 2010). One major explanation for sex centers on the ubiquitous evolutionary conflict between all organisms and their parasites and pathogens (Salathé et al. 2008; Lively 2010). These natural enemies generate time-lagged, frequency-dependent selection that is predicted to limit the success of any common genotype (Jaenicke 1978; Hamilton 1980). In this view, sex

is favored because it generates an endless succession of novel genotypes that are initially rare, and therefore resistant to exploitation by the dominant pathogens. Under this "Red Queen" hypothesis (Bell 1982), obligately asexual lineages disappear because they cannot generate variation sufficiently rapidly to stay ahead of coevolving enemies, whereas sexual lineages are able to "run" fast enough to stay in the same place. Like all animals, the bdelloid rotifers suffer from a range of parasites. In particular, over 50 species of deadly fungi and water moulds have been described as specific endoparasites or predators of this group (Barron 2003). Why bdelloids have not been driven extinct under an escalating burden of disease is difficult to explain under the Red Queen hypothesis. However, this inconsistency may be resolved by considering the consequences of desiccation-tolerance. Theoretical simulations of Red Queen dynamics demonstrate that populations of asexual hosts can withstand rapidly-evolving antagonists if several special conditions are met. Specifically, if hosts inhabit a spatially-structured metapopulation, disperse among patches rapidly, and do so during a parasite-free phase of their life history, then immigration can replace recombination as a source of effectively novel genotypes. When hosts are decoupled from coevolving parasites in this way, the Red Queen hypothesis predicts a reduced selective advantage to sex, so that persistent asexuality may be favored (Ladle et al. 1993; Judson 1995; Judson 1997; Sasaki et al. 2002; Gandon & Otto 2007).

Intriguingly, bdelloid rotifers may be the most likely of all animal groups to meet the necessary conditions for spatial and temporal escape. They are among the smallest metazoans (typically <300μm in length), and their extraordinary desiccation-tolerance allows them to thrive in tiny limnoterrestrial habitat patches, including the film of water surrounding moss leaves or particles of soil (Ricci 1987). Anhydrobiotic tuns can be dispersed by wind, which allows bdelloids to colonize isolated patches quickly,

migrate among fragmented microhabitats frequently, and perhaps even to travel on an intercontinental scale (Jenkins & Underwood 1998; Cáceres & Soluk 2002; Fontaneto et al. 2008). If parasites are unable to accompany their hosts, desiccated aerial dispersal would decouple bdelloids from their coevolving enemies in space. Even if they do not disperse, desiccated bdelloids can remain viable for many times the lifespan of a hydrated individual, allowing their genotypes to be stored temporarily beyond the reach of parasites tracking the active population (Guidetti & Jöhnson 2002; Ellner & Hairston 1994). As the hydration profile of a heterogenous habitat patch fluctuates, these "banked" genotypes will be intermittently restored to the population, representing a source of effective genetic novelty. If parasites are unable to tolerate desiccation to the same degree as their hosts, or for as long, then bdelloid populations may also become decoupled from antagonists in time. The unusual physiology and ecology of bdelloids thus offers both spatial and temporal escape from coevolving parasites: a powerful combination of factors that substantially reduces the advantage of sex in theoretical models derived from the Red Queen hypothesis (Sasaki 2002). If the above assumptions hold, therefore, the ancient asexuality of the bdelloids could be reconciled with a coevolutionary interpretation of sexual reproduction (Normark et al. 2003).

Wilson and Sherman (2010) provided initial evidence for the efficacy of anhydrobiosis against parasites in laboratory trials. Cultured populations of the bdelloid *Habrotrocha elusa elusa* (Milne 1916) were exposed to infectious spores (condia) of the lethal fungal parasite *Rotiferophthora angustispora* (Barron 1985), which belongs to the most common and speciose genus of bdelloid parasites (Barron 1991). The infected populations were desiccated for between 7 and 21 days, and screened for parasites after rehydration. Without desiccation, control populations were all rapidly exterminated, as were populations rehydrated after 7 or 14 days.

However, after 21 days of desiccation the fungus was absent from 60% of rehydrated populations and never returned; after 28 and 35 days, this fraction rose to 85% and 90.5% respectively. These rehydrated bdelloid populations experienced substantially increased growth relative to the infected controls. In a second experiment, a heavily-infected population of *H. elusa* was desiccated on a natural substrate (sterilized moss and soil), and placed in a wind chamber. Fans blew material from this 'source' dish toward empty, sterile 'target' dishes which were rehydrated after just 7 days. Rotifer tuns established populations in over 70% of the target dishes, and 63.6% of these newly-founded populations were free of parasites. In contrast, when wet material was "dispersed" by pipette from an identical infected population that had not been desiccated, rotifer populations in all control dishes were exterminated by the fungus. These experiments show that *H. elusa* is more tolerant of extended desiccation than *R. angustispora*, and that even a short period of desiccation is sufficient to escape from this parasite if the bdelloid host is permitted to disperse to new habitats while dry.

These results strengthen the hypothesis that anhydrobiosis and wind dispersal decouple bdelloids from their coevolving natural enemies in both space and time. However, there are several important limitations. First, the original experiments paired only one bdelloid species with one fungus species. It is vital to examine whether the findings extend to other species or genera of hosts or parasites, and whether they hold for rotifer populations afflicted with multiple parasites simultaneously, as is the case in nature. Second, although the observed increase in bdelloid population growth after extended anhydrobiosis appeared to be due to the destruction of fungal parasites, some bdelloid species are known to show enhanced fecundity after recovering from anhydrobiosis even when fungal parasites are not present Ricci & Caprioli (2005) suggested that rehydration may serve as a cue to invest heavily in reproduction in order to colonize a presumably empty habitat. It is therefore important to control for

this effect when assessing whether the removal of parasitic fungi significantly improves growth in rehydrated bdelloid populations . Finally, previous wind dispersal trials were limited to 7 days, whereas natural durations of desiccation and dispersal are likely to be longer; by extending the duration of these trials, the combined effect of spatial and temporal dispersal can be investigated. Here, the methodology of Wilson and Sherman (2010) is adapted and extended in three experiments that address these outstanding questions in multiple species of bdelloids and parasites.

Materials

Rotifers

Monoclonal populations of three bdelloid species each originated with a single individual isolated from natural material. *H. elusa elusa* and *H. bidens* (Gosse 1851) were both isolated from moss collected in June 2006 from the Mundy Wildflower Garden in Ithaca, NY, USA (42°27'01.50"N; 76°28'02.49"W). *Adineta vaga* (Davis 1873) was isolated from moss growing on a tree, collected in April 2007 on the campus of Cornell University in Ithaca, NY, USA (42°26’51.65”N; 76°28’35.00”W). Stock populations were maintained in non-axenic long-term serial batch culture in 35mm Petri dishes over Czapek’s 0% agar, as described by Wilson and Sherman (2010) and Barron (1985). The growth medium was rainwater, collected in glass flasks on the roof of Mudd Hall, Cornell University, Ithaca, NY (42°26'50.35"N; 76°28'44.80"W), filtered through Whatman Grade 1 paper and then sterilized by autoclave. In neither the stock cultures nor in any of the experiments described below was any explicit food source provided to the rotifers, nor were the dishes cleaned, nor was the medium changed. Rotifers nevertheless made adequate growth, and frequently reached population sizes of several hundred individuals, apparently feeding on adventitiously co-cultured bacteria that subsist on little more than inorganic ions,

organic compounds from rainwater, and the molecular constituents of agar itself. Rotifer population dynamics on each dish typified the standard microbiological growth curve, with lag, log, stationary and decline phases occurring over the course of approximately 2 months. All populations eventually died off as resources in the small dishes ran out, or waste products accumulated and hindered bacterial growth. However, the relatively brief window of growth provided sufficient time for differences among experimental treatments to become clear. In many ways, dynamics in these small and resource-limited dishes might be expected to approximate those found in the tiny ephemeral rainwater patches that bdelloids frequently colonize in nature.

Fungal Parasites

Five fungal species were isolated from natural areas close to the source of the bdelloids, using methods described by Barron (1985). These isolates are available from the USDA Agriculture Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF), and their accession numbers in that collection are indicated. *H. spirosporum* (ARSEF 8821; Barron 1986) and *R. ovispora* (ARSEF pending; Barron 1980) were both isolated from compost collected on the Cornell University campus (42°26’51.85”N; 76°28’36.50”W) in May 2008. *R. angustispora* (ARSEF 8991) and *R. cylindrospora* (ARSEF 8992; Barron 1985) were both isolated from moss and soil collected near the shore of Cayuga lake (42°27'32.46"N 76°29'37.51"W) in October 2008. *R. globospora* (ARSEF 8995; Barron 1991) was isolated from soil collected from the Mundy Wildflower Garden (42°27'02.80"N; 76°28'09.49"W) in October 2009. These species share an endoparasitic mode of attack: conidia are ingested by rotifers and germinate in the esophagus to produce assimilative hyphae that digest the host’s tissues. After several days, hyphae penetrate the integument and, upon contact

with air, produce conidiophores bearing hundreds of fresh conidia that fall back into the water to restart the infection cycle.

Wind Chamber

The wind chamber consisted of a rectangular, sealed Plexiglas box (1.2 x 0.4 x 0.76 m), containing five 48V fans (ETRI Co.). These generated a turbulent flow with a mean velocity of 0.97 m·s-1 (± 0.43 SD) and maximum gusts of 2.24 m·s-1 (a 'light breeze' on the Beaufort Scale), measured using a hot-wire anemometer (Testo 425). The chamber was kept in a room whose relative humidity (R.H.) was maintained at approximately 40% by humidifiers and dehumidifiers linked to hygrostats. In Experiments One and Two, the infected 'source' dish was placed in the center of the chamber floor, at the turbulent convergence of the airflows from the fans. The floor was Styrofoam, into which 16 circular recesses had been drilled to accommodate 'target' dishes at a distance of approximately 30cm from the source plate.

Methods

Experiment One

Wilson and Sherman (2010) reported significantly increased growth of bdelloid populations after extended desiccation relative to continually hydrated control populations. Two factors could have contributed to this outcome. Most obviously, growth may have been promoted following the destruction of deadly fungal parasites in these experiments by anhydrobiosis, which provided a 'temporal escape' for the hosts. However, studies of the genus *Adineta* suggest that bdelloids sometimes show a burst of reproduction after recovery from anhydrobiosis even in the absence of fungal parasites, perhaps as an adaptation to rapidly fill a habitat patch now devoid of desiccation-sensitive competitors (Ricci & Caprioli 2005). To distinguish these two

effects, and to broaden the investigation of temporal escape to a second rotifer genus and another parasite species, the growth of *A. vaga* populations was measured in the presence and absence of the fungal parasite *R. globospora*, with and without four weeks of desiccation. If the growth-enhancing effect of desiccation were due chiefly to the removal of fungal parasites, it would be most prominent in the infected dishes; if it were independent of parasitism, it would be seen to a similar degree in all dishes.

Populations of *A. vaga* (n=48) were established by transferring single individuals to 35mm Petri dishes containing 1.0mL of filtered, sterilized rainwater over 0% Czapek's agar. After 9 days, the mean population size was 31.5 individuals (± 19.6 s.d.). At this point, approximately 50 conidia of *R. globospora* were added to 24 of the dishes. After a further 5 days, 12 infected and 12 uninfected dishes were desiccated by removing their lids and exposing them to 40% R.H. Desiccation was achieved in 24 hours: at this point, CoCl2 paper touched to the dishes no longer changed color, and rotifers crumbled when scraped with a fine needle rather than being moved or punctured. The desiccated plates were maintained at 40% RH for 28 days, then rehydrated with 1.0mL of filtered, sterilized rainwater after a 2-hour "prehydration" period during which the relative humidity was increased to ~55% by placing a dish of distilled water nearby. The other 12 infected and 12 uninfected dishes remained hydrated throughout the experiment. Every 3-4 days, living and infected rotifers in all hydrated dishes were counted. Counting resumed for desiccated dishes 3 days after rehydration. Discounting the time spent in anhydrobiosis, all groups of dishes were monitored for 45 days.

Experiment Two

Wilson and Sherman (2010) reported that *H. elusa* populations can be freed from infection by *R. angustispora* following 7 days of desiccated dispersal in a wind

chamber. To extend this result, the experiment was repeated for a second bdelloid species in the genus *Habrotrocha* (*H. bidens*), paired with the parasite *Harposporium spirosporum*. This genus contains the second highest number of bdelloid parasites behind *Rotiferophthora*. A heavily infected population of *H. bidens* was dried onto a natural substrate and placed in a wind chamber, where desiccated material was blown to sterile 'target dishes'. Target dishes were collected from the chamber after 7 days, rehydrated and monitored for newly-founded rotifer populations and *Harposporium*. In order to assess the effect of combining temporal and spatial escape, further batches of target dishes were collected and rehydrated after 14 and 21 days,. In all cases, growth was compared with populations founded by wet transfer of an equivalent mass of infected material. It was predicted that growth should be significantly stronger and parasitism less frequent in the populations founded by wind dispersal, especially after extended durations of desiccation.

The initial population of *H. bidens* was founded by placing a single rotifer onto a 100mm Petri dish lined with 0% Czapek's agar, and adding 6.0mL of sterilized, filtered rainwater. After 30 days, rotifers on a randomly selected 4cm^2 area of the dish were counted on a grid, and the dish was estimated to contain a total of ~3000 individuals. At this time it was inoculated with approximately 3000 conidia of *H. spirosporum* suspended in sterile distilled water. Conidial density was controlled by staining a 10uL sample of the suspension with lactic acid-cotton blue, counting it with a hemocytometer, then adjusting the volume of the inoculum accordingly. After allowing 2 days for new infections to occur, 0.8g of sterilized, milled *Sphagnum* moss was added to the plate, along with 0.4g of sterilized soil. After 24 hours, the agar was removed from the plate, leaving the rotifers and fungi attached to the natural substrate. At this point, infected wet dispersal control dishes were established, as described below. The lidless plate was then placed in the wind chamber, where it dried

completely after 16 hours, as indicated by $CoCl_2$ test paper. A sterile spatula was used to initiate crumbling of the dried substrate, which then proceeded passively through the action of the artificial wind. The 16 recesses in the wind chamber were filled with empty 'target' Petri dishes (35mm) of known mass, lined with sterile, desiccated Czapek's 0% agar. After 7 days, these were removed, re-weighed and hydrated with 1.0mL of sterilized, filtered rainwater. A fresh set of target dishes replaced them in the wind chamber. This sampling process was repeated at 14 and 21 days.

In order to establish a growth rate for uninfected, wet-dispersed rotifer populations, a second population of *H. bidens* was established in another 100mm Petri dish. No conidia were added at 30 days, but moss and soil were added and the agar was removed as before. The distal 1cm of a pipette tip was removed to permit aspiration of substrate particles, and 0.197mL of material from this uninfected dish was transferred by pipette to 20 Petri dishes (35mm) lined with sterile, desiccated 0% Czapek's agar. This volume was calibrated so that it contained approximately 4.0 mg of rotifer-moss-soil suspension by dry mass; comparable to the mass expected to accumulate in target dishes in the wind chamber every 7 days. A further 0.803mL of sterile, filtered rainwater was added so that these uninfected, wet dispersal control dishes matched the total volume of the experimental target dishes (1mL). The same process was used to create a set of 16 infected wet dispersal control dishes: each was founded with 0.197 mL of suspension taken from the infected source dish before it was dried, as described above.

The numbers of living and infected bdelloids were counted for each hydrated set of dispersal dishes approximately every 5-7 days for 40 days.

Experiment Three

This experiment involved an extended wind-dispersal design essentially identical to that of Experiment Two, but it paired the bdelloid *H. elusa* with four species of parasites simultaneously, in order to determine whether all four enemies could be eluded, or whether some were resistant to desiccation and frequently accompanied the rotifers over multiple weeks of desiccation and spatial dispersal.

A population of *H. elusa* was reared as described above for *H. bidens*. After 30 days, it was estimated to contain 6500 individuals. At this stage, the population was inoculated with approximately 700 conidia of each of the following parasites: *H. spirosporum*; *R. angustispora*; *R. cylindrospora*; *R. ovispora*. These are all "relatively common" species (Barron 1985), but were also selected because their spores are easily distinguished. The fungi spread in the dish for 7 days, at which point their relative incidences in the source dish were compared by randomly selecting 8 areas of the dish (1cm x 1cm squares) and examining all rotifer corpses in those areas (n=60) to determine which parasite killed them. Moss and soil were then added as described above, the agar was removed, and 25 infected wet dispersal control dishes were established. A second source dish, uninfected but otherwise identical, was used to create 10 uninfected wet dispersal control dishes. The infected source dish was placed in the wind chamber as before, and target dishes with debris were collected, weighed and hydrated every 7 days for 3 weeks.

Each set of hydrated dishes was monitored approximately every 5-7 days for 60 days. In addition to counting the numbers of living and infected rotifers, once the rotifer population in a dish was judged to have been exterminated (>95% mortality), each dead rotifer was examined to determine which parasite killed it. Ratios of parasite incidence were compared among dishes to determine whether some parasite

species were disproportionately represented among the infections that returned after desiccation.

Statistical Analysis

For each treatment group, two quantitative outcomes were calculated and analyzed. First, the relative extent and duration of rotifer population growth was assessed for each dish by measuring the total area underneath its growth curve. Here, one "unit" represents one rotifer present on one day: one clonal population is judged to be more successful than another if it can maintain a higher density of individuals over a longer period of time. A natural log transformation was applied to these values to ensure normality, and ANOVA was applied. Where the effect of treatment group was significant, Tukey post-hoc comparisons were conducted to determine which groups differed. In the Results section, any discussion of significant differences following ANOVA refers to these post-hoc tests. The second measure was the percentage of rotifers per dish that had been killed by parasites at the end of the monitoring period. In the second and third experiments, each dish ended with a value of either 0% or >95% ('extermination'), and so nonparametric tests of binary proportions (e.g. χ^2) could be used. Where these were significant, Tukey-adjusted post-hoc tests were used to identify significant differences between individual pairs of proportions (Zar 1999). Dishes in the first experiment showed a greater range of final infection percentages; these were therefore arcsine transformed and ANOVA was applied.

Results

Experiment One

There were highly significant differences in population growth among the 4 treatment groups (Fig. 2.1, ANOVA: $F_{3, 44} = 55.43$, $p < 0.0001$; $R^2 = 77.7\%$), driven almost entirely by a large difference between the infected control group and all other groups (Fig. 2.1). Without desiccation, *R. globospora* exterminated all 12 populations of *A. vaga* (Fig. 2.1 A). After 28 days of anhydrobiosis, a significantly lower proportion of rotifer populations succumbed to parasitism (4 of 12 dishes; Fisher's Exact Test: $p = 0.0013$), resulting in significantly higher mean growth than the infected controls. Indeed, post-hoc pairwise tests revealed no significant differences in total population growth among the uninfected controls, desiccated controls and the infected, desiccated dishes. This should not be interpreted as a failure to replicate earlier results (e.g. Ricci & Caprioli 2005): anhydrobiosis did seem to promote reproduction in the uninfected control populations, which grew very rapidly in the 2 days immediately after rehydration, and briefly reached almost twice the maximum density of their undried counterparts. However, due to the limited availability of resources in the small dishes, the rehydrated populations entered their decline phase more rapidly thereafter, and their numbers fell off steeply, so that there was no significant difference in the overall areas under the growth curves for the dried and undried control groups, as was seen for the infected groups (Fig. 2.1C).

Experiment Two

The growth of *H. bidens* populations differed significantly among the 5 treatment groups (Fig. 2.2; ANOVA: $F_{4, 73} = 27.97$, $p < 0.001$; $R^2 = 42\%$), and the final proportions of infected rotifers differed significantly among the 4 inoculated groups (Fig. 2.2; ANOVA: $F_{3, 72} = 66.84$, $p < 0.001$; $R^2 = 77.6\%$). Populations

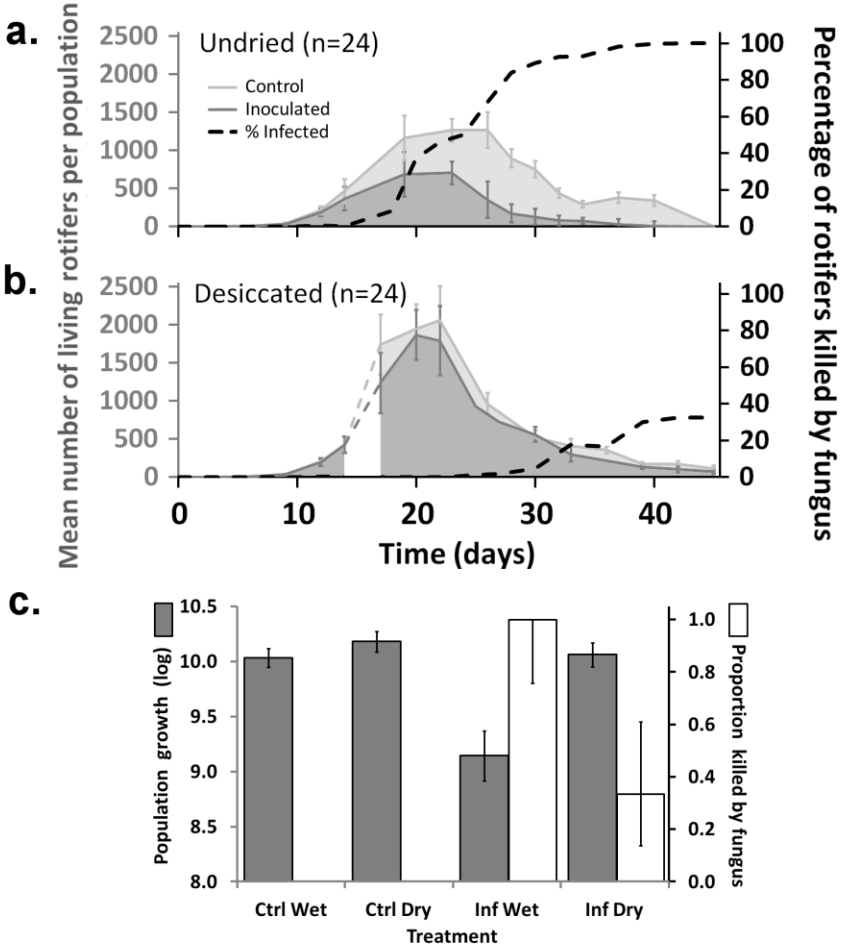


Figure 2.1 After 28 days of in situ desiccation, the fungal parasite *R. globospora* disappeared from two thirds of infected *Adineta vaga* populations, resulting in significantly increased growth relative to undried infected controls. Desiccation per se did not lead to significant growth increases relative to undried uninfected controls. (**a**) Growth curves (± 95% CI) for uninfected rotifer populations, and populations inoculated at 9 days with conidia of *R. globospora* (mortality curve also shown for the latter). (**b**) Corresponding growth and mortality curves for infected and uninfected dishes desiccated at day 14. Dashed lines between day 14 and 'day 18' encompass a 24-hour dehydration period, 28 days of desiccation, 2 hours of prehydration and a 3 day post-rehydration recovery period. Only during the dehydration and recovery periods was rotifer activity or reproduction possible, therefore only those 4 days were represented explicitly as distance on the x-axis, or taken into account when calculating population growth. (**c**) Summary of treatment differences.

originating by wet dispersal from the infected source dish experienced significantly less growth than those originating from the uninfected source dish. Without desiccation, parasite spores accompanied host populations into every new dish.

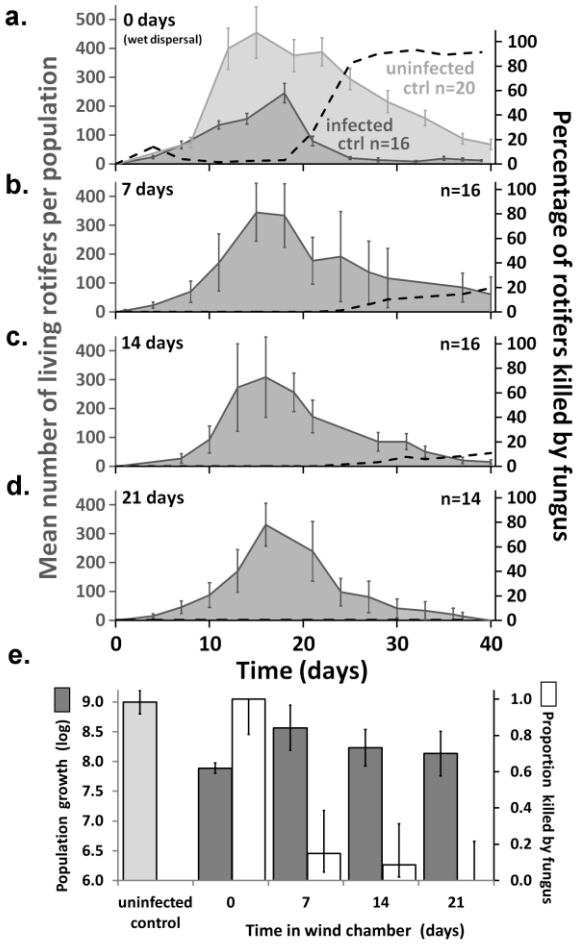


Figure 2.2 The fungal parasite *Harposporium spirosporum* is eliminated from populations of *Habrotrocha bidens* during desiccated wind dispersal. (**a**) Growth curves (± 95% CI) for rotifer populations founded by wet transfer of material from a source dish infected with *H. spirosporum*, versus an uninfected source dish. The proportion of infected rotifers in each dish was used to construct a mean mortality curve. (**b-d**) Growth and mortality curves for rotifer populations founded from the same infected source dish by dispersal in a wind chamber combined with 7, 14 and 21 days of anhydrobiosis. (**e**) Statistical summary of treatment differences. Population growth was quantified for each dish as the natural logarithm of the total area under its growth curve; shaded bars show the mean for each treatment group (± 95% CI). Open bars show the mean mortality caused by parasites for dishes in each group after 40 days (± 95% CI).

Founding rotifers ingested these spores and became inactive for a 3-5 day incubation period, during which they made no contribution to population growth. Once these infections produced conidia, a second wave of hosts was inactivated. Despite some initial reproduction by uninfected rotifers, the populations crashed after day 20 as increasing numbers of infections matured and sporulated. By 30 days, over 90% of rotifers had been killed by the parasite, and the populations were smaller than those in the control group by an order of magnitude.

The mean mass of material accumulating in the wind-dispersed target dishes was 4.73 mg, with high variability (± 4.8 mg s.d.). Dishes collected at 7, 14 and 21 days did not differ significantly in the mass deposited (ANOVA: $F_{2, 40} = 1.89$, $p = 0.164$). Rotifers established populations in 16 (100%) of the target dishes collected after 7 days and 14 days, and in 14 (87.5%) of the dishes collected after 21 days. In the 7-day group, *H. spirosporum* appeared in 4 of 16 dishes, but it failed to exterminate these and its mean incidence at 40 days was only 19.5%: significantly lower than in the infected control group. In the 14-day group, the parasite colonized (but failed to exterminate) 2 of 16 dishes. Its final incidence (10.9%) was significantly lower than the infected control group, but not significantly different from the 7-day group. In the 21-day group, the parasite was completely absent from all 14 dishes with rotifers. The mean population growth of all wind-founded dishes was significantly greater than that of the infected control group, but there were no significant differences among the 7-, 14- and 21-day groups. Neither the population growth nor the incidence of parasitism was significantly correlated with the mass deposited on dishes.

Experiment Three

There were highly significant differences in population growth among the 5 treatment groups (Fig. 2.3, ANOVA: $F_{4, 80} = 20.77$, $p < 0.0001$; $R^2 = 48.5\%$). The proportions of dishes exterminated by parasites differed significantly among the 4 inoculated groups (Fig. 2.3, $\chi^2 = 36.46$, d.f. = 3, n = 72, $p < 0.0001$). Populations of *H. elusa* founded by wet transfer from an infected source dish were all exterminated within 18 days; their growth was eclipsed by that of the uninfected controls (Fig. 3a, e). Rotifers colonized all 16 of the 7-day wind dispersal dishes. Parasites exterminated 9 of these populations (56.3%); a significantly lower incidence than in the infected wet dispersal controls. The population growth of the 7-day dishes was significantly higher than that of the infected controls, although it remained significantly lower than the growth of the uninfected controls. Rotifers colonized 15 of 16 dishes in the 14-day group (93.8%), and all 16 dishes in the 21-day group. Parasites exterminated 3 populations in each group (20% and 18.8% respectively). These percentages were statistically indistinguishable, as was the population growth experienced by the two groups. Both groups had a significantly lower incidence of parasitism than the 7-day group and the infected control group. Their population growth was significantly higher than that of the infected control group; indeed, it was not significantly different from that of the uninfected control group in pairwise post-hoc tests. The mean mass of material accumulating in the wind-dispersed target dishes was 3.6 mg (± 1.9 mg s.d.). Dishes collected after 7, 14 or 21 days did not differ significantly in the mass deposited (ANOVA: $F_{2, 47} = 1.18$, $p = 0.316$).

Seven days after inoculating the initial source dish with equal numbers of conidia, the ratio of deaths caused by the four parasite species differed significantly from a null expectation of 1:1:1:1, (Fig. 2.4; $\chi^2 = 8.93$, d.f. = 3, n = 60, $p = 0.03$). *H. spirosporum* was underrepresented, probably because its incubation time is

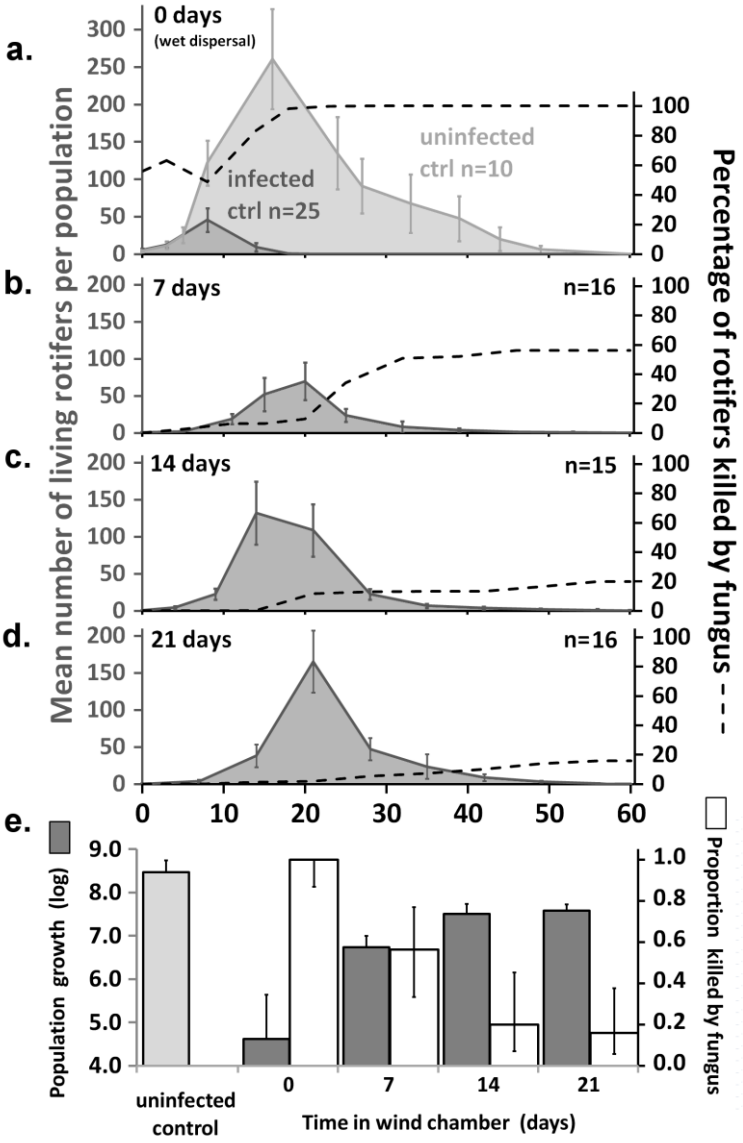


Figure 2.3 Populations of *Habrotrocha elusa* escape from multiple parasite species during desiccated dispersal by wind. (**a**) Growth curves (± 95% CI) for rotifer populations founded by wet transfer of material from a source dish infected with *H. spirosporum*, *R. angustispora*, *R. cylindrospora* and *R. ovispora*, versus an uninfected source dish. Mortality curves include deaths from all parasites; see Fig. 3 for a more detailed breakdown. (**b-d**) Growth and mortality curves for rotifer populations founded from the same infected source dish by desiccated dispersal in a wind chamber. (**e**) Statistical summary of treatment differences (see **Fig. 2**). Open bars show the final proportion of dishes that had been exterminated by parasites at 60 days (± 95% CI).

2 days longer than that of the *Rotiferophthora* species, which also helps to explain the slow progress this parasite made during Experiment Two. When analyzing parasite ratios in wet and wind dispersal treatment groups, corpses were no longer appropriate units of independent replication: multiple corpses are generated by a single instance of a parasite dispersing to a dish. To avoid pseudoreplication, the units of interest were dishes rather than corpses (i.e. how many dishes contained at least one instance of each of the four parasite species). Most of the infected wet dispersal dishes contained more than one parasite, which was never true of a wind dispersal dish. Wet dispersal dishes had a significantly different parasite ratio from the original source dish (Fig. 2.4; $\chi^2 = 17.1$, d.f. = 3, n = 85, $p = 0.001$). *H. spirosporum* was overrepresented, presumably having had more time to incubate and spread. *R. cylindrospora* and *R. ovispora* were underrepresented, perhaps because they were either less aggressive, or less readily dispersed in water. No significant differences in parasite species ratios were detected in any of the wind dispersal groups versus either the original source dish or the wet dispersal dishes. However, the power of contingency tests to detect such differences is low given the small sample sizes involved.

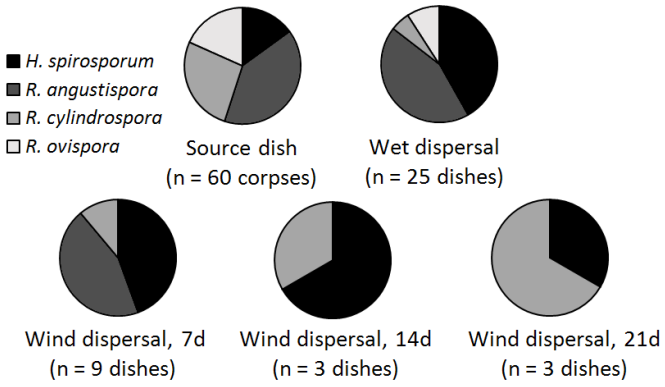


Figure 2.4 Relative representation of four parasite species among rotifers killed (or dishes exterminated) in the various treatment groups of Experiment Two. Only the source dish and the wet dispersal dishes differed significantly in the ratio of parasite species.

Discussion

Wilson and Sherman (2010) reported that the bdelloid rotifer *H. elusa* can shed a lethal fungal parasite (*R. angustispora*) during periods of extended desiccation, and disperse by wind to create parasite-free populations after even short periods of desiccation. The experiments described here demonstrate that parasite evasion during anhydrobiosis is not limited to this pair of species. In Experiment One, four weeks of in situ desiccation were sufficient to remove *R. globospora* from 66% of populations of the cosmopolitan bdelloid species *A. vaga*, demonstrating that anhydrobiotic escape extends to a second bdelloid family and a second *Rotiferophthora* species. This experiment also examined whether anhydrobiosis itself might be partially responsible for increased rotifer population growth in rehydrated dishes, rather than the impact of parasite removal. Representatives of the genus *Adineta* have previously shown a "rebound" effect in growth following recovery from desiccation (Ricci & Caprioli 2005). In Experiment One, desiccated dishes in the infected treatment group experienced more growth than continually hydrated infected dishes; in fact, their growth was statistically indistinguishable from that of uninfected dishes. Importantly, however, there was no corresponding increase in growth when uninfected populations were desiccated and rehydrated, so the growth benefits to the infected group were due to the removal of parasites, not anhydrobiosis per se. Reproduction in the uninfected dishes did rebound sharply just after rehydration, but the limited carrying capacity of the small agar dishes minimized any impact on the long-term growth outcome. Thus, even in a genus with a clear propensity for a post-anhydrobiotic surge of reproduction, a bout of desiccation and rehydration had little overall impact on the area under the total population growth curve when resources were limited. Given this result, it is unlikely that rebound effects can explain the differences in growth outcomes reported in the other experiments described here, or those of Wilson and Sherman (2010), since

in every case, small resource-limited dishes were used, and the measure of growth was the total area under the curve. As this experiment shows, effects are more likely due to the removal of fungal parasites, which can exterminate populations and arrest growth before the point where resources become limiting.

After 7 days of desiccated dispersal in a wind chamber in Experiment Two, newly founded populations of *H. bidens* had largely shed *H. spirosporum*, and exhibited significantly enhanced growth relative to infected populations dispersing in water. Increasing the duration of desiccated dispersal to 14 or 21 days provided little additional benefit to population growth, since the parasite was already almost entirely eliminated after 7 days. *Harposporium* is the second most speciose fungal group preying on bdelloid rotifers, and its susceptibility to desiccation provides further evidence that these ancient asexuals can escape important biological enemies in space and time.

In Experiment Three, *H. elusa* was challenged with four fungal parasites simultaneously. Their combined incidence was approximately halved in populations founded after 7 days of wind dispersal, just as Wilson and Sherman (2010) reported for *R. angustispora* alone. These populations experienced significantly improved growth relative to wet-dispersed controls, but could not quite match the growth of parasite-free control populations. After 14 or 21 days in the wind chamber, however, the influence of parasites on rotifer population growth was minimal; indeed, the most aggressive parasite (*R. angustispora*) had disappeared entirely. Rotifer populations in these groups experienced growth that was statistically indistinguishable from that of uninfected control populations. Examining the relative representations of the four parasite species yielded no evidence that any had a relatively higher resistance to desiccation or superior capacity for aerial dispersal, but this test lacked power due to the small sample size.

In the experiments described by Wilson and Sherman (2010), conidia of *R. angustispora* were permitted 36-72 hours to establish themselves in host populations before they were desiccated. It might be argued that this is insufficient time for the parasite to complete its lifecycle: hyphae typically take at least 30 hours to emerge after spore ingestion, conidiophore formation requires a further 6-12 hours, and thick-walled dictyochlamydospores (whose function is not clear) may not be fully formed for another day or more. If one or more of these structures is linked to desiccation tolerance, the original experiments might not have captured the true ability of the fungus to resist dehydration. However, Experiment Three addresses this objection: the parasites (which included *R. angustispora*) were allowed more than 7 days to colonize the source dish, and they spent a further 16 hours growing while the dish desiccated in the wind chamber. During the pre-desiccation survey of parasite species ratios, all the fungal structures listed above were seen in abundance for all species (except that *H. spirosporum* does not make dictyochlamydospores). The outcome of Experiment Three indicates that parasites do no better even if we allow several full infection cycles to elapse, and all fungal structures to develop fully prior to desiccation.

Taken together, these results reinforce and extend the evidence for spatiotemporal decoupling of bdelloid rotifers from coevolving parasites. Three bdelloid species in two genera are now known to shed fungal enemies during desiccation and artificial wind dispersal. It is too soon to conclude that all parasites of bdelloids are susceptible to desiccation: even within the fungi, several taxonomically distinct groups of bdelloid parasites remain to be examined (Barron 2003), and little is known about viral or bacterial parasites. However, it is surely noteworthy that five common fungal parasites in the two most speciose described genera are unable to tolerate desiccation or aerial dispersal to the same degree as their bdelloid hosts. This

might reflect high evolutionary or physiological barriers to anhydrobiosis, although there are certainly many fungi with excellent tolerance for physical extremes (Magan 2007). Alternatively, there may not be strong selection on these parasites to develop desiccation resistance: if they typically thrive as "sit-and-wait" opportunists in reliably damp habitat patches that receive diverse bdelloid immigrants, suitable hosts may arrive sufficiently frequently that parasites are not favored to evolve the physiology needed to follow a single host species through space and time. In turn, this would prevent them from eliminating a host genotype globally, and rapidly dispersing hosts could persist. This scenario would fit well with theoretical models of Red Queen dynamics in a metapopulation (e.g. Ladle 1993; Judson 1995).

The anomalous success of the asexual bdelloids has important logical connections to the enigma of sex more generally. A convincing hypothesis for the maintenance of sexual reproduction should also be able to explain or at least accommodate the ancient asexuality of these rotifers (Butlin 2002). This study extends the evidence that bdelloid rotifers match a theoretical scenario based on the Red Queen hypothesis, in which asexual hosts can persist if they become decoupled from parasites in space and time. If parasites and pathogens do indeed contribute to the typical failure of asexual lineages, the bdelloids may have avoided this fate because they can travel extremely rapidly, or lie dormant for extended periods, in a desiccated state that their major enemies cannot tolerate. Therefore, their ancient asexuality may actually be consistent with a coevolutionary interpretation of sexual reproduction, even as it remains "something of an evolutionary scandal" under alternative views.

REFERENCES

Agrawal, A. F. 2006. Evolution of sex: why do organisms shuffle their genotypes? Current Biology 16: R696-R704

Arkhipova, I. R. & M. Meselson, 2000. Transposable elements in sexual and anciently asexual taxa. Proceedings of the National Academy of Sciences USA 97: 14473-14477.

Arkhipova, I. R. & M. Meselson, 2005. Deleterious transposable elements and the extinction of asexuals. Bioessays 27: 76-85.

Barraclough, T. G., Fontaneto, D., Ricci, C. & E. A. Herniou, 2007. Evidence for inefficient selection against deleterious mutations in cytochrome oxidase I of asexual bdelloid rotifers. Molecular Biology and Evolution 24: 1952-1962.

Barron, G. L. 1980. Fungal parasites of rotifers: two new verticillate endoparasites with aerial conidiophores. Canadian Journal of Botany 58: 432-438.

Barron, G. L. 1985. Fungal parasites of bdelloid rotifers: Diheterospora. Canadian Journal of Botany 63: 211-222.

Barron, G. L. 1986. A new Harposporium parasitic on bdelloid rotifers. Canadian Journal of Botany 64: 2379-2382.

Barron, G. L. 1991. A new genus, Rotiferophthora, to accommodate the Diheterospora-like endparasites of rotifers. Canadian Journal of Botany 69: 494-502.

Barron, G. L. 2003. Fungal parasites and predators of rotifers, nematodes, and other invertebrates. In Mueller, G. M., Bills, G. F. & M. S. Foster (eds) Biodiversity of fungi: inventory and monitoring methods. Elsevier, New York: 435-450.

Bell, G. 1982. The Masterpiece of Nature. Berkeley: University of California Press.

Burt, A. 2000. Sex, recombination and the efficacy of selection: was Weismann right? Evolution 54: 337-351

Butlin, R. 2002. The costs and benefits of sex: new insights from old asexual lineages. Nature Reviews Genetics 3: 311-317.

Cáceres, C. E. & D. A. Soluk, 2002. Blowing in the wind: a field test of overland dispersal and colonization by aquatic invertebrates. Oecologia 131: 402-408.

Comai, L. 2005. The advantages and disadvantages of being polyploidy. Nature Reviews Genetics 6: 836-846.

Davis, H. 1873. A new *Callidina*; with the results of experiments on the desiccation of rotifers. Monthly Microscopical Journal, London 9: 201-209.

Dolgin, E. S. & B. Charlesworth, 2006. The fate of transposable elements in asexual populations. Genetics 174: 817-827.

Fontaneto, D., Barraclough, T. G., Chen, K., Ricci, C. & E. A. Herniou, 2008. Molecular evidence for broad-scale distributions in bdelloid rotifers: everything is not everywhere but most things are very widespread. Molecular Ecology 17: 3136-3146.

Gandon, S. & S. P. Otto, 2007. The evolution of sex and recombination in response to abiotic or coevolutionary fluctuations in epistasis. Genetics 175: 1835-1853.

Gladyshev, E. & M. Meselson, 2008. Extreme resistance of bdelloid rotifers to ionizing radiation. Proceedings of the National Academy of Sciences USA 105: 5139-5144.

Gladyshev, E. A. & I. R. Arkhipova, 2010. Genome structures of bdelloid rotifers: shaped by asexuality or desiccation? Journal of Heredity 101: S85-S93.

Gladyshev, E. A., Meselson, M. & I. R. Arkhipova, 2008. Massive horizontal gene transfer in bdelloid rotifers. Science 320: 1210-1213.

Gosse, P. H. 1851. A catalogue of Rotifera found in Britain; with descriptions of five new genera and thirty-two new species. Annals and Magazine of Natural History, London (Ser. 2) 8: 197-203.

Guidetti, R. & K. I. Jönsson, 2002. Long-term anhydrobiotic survival in semi-terrestrial micrometazoans. Journal of Zoology 257: 181-187.

Hamilton, W. D. 1980. Sex versus non-sex versus parasite. Oikos 35: 282-290.

Hickey, D. A. 1982. Selfish DNA: a sexually-transmitted nuclear parasite. Genetics 101: 519-531.

Jaenicke, J. 1978. An hypothesis to account for the maintenance of sex within populations. Evolutionary Theory 3: 191-194.

Jenkins, D. G. & M. O. Underwood, 1998. Zooplankton may not disperse readily in wind, rain, or waterfowl. Hydrobiologia 387/388: 15-21.

Judson, O. P. & B. B. Normark, 1996. Ancient asexual scandals. Trends in Ecology and Evolution 11: 41-46.

Judson, O. P. 1997. A model of asexuality and clonal diversity: cloning the Red Queen. Journal of Theoretical Biology 186: 33-40.

Judson, O.P. 1995. Preserving genes: a model of the maintenance of genetic variation in a metapopulation under frequency-dependent selection. Genetical Research, Cambridge 65: 175-191.

Kondrashov, A. S. 1982. Selection against harmful mutations in large sexual and asexual populations. Genetical Research 40: 325-332.

Kondrashov, A. S. 1993. Classification of hypotheses on the advantage of amphimixis. Journal of Heredity 84: 372-387.

Ladle, R. J., Johnstone, R. A. & O. P. Judson, 1993. Coevolutionary dynamics of sex in a metapopulation: escaping the Red Queen. Proceedings of the Royal Society B. 253: 155-160.

Lively, C. M. 2010. A review of Red Queen models for the persistence of obligate sexual reproduction. Journal of Heredity 101: S13-S20.

Lively, C.M. & D. G. Lloyd, 1990. The cost of biparental sex under individual selection. The American Naturalist 135: 489-500.

Magan, N. 2007. Fungi in extreme environments. In C. P. Kubicek & I. S. Druzhinia (eds) The Mycota. IV. Environmental and microbial relationships. Springer Verlag, Berlin: 85-103.

Mandegar, M. A. & S. P. Otto, 2007. Mitotic recombination counteracts the benefits of genetic segregation. Proceedings of the Royal Society of London B. 274: 1301-1307.

Mark Welch, D. B. & M. Meselson, 2000 Evidence for the evolution of bdelloid rotifers without sexual reproduction or genetic exchange. Science 288: 1211-1215.

Mark Welch, D. B., Mark Welch, J. L. & M. Meselson. 2008. Evidence for degenerate tetraploidy in bdelloid rotifers. Proceedings of the National Academy of Sciences USA 105: 5145-5149.

Mark Welch, D. B., Ricci, C. & M. Meselson, 2009. Bdelloid rotifers: progress in understanding the success of an evolutionary scandal. In Schön, I., Martens, K. & P. Dijk (eds), Lost Sex. Springer, New York: 259-279.

Mark Welch, M. & M. S. Meselson, 2001. Rates of nucleotide substitution in sexual and anciently asexual rotifers. Proceedings of the National Academy of Sciences USA 98: 6720-6724.

Maynard Smith, J. 1978. The evolution of sex. Cambridge University Press.

Maynard Smith, J. 1986. Contemplating life without sex. Nature 324: 300-301.

Milne, W. 1916. On the bdelloid Rotifera of South Africa. Journal of the Quekett Microscopical Club 13: 47-83.

Normark, B. B., Judson, O. P. & N. A. Moran, 2003. Genomic signatures of ancient asexual lineages. Biological Journal of the Linnean Society 79: 69-84.

Ricci, C. & D. Fontaneto, 2009. The importance of being a bdelloid: ecological and evolutionary consequences of dormancy. Italian Journal of Zoology 76: 240-249.

Ricci, C. & M. Caprioli, 2005. Anhydrobiosis in bdelloid species, populations and individuals. Integrative and Comparative Biology 45: 759-763.

Ricci, C. N. 1987. Ecology of bdelloids: how to be successful. Hydrobiologia 147: 117-127.

Rice, W. R. 2002. Experimental tests of the adaptive significance of sexual recombination. Nature Reviews Genetics 3: 242-251.

Salathé, M., Kouyos, R. D. & S. Bonhoeffer, 2008. The state of affairs in the kingdom of the Red Queen. Trends in Ecology and Evolution 23, 439-445.

Sasaki, A., Hamilton, W. D. & F. Ubeda, 2002. Clone mixtures and a pacemaker: new facets of Red-Queen theory and ecology. Proceedings of the Royal Society B. 269: 761-772.

Schaack, S., Pritham, E. J., Wolf, A. & M. Lynch, 2010. DNA transposon dynamics in populations of Daphnia pulex with and without sex. Proceedings of the Royal Society of London B. In Press.

West, S. A., Lively, C. M. & A. F. Read, 1999. A pluralist approach to sex and recombination. Journal of Evolutionary Biology 12: 1003–1012.

Wilson, C. G. & P. W. Sherman, 2010. Anciently asexual bdelloid rotifers escape lethal fungal parasites by drying up and blowing away. Science 327: 574-576.

Zar, J. H. 1999. Biostatistical Analysis, 4th ed. Prentice Hall, London: 564.

CHAPTER 3

OUTRUNNING THE RED QUEEN: BDELLOID ROTIFERS DISPERSE RAPIDLY AND INDEPENDENTLY OF DEADLY FUNGAL PARASITES IN A NATURAL METAPOPULATION

Abstract

Sexual reproduction is nearly ubiquitous in nature despite significant costs. The Red Queen hypothesis suggests that sex predominates because obligately asexual lineages are consistently extinguished by relentlessly evolving parasites and pathogens. However, theoretical models of coevolution predict that asexuality can persist in one special case: when host organisms inhabit spatially structured metapopulations and disperse among habitat patches rapidly and independently of biological enemies. We examined whether these criteria are fulfilled by bdelloid rotifers, a class of freshwater invertebrates that has survived without sex for millions of years. Bdelloids thrive in ephemerally moist microhabitats, withstanding periods of desiccation by contracting into tiny anhydrobiotic propagules that can disperse widely on the wind to colonize new patches. To determine whether this unusual ecology decouples populations from coevolving fungal parasites, we established sterile habitat patches at a natural wetland site and assessed colonization by rotifers and fungal parasites for five weeks. Bdelloids colonized elevated habitat patches more rapidly than other microfauna, and these wind-founded populations had a tenfold lower incidence of parasitism than populations at ground level. Extended desiccation of material from infected habitats also reduced parasitism significantly. Results support the hypothesis that desiccation-tolerance and wind dispersal allow the anciently asexual bdelloids to "outrun" the Red Queen in space and time.

Introduction

Why sexual reproduction predominates over asexual reproduction is a long-standing puzzle in evolutionary biology [1-4]. This is because sex requires investment in males, who do not contribute to population growth because they cannot produce eggs. Asexual females transmit their genes up to twice as efficiently by producing independently reproductive clonal daughters. Asexuals also avoid the energy, time, and health costs associated with mating, and the risk of disrupting a successful genotype during recombination [3]. Given these advantages, it is surprising that less than 1% of animal and plant species are obligately asexual [5]. Clonal lineages arise occasionally in most taxonomic groups, but their "twiggy" distribution at the tips of phylogenetic tree branches implies that they generally disappear before they can radiate [6,7].

Rotifers of the class Bdelloidea are a famous exception. These microscopic freshwater invertebrates have existed for at least 35 million years and today number more than 450 species [8,9], but no males, sperm, or indications of meiosis have been found despite close observation for more than three centuries [10]. Several lines of molecular evidence also support the inference that bdelloid rotifers abandoned sexual reproduction tens of millions of years ago, after diverging from their facultatively sexual sister clade, the monogonont rotifers [11,12].

Many hypotheses have been proposed to explain why, in general, sexual lineages survive and speciate whereas asexual lineages rapidly die out [3,4,13]. Any of these hypotheses would be strengthened if it could also explain how the anciently asexual bdelloids have flourished and proliferated, contrary to the prevailing pattern [14]. A prominent contender is the Red Queen hypothesis, which proposes that sex is maintained because it facilitates adaptation to rapidly evolving parasites and pathogens [15-19]. In theoretical simulations, these rapidly evolving natural enemies

relentlessly track common host genotypes and generate time-lagged, negative frequency-dependent selection that favours rare combinations of resistance or defence alleles [20,21]. Sex creates new genetic combinations each generation, thus ensuring a constant supply of rare genotypes while also preserving temporarily unfavourable alleles for subsequent recycling. By contrast, obligate asexuals must rely on mutations for novelty and variation, and therefore are slower to respond to coevolving parasites. Asexual genomes also are completely linked: if a temporarily unfavourable genotype is lost, it cannot be recovered by recombination after the focus of parasite-mediated selection has shifted elsewhere [20]. A gradual attrition of genetic diversity is predicted to drive the replacement of asexual lineages by sexual competitors that are better able to keep pace with antagonists.

Red Queen dynamics have been modelled extensively, demonstrating the plausibility of this hypothesis across a broad region of parameter space [17-22]. Moreover, key assumptions of the Red Queen model have been supported empirically in tractable invertebrates such as freshwater cladocerans and snails, where rapid evolutionary interactions with parasites can be observed in the field over relatively small spatial and temporal scales [23-27]. However, one piece of evidence appears to challenge the explanatory power of the Red Queen: bdelloid rotifers are afflicted by at least 50 described species of deadly fungal and oomycete parasites [28], but bdelloids have nevertheless endured as obligate asexuals. The Red Queen's account of sex can only be reconciled with these 'scandalous' asexual rotifers if they have unusual alternative ways to address or avoid relentlessly coevolving enemies [14,29].

According to a substantial body of theoretical work, asexuals can circumvent antagonistic coevolution under special ecological conditions [30-34]. These scenarios involve structured metapopulations, where parasites and hosts are allowed to migrate among numerous discrete habitat patches. Asexuality is stable if hosts disperse

independently of their parasites and at substantially higher rates, because these conditions reduce the ability of parasites to erode host genetic diversity. An asexual clone may be eliminated by co-adapted parasites in one patch, but it will persist if descendents have dispersed without parasitic accompaniment and colonized either pristine microhabitats with no parasites, or patches where the local parasites are poorly matched genetically. Like recombinant sexual offspring, immigrating clones represent novel, locally rare genotypes. By the time a naïve parasite evolves appropriate virulence factors toward recent immigrants, or an appropriately adapted parasite arrives at the patch, host propagules will have dispersed again, and new clones will have arrived. In principle, sex is not necessary if hosts can escape the Red Queen's genetic race by playing an endless game of stochastic "hide-and-seek" with their parasites and pathogens in space and time [35].

If these ecological models apply to bdelloid rotifers, their ancient asexuality could be accommodated under the Red Queen hypothesis. Indeed, there are several reasons to suspect that bdelloids are especially likely to satisfy the restrictive demographic and habitat conditions. Most species measure less than 0.4 mm when their bodies are maximally extended (<0.1mm in *Habrotrocha minuta*), which places them among the smallest of all metazoans [36,37]. Substantial populations can be supported in the tiniest freshwater habitat patches, such as the film of moisture that surrounds moss leaves and soil particles. Bdelloids are able to exploit these ephemeral microhabitats because they can withstand complete loss of cellular water at any life stage [38]. When their habitat dries out, adult bdelloids contract to less than half their full length and form compact anhydrobiotic ovoids called "tuns" [39]. When water returns, the dormant tuns rehydrate and the rotifers resume their life cycle as if the interruption had never occurred [40]. An individual rotifer might experience multiple

desiccation events in its lifetime, each typically lasting a few days or weeks. However, tuns have recovered after as long as nine years without water [38].

The bdelloids' capacity for anhydrobiosis has two important consequences for coevolutionary models that incorporate metapopulation structure. First, if bdelloids are more tolerant of extended desiccation than their parasites, anhydrobiotic tuns would represent a "bank" of dormant propagules whose genotypes are shielded from coevolving enemies for periods that may greatly exceed the typical generation time. Theory suggests that such temporal storage of genotypes can mitigate the erosion of genetic variation that occurs if all members of a cohort are synchronously subjected to selection by co-adapted parasites [33,41]. The second crucial dimension to the bdelloids' life history is that dry tuns can be transported for great distances by wind, attached to tiny substrate particles [42]. Each of these windborne propagules is capable of independent reproduction soon after it lands in water, permitting the rapid colonization of remote, isolated habitat patches. As a result, bdelloids have been found on every continent and in virtually every habitat containing fresh water thus far examined [43]. Closely-related clones have even been found thousands of miles apart [44], implying intercontinental dispersal. If coadapted parasites are unable to accompany rotifers on these aerial journeys, anhydrobiosis could conceivably allow for spatial as well as temporal escape.

Wilson and Sherman [35] provided initial evidence for these temporal and spatial effects of anhydrobiosis. They reported that a lethal parasitic fungus (*Rotiferophthora angustispora*) cannot withstand desiccation or disperse by wind to the same degree as its bdelloid host (*Habrotrocha elusa*). Five weeks of desiccation eliminated the fungus from over 90% of laboratory cultures of rotifers. When a heavily infected population was desiccated for just 7 days and tuns were dispersed by fans in a wind chamber, over 60% of newly-founded populations remained free of the

parasite. By contrast, the fungus completely exterminated all continuously hydrated control populations, including those where rotifers were allowed to disperse from an infected area by water. If the results of this experiment hold under natural conditions, then bdelloids would fulfil the assumption of independent dispersal that is required theoretically to outrun the Red Queen.

The purpose of the present study was to test this critical assumption of parasite-free dispersal in natural populations. There were four specific objectives. First, we compared the rates at which bdelloids and their fungal parasites colonized pristine ground-level habitats, by sterilizing small patches of natural soil and monitoring how long it took for host and parasite populations to return to their initial levels. We predicted that rates of host and parasite immigration would be similar as long as both organisms were able to use groundwater as a dispersal medium. Second, we compared the rates at which bdelloids and parasites colonized elevated microhabitats, by suspending sterile dishes 30cm above the ground. We predicted that bdelloid tuns, carried by wind, would immigrate independently and more rapidly than their parasites. Third, we investigated whether extended desiccation of freshly collected natural soil samples could reduce bdelloids' fungal parasite loads, even without dispersal. We predicted that a simulated drought would provide a temporal escape, cleansing parasites from an infected habitat patch and allowing any recovering or immigrating bdelloids to thrive there. Finally, we compared bdelloids with nematodes, tardigrades, and monogonont rotifers in their abilities to colonize terrestrial and elevated microhabitats. These other microfauna also have desiccation-tolerant stages, and presumably share the potential to outlast their parasites in time. However, since only bdelloids are anciently asexual, we predicted that they would outpace the other taxa in the spatial dimension, and colonize our elevated habitats more frequently and rapidly.

Methods and Materials

(a) *Study sites*

Our field study was conducted in September and October of 2009 at Cornell University's Experimental Ponds Facility, situated in a rural area north of Ithaca, New York, USA (42º 30'09" N, 76º26'14" W). At that location, 40 shallow man-made ponds (20 x 20 x 2.4m deep) are arranged in a 4x10 grid over an area of approximately 150 x 350m. All experiments and sampling procedures (below) were replicated for each pond, at a focal site approximately 1.5m back from the centre of its western shoreline. Bdelloids do not require permanent bodies of water, but we chose this protected wetland to increase the likelihood that a broad range of microfauna (and parasite) species would be represented.

(b) *Sampling and experimental design*

At each of the 40 focal sites, we excavated a disc of soil approximately 150mm in diameter and 30mm deep, placed it in a plastic bag, and transported it to the laboratory along with any associated vegetation (moss, lichen, grass, etc.). We screened (as described below) 0.1g of material from the surface of each disc as an initial control to establish the baseline discovery rate of bdelloids, their parasites, and other microfauna. The material in each disc was then sterilized by autoclaving for 90 minutes [45]. Two further 0.1g sub-samples were removed from the autoclaved material. The first was screened to confirm that all animal populations had been exterminated, and the second was added to a dish that had been "baited" [46] with large, laboratory-cultured populations of two common species of bdelloid rotifers (*Adineta vaga* and *Habrotrocha elusa*). After 3, 4 and 5 weeks, these baited dishes were screened to confirm that no parasitic fungi appeared.

Each sterilized soil disc was returned to the field site and re-embedded at its original spot in a plastic dish (150mm diameter x 35mm deep) with multiple holes in the bottom and sides to connect it with the surrounding groundwater and prevent waterlogging during rainstorms. The rim of the dish was flush with the ground surface. For the next 5 weeks, we collected a sample (~1g) of surface material from each dish once per week. Each sample was bagged and returned to the laboratory, where a sub-sample (0.1g) was screened for microfauna and parasites. On the fifth week we also collected undisturbed surface soil from 30cm away and, in the laboratory, screened a 0.1g sub-sample as a terminal control to see if the prevalence of bdelloids, their parasites, and other microfauna at the location had changed during the experiment relative to our initial baseline.

In order to study aerial dispersal, at each of the 40 sites we created an elevated, barren habitat patch. A plastic filter funnel was mounted on top of a stake, 30cm above the soil surface and 30cm away from the sterile soil patch on the ground. The catchment area of the funnel (150mm in diameter) was equal to that of the soil patch. The funnel mouth was open to the wind and rain, but it was too high above the ground to receive water splashed up from the soil during rainstorms [47]. Into the mouth of each funnel we recessed a lidless 88mm Petri dish of known mass, containing a layer of desiccated Czapek's 0% agar. This substrate promotes the growth of fungal parasites of rotifers [28], and would not be washed away by rain. The Petri dish was glued at a 15º angle, so that up to 14mL of rainwater could pool on one side, creating a habitat patch with a depth gradient. Excess water spilled over the lower edge of the dish and down the funnel spout. For 5 weeks, the funnels trapped particles that were borne on the wind and rain (or, perhaps, on birds or insects); these accumulated in the target dish along with any associated microorganisms. At the end of this period, the Petri dishes were removed from the funnels and returned to the laboratory where they

were re-weighed, and the deposited material was screened for bdelloids, their parasites, and other microfauna.

To investigate effects of extended desiccation on microfauna and bdelloid parasites, a portion of the initial control sample from each site was dried in a plastic weighing dish and maintained at 40% relative humidity for 5 weeks, after which a 0.1g sub-sample was rehydrated with filtered, sterilized rainwater, and screened. These procedures were repeated for material from the terminal control sample at each site.

(c) *Screening and data analysis*

Each 0.1g sub-sample was suspended in 0.5mL of sterile distilled water by vigorous shaking for 30 seconds. The resulting slurry was transferred to the center of an 88mm Petri dish with a thin layer of Czapek's 0% agar [46]. After 24 hours, an additional 2mL of sterile distilled water was added to keep the agar surface moist, and the dishes were stored at room temperature in a transparent, humid box (98% relative humidity, 12L:12D). At 3, 4 and 5 weeks, each dish was scanned exhaustively under a compound microscope (at 100x magnification) for bdelloid or monogonont rotifers, nematodes, and tardigrades. Each of these groups was scored as "present" on a dish if at least one active individual was seen (i.e., the presence of eggs or tuns alone was insufficient). Bdelloids were identified to the family level, and examined for infections. We did not attempt to enumerate all the bdelloid species in our screened samples because they contained prohibitively large numbers of animals for such close taxonomic work; moreover, species identification of bdelloids is challenging [48] and assessing rotifer species diversity was not the focus of this investigation.

Fungal infections typically presented as hyphae or spores emerging from a dead rotifer, and they could usually be identified as belonging to one of the known

species of bdelloid enemies. When there was doubt about whether a dead bdelloid had indeed been killed by an apparently associated fungus, the corpse was transferred to a dish baited with cultured *Adineta vaga* and *Habrotrocha elusa*, and parasitism was confirmed only if a new infection cycle could be initiated.

Wind-dispersed material was screened in the same Petri dishes that had served as elevated habitat patches in the field, since they already were lined with the appropriate agar for microfaunal and fungal growth. These were collected on a dry day and returned to the laboratory, where they were detached from the funnels and re-weighed. The mean mass of debris deposited by wind during the five weeks of the experiment was 0.137g (± 0.077 s.d.). This was 37% higher than the 0.1g screened in other sub-samples (which had been selected a priori to match the mass deposited in an earlier wind trial), but the extra material only increased the chance that at least one parasite would be present in the elevated dishes, setting a more conservative bar for the prediction that wind dispersal facilitates parasite evasion. Filtered, sterilized rainwater was added to each dish to restore the standard moisture level for dishes during screening, and their lids were replaced. They were examined for microfauna and fungi 3, 4 and 5 weeks later as described above.

Our methods of screening could not detect a bdelloid parasite in a sample that contained no bdelloids (either by chance or because they had already been exterminated), potentially resulting in an underestimate of the total number of samples that contained parasites. To eliminate this problem, we compared relative incidences of parasitism across only those samples that contained bdelloids. In addition, to assess the extent of underestimation, we used baited dishes to screen another 0.1g of the terminal control material collected from each site. These were incubated like the others and examined at 3, 4 and 5 weeks for parasites. We also performed baited screening on the terminal samples that had been desiccated and rehydrated, to check

whether the effects of dehydration were still evident when parasites could be detected regardless of whether a sample had any naturally occurring bdelloids.

We used nonparametric statistical tests to analyse the binary (presence or absence) data for the various groups of microfauna and bdelloid parasites. We established their proportional incidence across the 40 independent sites (ponds) that served as our units of replication, and tested our initial hypotheses by comparing these proportions between the appropriate treatments or timepoints. All p-values represent Fisher's exact test unless otherwise stated; unplanned contrasts are indicated by the use of a Bonferroni adjustment. Error bars and ranges indicate standard deviations.

Results

(a) *Immigration of bdelloids and parasites to different microhabitats*

Bdelloid rotifers were present in 42.5% (±7.8 s.d.) of the 40 initial control samples, and 47.5% (±7.9) of the 40 terminal control samples. The initial incidence of fungal parasitism among samples containing bdelloids (Fig. 3.1) was 23.5% (±10.3), and the incidence five weeks later was 26.3% (±10.1), implying minimal changes in the prevalence of bdelloids or their parasites at the field location during our experiment.

Sterilization was effective: none of the autoclaved samples contained living animals, and no fungi appeared in the baited test dishes. Once the sterilized ground-level habitat patches were returned to the field sites, the incidence of bdelloid rotifers began to increase significantly (binary logistic regression: $\log(p/(1-p))= -1.01+0.248*\text{week}$; d.f.=1, n=240, G=7.09, $p = 0.008$), reaching a maximum of 62.5% (± 7.7) at Week 3; in Weeks 4 and 5 there was a slight decrease to 45% (± 7.9), a level closer to the baseline found in the initial and terminal controls. Parasites of bdelloid

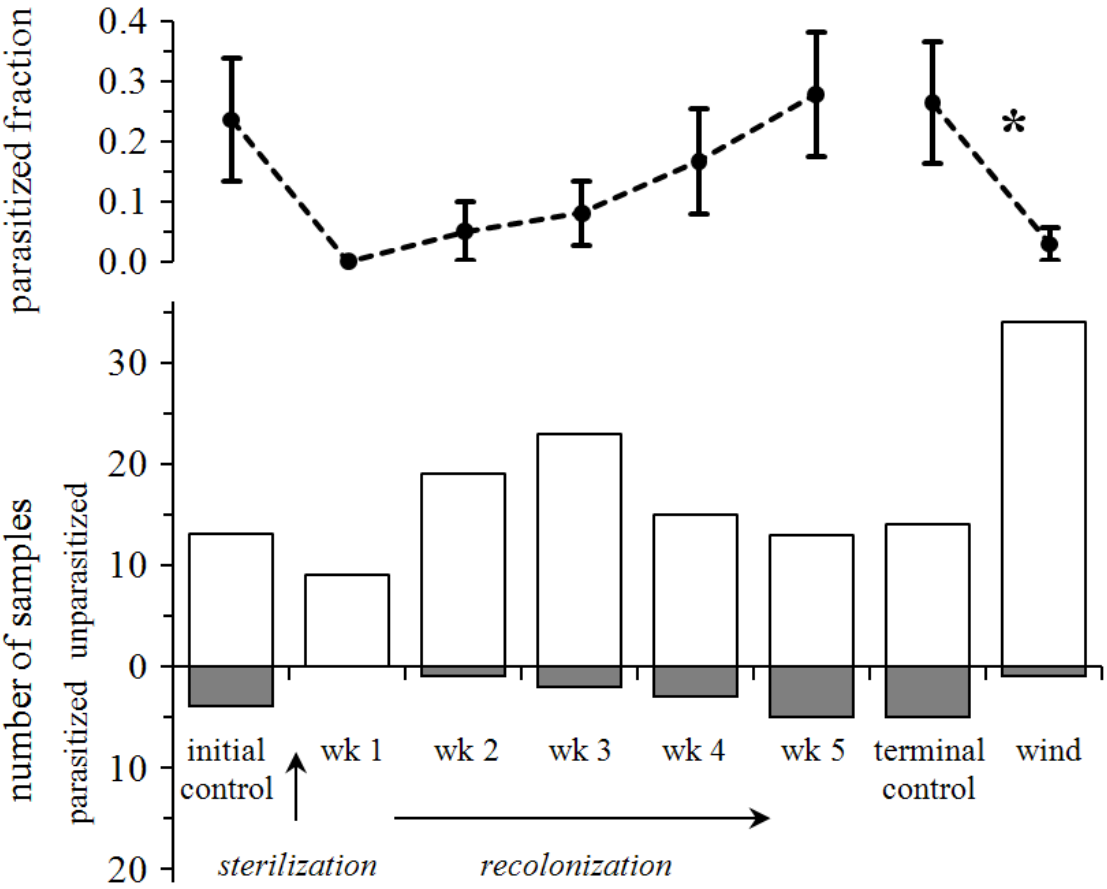


Figure 3.1 Occurrence of bdelloid rotifers and fungal parasites in samples collected during a sterilization and recolonization field experiment. Bars indicate the number of samples containing bdelloid rotifers (and whether those samples also contained fungal parasites). The proportional incidence of fungal parasitism among samples is plotted above; error bars indicate standard deviations of these proportions. * : $p<0.02$, Fisher's Exact Test, n=54.

rotifers were not detected in Week 1, but their incidence increased consistently and significantly thereafter (Fig. 3.1; binary logistic regression: $\log(p/(1-p))= -4.69+0.755*\text{week}$; d.f.=1, n=90, G=7.06, $p = 0.008$), reaching the baseline level at Week 5 (27.8% ± 10.3). Thus, bdelloids and their fungal parasites returned to their baseline levels of abundance in less than five weeks.

Results from the elevated habitats were markedly different. After 5 weeks, bdelloid rotifers were present in 87.5% (± 5.2) of these dishes (Fig. 3.1), which is significantly higher (by nearly two times) than their incidence in either the initial or terminal control samples, or in the samples collected after five weeks of terrestrial recolonization ($p < 0.001$ in all cases). Although the mass of material screened from the elevated dishes was on average 37% greater than that screened for terrestrial samples, bdelloid parasites were present in only one elevated-dish sample (2.9% ± 2.8), a significantly lower incidence (by nearly an order of magnitude) than either the initial or terminal control samples ($p = 0.034$; $p = 0.0168$ respectively), or the samples collected after five weeks of terrestrial recolonization ($p = 0.0139$).

When terminal control samples were screened using 40 dishes that had been pre-baited with rotifers, the absolute number of parasitized samples rose slightly (Fig. 3.2). However, given that all 40 dishes contained rotifers, the relative incidence of parasitism was still only 35% (± 7.5), which is not a significant proportional increase over the unbaited control samples ($p = 0.326$). Therefore, although baiting reveals more parasites, the relative incidence of parasitism could be estimated from samples with naturally occurring bdelloids only: there was not a large fraction of screened dishes in which parasites went undetected due to the extermination of their hosts. Interestingly, however, baited and unbaited dishes sometimes revealed different parasite species in the same infected sample, implying that the identity of the available bdelloid hosts affected which parasites were observed.

Extended desiccation had adverse effects on the bdelloids' parasites but not the rotifers themselves. In the initial and terminal samples that were desiccated for 5 weeks and rehydrated, the mean incidence of bdelloids was 77.9% (± 4.7). This was actually a significant increase in rotifer prevalence over the control samples that were not desiccated (Fig. 3.2; $p < 0.001$). By contrast, the incidence of parasitism in the

rehydrated samples was reduced to 6.7% (±3.2) from 25% (±7.2) in the non-desiccated samples (Fig. 3.2; $p = 0.015$). When the rehydrated terminal samples were screened on pre-baited dishes, the incidence of parasites was still only 5.4% (±3.7), again significantly lower than the 35% (±7.5) found in the non-desiccated, baited samples ($p = 0.0016$).

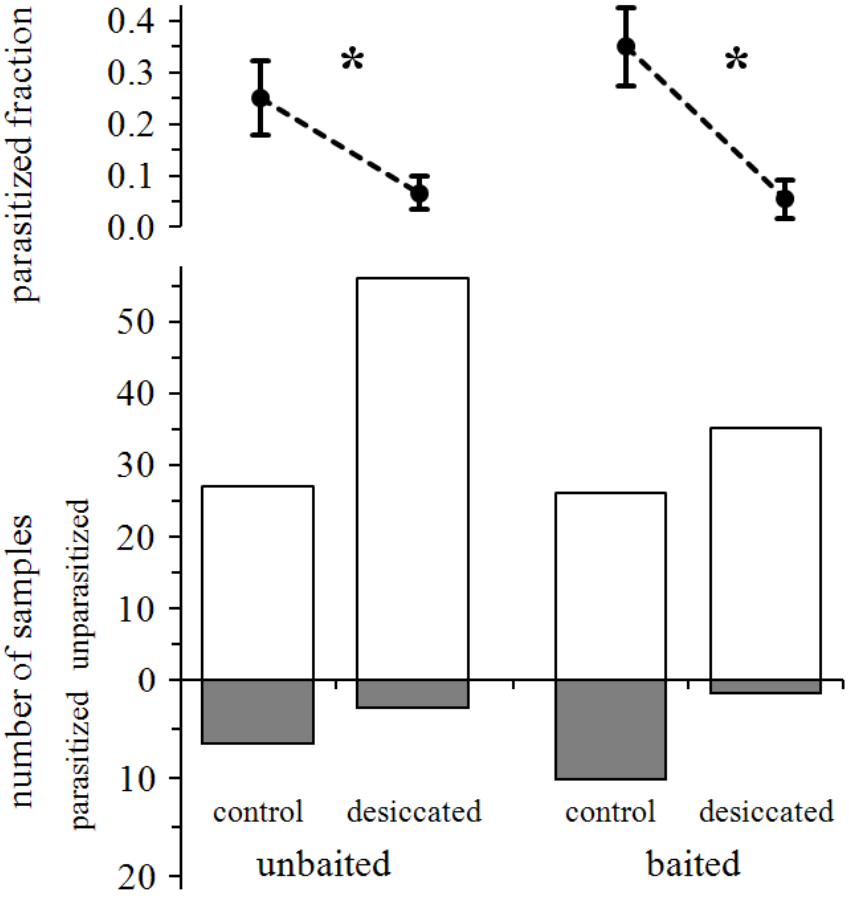


Figure 3.2 Five weeks of desiccation significantly reduced the incidence of fungal parasites in samples of natural material containing bdelloid rotifers. 'Baited' plates were supplemented with lab-cultured rotifers before screening in order to enhance the detection of parasites. Error bars indicate standard deviations of the proportions of samples that contained at least one parasite. * : $p<0.002$, Fisher's Exact Test; n=96 (unbaited); n=77 (baited).

(b) *Parasite diversity*

By the end of the screening period, we had observed fungal parasites of bdelloids in 41 of our samples. In six of these, two parasite species were detected, for a total of 47 records of parasitism (Table 3.1). These fungi represented 14 species from five genera. *Rotiferophthora* was represented most frequently (8 species, 44.7% of all parasite records). All five genera are lethal to their hosts, and bdelloid populations in parasitized screening dishes were rapidly depleted once an epidemic began. Indeed, several species of *Rotiferophthora* and *Harposporium* were so aggressive that they eliminated all bdelloids in their dishes during the three weeks of observation. No significant differences were detected in the representation of the five fungal genera among treatments, except that the few parasites recorded from desiccated (N=6) and elevated (N=1) samples belonged disproportionately to *Triacutus* (4 of those 7 records; 57.1%). This was a significant overrepresentation ($p = 0.0045$ after Bonferroni adjustment), given that *Triacutus* occurred just once among 40 parasite records from ground-level samples that had not been desiccated (2.6%; Table 3.1).

Table 3.1 Fungal parasite species recorded attacking bdelloid rotifers during the field experiment.

treatment	parasite species	frequency
initial (control)	*Pseudomeria mucosa*	2
	Rotiferophthora cylindrospor	2
(desiccated)	*P. mucosa*	1
terminal (control)	*P. mucosa*	2
	R. cylindrospora	1
	R. attenuata	1
	R. ovispora	1
	Triacutus subcuticularis	1
(desiccated)	*Pochonia sp.*	1
	T. subcuticularis	2
(baited)	*Harposporium spirosporum*	2
	H. botuliforme	1
	H. cocleatum	1
	Pochonia sp.	1
	P. mucosa	7
	R. barronii	1
	R. cylindrospora	1
	R. denticulospora	1
	R. globospora	1
	R. intermedia	2
(desiccated, baited)	*R. guttulaspora*	1
	T. subcuticularis	1
week 1	-	-
week 2	*P. mucosa*	1
week 3	*R. intermedia*	2
week 4	*P. mucosa*	1
	R. denticulospora	2
week 5	*P. mucosa*	2
	R. cylindrospora	1
	R. denticulospora	2
	R. globospora	1
wind	*T. subcuticularis*	1

(c) *Microfauna in elevated and terrestrial samples*

In addition to bdelloid rotifers, we recorded the presence of monogonont rotifers, nematodes and tardigrades in the initial and terminal control samples, in the samples that had been desiccated for 5 weeks, and in the elevated habitat samples, as well as in the sterilized terrestrial habitat patches. Representatives of all three major bdelloid families occurred in our samples (Table 3.2). Habrotrochidae (which includes many soil-dwelling species) was most common in both the elevated and terrestrial samples, followed by Adinetidae and Philodinidae. Frequency distributions of the families did not differ significantly between control and desiccated soil samples (χ^2 = 3.36, d.f. =2, p = 0.186), nor between control samples and the immigrants to terrestrial patches (χ^2 = 1.24, d.f. =2, p = 0.537). The elevated-habitat colonists had a more equal distribution among families than immigrants to terrestrial patches (χ^2 = 7.99, d.f. =2, p = 0.018), due to increased abundance of Adinetidae, and reduced abundance of Habrotrochidae. No species of the fourth bdelloid family (Philodinavidae) were seen during our study, which is unsurprising since this unusual group contains fewer than 10 described species, and these are seldom recorded [49].

Nematodes were present in 63.8% (±5.4 s.d.) of initial and terminal control samples, which was significantly higher than the incidence of bdelloids in the same samples (Fig. 3.3, p = 0.026). Like the bdelloids, nematodes quickly recolonized the sterilized soil samples. Their incidence increased significantly over the five week study period (binary logistic regression: log(p/(1-p))= -0.744+0.295*week; d.f.=1, n=240, G=10.09, p = 0.001), reaching baseline levels by week 4 (65% ±7.5). Compared to the controls, the proportion of samples containing nematodes after desiccation was reduced significantly, to 37.7% ± 5.5 (Fig. 3.3, p = 0.0014). This is also significantly lower than the proportion of desiccated samples that contained bdelloids

Table 3.2 Numbers of screened dishes containing representatives of each of the three major bdelloid families. Percentages indicate the relative representation of families among all records for each treatment.

samples	bdelloid family	frequency	%
control	Habrotrochidae	29	36.3
(initial & terminal, n=80)	Adinetidae	12	15.0
	Philodinidae	10	12.5
desiccated	Habrotrochidae	52	65.0
(initial & terminal, n=80)	Adinetidae	27	33.8
	Philodinidae	8	10.0
terrestrial recolonization	Habrotrochidae	72	36.0
(weeks 1-5, n=200)	Adinetidae	29	14.5
	Philodinidae	15	7.5
wind colonization	Habrotrochidae	29	72.5
(n=40)	Adinetidae	28	70.0
	Philodinidae	14	35.0

(Fig. 3.3, $p < 0.001$). Nematodes occurred in just 7.5% (±4.2) of the elevated dishes, which is even lower than their incidence in the desiccated samples (Fig. 3.3, $p < 0.001$), and once again significantly lower than the incidence of bdelloids in the same elevated samples (Fig. 3.3, $p < 0.001$).

Monogonont rotifers were present in 11.25% (±3.5) of the control samples, 3.9% (±2.2) of the desiccated samples, and 7.5% (±4.2) of the elevated samples. These occurrence rates do not differ statistically ($p = 0.172$), and all are significantly lower than the corresponding incidences of bdelloids (Fig. 3.3, $p < 0.001$ in all cases).

There was no significant increase in monogonont frequency during the five weeks of terrestrial recolonization (binary logistic regression: d.f.=1, n=240, G=0.83, p = 0.362); their incidence fluctuated, and it was never higher than the 17.5% (±6.0) recorded in the second week. Monogononts favour permanent aquatic habitats [43], and although all of our sampling sites were near ponds, our terrestrial and elevated microhabitat patches were only ephemerally moist and were seldom colonized by monogononts.

Living tardigrades were only seen in a few of the elevated dishes (10% ± 4.7), which is a significantly lower incidence than bdelloids ($p < 0.001$). They were not found in any of the terrestrial or desiccated samples.

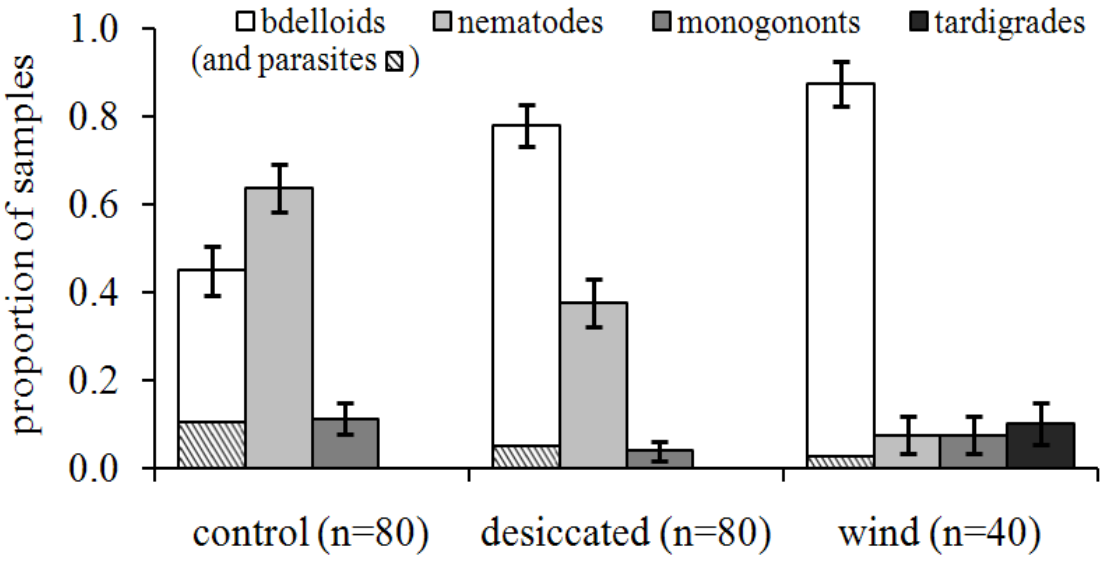


Figure 3.3 Proportional incidence of four groups of microfauna in control, desiccated and wind-dispersed samples. Hatched bars indicate samples containing both bdelloid rotifers and their fungal parasites. Error bars indicate standard deviations.

Discussion

In the field, bdelloid rotifers and their fungal parasites rapidly recolonized patches of sterilized soil and vegetation at ground level, and reached their original frequencies of occurrence in less than 5 weeks. During the same period of time, bdelloids successfully colonized sterile elevated habitat patches of equivalent catchment area, reaching nearly double their initial and final frequencies in the terrestrial samples. However, the bdelloids' fungal parasites rarely immigrated to these elevated habitat patches, and they were underrepresented by a factor of ten relative to their frequencies in the terrestrial samples at the beginning and end of our experiment.

These differences can be attributed in part to desiccation-tolerance: when natural soil samples were dried under controlled conditions for five weeks and rehydrated, the proportion of screened dishes that contained bdelloid rotifers increased by a factor of 1.5, whereas the incidence of parasites declined by a factor of four. Bdelloids can tolerate longer periods of desiccation than their parasites [35], which explains the scarcity of fungal enemies in dried samples. In turn, removal of lethal parasites helps to account for the increased frequency of bdelloids in the dried samples, together with reduced competition from more desiccation-sensitive microfauna, and an intriguing physiological mechanism whereby anhydrobiosis appears to enhance bdelloid fecundity. Ricci and Caprioli [40] suggested that this latter effect may be due to removal or inhibition of cryptic parasites such as viruses or bacteria, or to adaptive allocation of resources to reproduction in rehydrated animals, which are likely to find themselves in an unoccupied microhabitat.

Bdelloids almost certainly arrived in our elevated habitats as desiccated tuns attached to microdebris, and these patches were themselves subject to frequent natural desiccation during the experiment. However, due to intermittent rainfall they never

dried out for longer than a week, so the marked reduction in parasites in the elevated samples (at least as great as that caused by five weeks of controlled desiccation: Fig. 3.3) must have been due in large measure to the poor wind-dispersal abilities of the fungi. By physically dissociating uninfected hosts from parasite spores (which are primarily waterborne), wind dispersal permits desiccated bdelloids to escape in space as well as time [35]. It is possible that parasites would eventually have appeared in our elevated habitats, as increasing amounts of dry material accumulated or exceptionally powerful gusts of wind transported viable spores in moist substrate. However, our results show that tuns can disperse independently of fungal parasites on an ecologically meaningful timescale. Given the short generation times of rotifers, five weeks is more than sufficient for a single bdelloid to establish a large population and generate hundreds of new propagules. Even if fungal infections eventually appeared, an extended desiccation event could purge them, or at least allow uninfected tuns to escape.

Our results extend and broaden those of a previous investigation of anhydrobiotic escape, which focused on one bdelloid-fungus species pair placed in an artificial wind chamber for a week [35]. The present study was conducted in the field over a period of 5 weeks, and demonstrates that species from three bdelloid families can disperse independently of fungi belonging to 5 genera, including the two most common and speciose parasite groups (*Rotiferophthora* and *Harposporium*). These results highlight the efficacy of wind in enabling bdelloid tuns to escape their parasites. Dispersal in water does not produce the same effect, as illustrated by the rapid return of parasites to our terrestrial microhabitats (Fig. 3.1).

In this study, one parasite (*Triacutus subcuticularis*) appeared to be more difficult for bdelloids to evade. This unusual fungal endoparasite is the only species in its genus, whose higher taxonomic affiliations are uncertain [50]. Uniquely, the

infected bdelloid host may remain alive and active for many days while yeast-like hyphal segments invade its pseudocoel, before it eventually dies and the parasite sporulates. Although *T. subcuticularis* occurred in only 5 samples, 3 of these had been desiccated and one was in an elevated microhabitat. The disproportionate occurrence of *T. subcuticularis* in these harsh treatment groups suggests that it has a higher tolerance for desiccation than other, more common and aggressive fungal parasites, and may even benefit from their absence.

The relative representation of the three major families of bdelloids did not vary significantly among our experimental treatments (Table 3.2), indicating that the ability to survive desiccation and disperse rapidly on the wind is widespread in these rotifers and that our results were not skewed by a restricted cluster of highly derived wind-dispersal specialists. It is probable that aerial dispersal is tied closely to the capacity for anhydrobiosis itself, which appears to be ancestral to the class Bdelloidea [51]. Only a few scattered species of bdelloids have entirely lost the ability to withstand desiccation, likely due to relatively recent specialization for a fully aquatic lifestyle (Ricci 1998).

Anhydrobiosis is not unique to bdelloids: many species of tardigrades and nematodes also can tolerate complete desiccation at any life stage [37]. We have not investigated whether these animals, like bdelloids, can outlast their fungal enemies over time; this seems probable given that similar parasite species attack all three groups [52]. However, our investigation of dispersal ecology indicates that bdelloids have an advantage in the spatial dimension. Nematodes were the most abundant microfaunal group in the control samples of soil and vegetation from our sites, and they rapidly recolonized the sterilized ground-level habitat patches. However, their abundance was significantly reduced in the samples subjected to extended desiccation, and they were even scarcer in the elevated microhabitats. Apparently the nematodes

in this habitat are less resistant to extended desiccation than the bdelloids, and less capable of aerial colonization. Compared to coiled, desiccated nematodes, bdelloid tuns are compact and regular in shape [37-39], which may facilitate ensconcement in fibrous or crenellated debris and help them to endure the physical and mechanical stresses of wind transport. In addition, anhydrobiosis does not arrest ageing in nematodes as it does in bdelloids, so desiccated nematodes arriving by wind will have relatively less remaining lifespan and reproductive potential compared to bdelloid colonists undertaking an equivalent journey [40].

Tardigrades appeared in a smaller number of elevated dishes than bdelloids. Their greater mass probably hinders their capacity for long-distance dispersal on the wind: a desiccated tardigrade is about four times heavier than a desiccated bdelloid [53]. Furthermore, all tardigrades had died by the second week of the screening period, whereas populations of the other microfaunal taxa persisted throughout screening (unless exterminated by parasites). Dead tardigrades also were seen occasionally in terrestrial samples. Tardigrades typically have more specific substrate and food requirements than bdelloids in culture, and they are particularly sensitive to anoxia [54,55]. Their failure to thrive in our observation dishes is consistent with evidence that microhabitat and food requirements limit tardigrade colonization as well as dispersal potential [56,57].

The extraordinary vagility of bdelloid rotifers by wind has been noted in previous studies of microfaunal dispersal. Over 12 months, Jenkins and Underwood [42] collected samples from two windsocks. The first was 150m downwind from a pond, at an elevation of 2.5m. The only animals recovered were bdelloid rotifers (in about 60% of samples). The second windsock was mounted on top of a building 1km from the nearest upwind body of standing water, at an elevation of 16m; again, only bdelloid rotifers were recovered (in 9% of samples). Caceres and Soluk [58] tracked

the colonization of pristine aquatic mesocosms 10-200m from small ponds, shielded by 0.5mm mesh so that colonization occurred primarily by wind and rain. Bdelloids were the first colonists, recorded after an average of 4.4 weeks, versus 5.6 for the next fastest colonist (a monogonont rotifer). Nkem and colleagues [59] found that bdelloid rotifers constituted 55.3% of animals in soil deposited by wind on frozen Antarctic lake surfaces; tardigrades accounted for 43.2%, and nematodes 1.5%. Sohlenius and Boström [57,60] sampled microfauna from "extremely isolated" ice-free patches in the Antarctic, where dispersal is primarily by wind, and climatic conditions are hostile. Bdelloids were present in 67% of samples; tardigrades in 40% and nematodes in 35%. Sohlenius and Boström [57, p.824] concluded that "rotifers apparently have a greater capacity for dispersal and can survive adverse environmental conditions better than nematodes." Results from this inhospitable climatic region are especially informative: Antarctic nematodes and tardigrades must be among the most hardy, desiccation-tolerant and dispersive representatives of their respective phyla, yet still the rotifers were more widespread. This difference was even more pronounced in our temperate wetland location, where many nematodes seemed unable to withstand desiccation at all.

The small size of bdelloid rotifers and their tolerance of desiccation at any life stage allows them to reach and exploit the most isolated, ephemeral and miniscule habitats, and to disperse among widely separated patches at an extraordinary rate relative to their generation time. Indeed, bdelloid rotifers may be unmatched by other animals or plants in these combined respects. Certain fungi, protists and prokaryotes undoubtedly possess equivalent or superior powers of dispersal, but our results demonstrate that the worst fungal parasites of bdelloids neither share their facility for wind dispersal, nor hitchhike with their hosts to new populations under natural conditions. Anhydrobiotic wind dispersal therefore enables these rotifers to satisfy a

crucial assumption of "hide-and-seek" models for the long-term maintenance of asexuality, in which hosts are disengaged from coevolving antagonists because of independent and highly asymmetric migration rates [30-34]. If Red Queen dynamics indeed play an important role in the maintenance of sexual reproduction, then spatiotemporal escape from deadly parasites and pathogens could be the key to understanding how bdelloid rotifers have persisted for millions of years without sex.

REFERENCES

1. Maynard Smith, J. 1978 *The evolution of sex*. Cambridge University Press.
2. Bell, G. 1982 *The masterpiece of nature*. Berkeley: University of California Press.
3. Agrawal, A. F. 2006 Evolution of sex: why do organisms shuffle their genotypes? *Curr. Biol*. **16**, R696-R704.
4. Otto, S. P. 2009 The evolutionary enigma of sex. *Am. Nat*. **174**, S1-S14.
5. Burt, A. 2000 Sex, recombination and the efficacy of selection: was Weismann right? *Evolution* **54**, 337-351.
6. Rice, W. R. 2002 Experimental tests of the adaptive significance of sexual recombination. *Nat. Rev. Genet*. **3**, 242-251.
7. Neiman, M., Meirmans, S. & Meirmans, P. G. 2009 What can asexual lineage age tell us about the maintenance of sex? *Ann. N.Y. Acad. Sci*. **1168**, 185-200.
8. Waggoner, B. M. & Poinar, G. O. 1993 Fossil habrotrochid rotifers in Dominican amber. *Experientia* **49**, 354-357.
9. Segers, H. 2007 Annotated checklist of the rotifers (Phylum Rotifera) with notes on nomenclature, taxonomy and distribution. *Zootaxa* **1564**, 1-104.
10. Birky, C. W. 2010 Positively negative evidence for asexuality. *J. Hered*. **101**, S42-S45.
11. Hur, J. H., Van Doninck, K., Mandigo, M. L. & Meselson, M. 2009 Degenerate tetraploidy was established before bdelloid rotifer families diverged. *Mol. Biol. Evol*. **26**, 375-383.
12. Mark Welch, D. B., Ricci, C. & Meselson, M. 2009 Bdelloid rotifers: progress in understanding the success of an evolutionary scandal. In *Lost sex* (eds. I Schön, K. Martens, P. Dijk), pp.259-279.New York: Springer.

13. Kondrashov, A. S. 1993 Classification of hypotheses on the advantage of amphimixis. *J. Hered*. **84**, 372-387.
14. Butlin, R. 2002. The costs and benefits of sex: new insights from old asexual lineages. *Nat. Rev. Genet*. **3**, 311-317.
15. Jaenicke, J. 1978 A hypothesis to account for the maintenance of sex within populations. *Evol. Theory* **3**, 191-194.
16. Hamilton, W. D. 1980 Sex versus non-sex versus parasite. *Oikos* **35**, 282-290.
17. Salathé, M., Kouyos, R. D. & Bonhoeffer, S. 2008 The state of affairs in the kingdom of the Red Queen. *Trends Ecol. Evol*. **23**, 439-445.
18. Neiman, M. and Koskella, B. 2009 Sex and the Red Queen. In *Lost sex* (eds. I Schön, K. Martens, P. Dijk), New York: Springer.
19. Lively, C. M. 2010 A review of Red Queen models for the persistence of obligate sexual reproduction. *J. Hered*. **101**, S13-S20.
20. Hamilton, W.D., Axelrod, R. & Tanese, R. 1990 Sexual reproduction as an adaptation to resist parasites (a review) Proc. Natl. Acad. Sci. USA **87**, 3566-3573.
21. Peters, A. D. & Lively, C. M. 2007 Short- and long-term benefits and detriments to recombination under antagonistic coevolution. *J. Evol. Biol.* **20**, 1206-1217.
22. Lively, C. M. 2010 Parasite virulence, host life history, and the costs and benefits of sex. *Ecology* **91**, 3-6.
23. King, K. C., Delph, L. F., Jokela, J. & Lively, C. M. 2009 The geographic mosaic of sex and the Red Queen. *Curr. Biol*. **19**, 1438-1441.
24. Jokela, J. Dybdahl, M. F. & Lively, C. M. 2009 The maintenance of sex, clonal dynamics, and host-parasite coevolution in a mixed population of sexual and asexual snails. *Am. Nat*. **174**, S43-S53.

25. Koskella, B. & Lively, C. M. 2009 Evidence for negative frequency-dependent selection during experimental coevolution of a freshwater snail and a sterilizing trematode. *Evolution* **63**, 2213-2221.
26. Wolinksa, J. & Spaak, P. 2009 The cost of being common: evidence from natural *Daphnia* populations. *Evolution* **63**, 1893-1901.
27. Decaestecker, E., Gaba, S., Raeymaekers, J. A. M., Stoks, R., Van Kerckhoven, L., Ebert, D. & De Meester, L. 2007 Host-parasite 'Red Queen' dynamics archived in pond sediment. *Nature* **450**, 870-874.
28. Barron, G. L. 1991 A new genus, *Rotiferophthora*, to accommodate the *Diheterospora*-like endparasites of rotifers. *Can. J. Bot*. **69**, 494-502.
29. Maynard Smith, J. 1986 Contemplating life without sex. *Nature* **324**, 300-301.
30. Ladle, R. J., Johnstone, R. A. & Judson, O. P. 1993 Coevolutionary dynamics of sex in a metapopulation: escaping the Red Queen. *Proc. R. Soc. B* **253**, 155-160.
31. Judson, O.P. 1995 Preserving genes: a model of the maintenance of genetic variation in a metapopulation under frequency-dependent selection. *Genet. Res. Camb*. **65**, 175-191.
32. Judson, O. P. 1997 A model of asexuality and clonal diversity: cloning the Red Queen. *J. Theor. Biol*. **186**, 33-40.
33. Sasaki, A., Hamilton, W. D. & Ubeda, F. 2002 Clone mixtures and a pacemaker: new facets of Red-Queen theory and ecology. *Proc. R. Soc. B* **269**, 761-772.
34. Gandon, S. & Otto, S. P. 2007 The evolution of sex and recombination in response to abiotic or coevolutionary fluctuations in epistasis. *Genetics* **175**, 1835-1853.
35. Wilson, C. G. & Sherman, P. W. 2010 Anciently asexual bdelloid rotifers escape lethal fungal parasites by drying up and blowing away. *Science* **327**, 574-576.

36. Donner, J. 1965 Ordnung Bdelloidea. *Bestimmungsbücher zur Bodenfauna Europas* **6**, 1-297.
37. Alpert, P. 2006 Constraints of tolerance: why are desiccation-tolerant organisms so small or so rare. *J. Exper. Biol*. **209**, 1575-1584.
38. Tunnacliffe, A. & Lapinski, J. 2003 Resurrecting Van Leeuwenhoek's rotifers: a reappraisal of the role of disaccharides in anhydrobiosis. *Phil. Trans. R. Soc. Lond. B* **358**, 1755-1771.
39. Ricci, C., Caprioli, M., Fontaneto, D. & Melone, G. 2008 Volume and morphology changes of a bdelloid rotifer species (*Macrotrachela quadricornifera*) during anhydrobiosis. *J. Morphol*. **269**, 233-239.
40. Ricci, C. & Caprioli, M. 2005 Anhydrobiosis in bdelloid species, populations and individuals. *Integr. Comp. Biol*. **45**, 759-763.
41. Ellner, S. & Hairston, N. G. 1994 Role of overlapping generations in maintaining genetic variation in a fluctuating environment. *Am. Nat*. **143**, 403-417.
42. Jenkins, D. G. & Underwood, M. O. 1998 Zooplankton may not disperse readily in wind, rain, or waterfowl. *Hydrobiologia* **387/388**, 15-21.
43. Ricci, C. N. 1987 Ecology of bdelloids: how to be successful. *Hydrobiologia* **147**, 117-127.
44. Fontaneto, D., Barraclough, T. G., Chen, K., Ricci, C. & Herniou, E. A. 2008 Molecular evidence for broad-scale distributions in bdelloid rotifers: everything is not everywhere but most things are very widespread. *Mol. Ecol*. **17**, 3136-3146.
45. Trevors, J.T. 1996 Sterilization and inhibition of microbial activity in soil. *J. Microbiol. Methods* **26**, 53-59.
46. Barron, G. L. 1985 Fungal parasites of bdelloid rotifers: *Diheterospora*. *Can. J. Bot*. **63**, 211-222.

47. Ghadiri, H. & Payne, D. 1988 The formation and characteristics of splash following raindrop impact on soil. *J. Soil. Sci*. **39**, 563-575.
48. Ricci, C. & Melone, G. 2000 Key to the identification of the genera of bdelloid rotifers. *Hydrobiologia* **418**, 73-80.
49. Ricci, C. & Melone, G. 1998 The Philodinavidae (Rotifera Bdelloidea): a special family. *Hydrobiologia* **385**, 77-85.
50. Barron, G. L. & Tzean, S. S. 1981 A subcuticular endoparasite impaling bdelloid rotifers using three-pronged spores. *Can. J. Bot*. **59**, 1207-1212.
51. Ricci, C. 1998 Anhydrobiotic capabilities of bdelloid rotifers. *Hydrobiologia* **387/388**, 321-326.
52. Glockling, S. L. & Yamada, F. 1997 A survey of fungi which kill microscopic animals in the dung of the amami rabbit. *Mycologist* **11**, 113-120.
53. Sohlenius, B. 1979 A carbon budget for nematodes, rotifers and tardigrades in a Swedish coniferous forest soil. *Holarctic Ecol*. **2**, 330-40.
54. Altiero, T. & Rebecchi 2001 Rearing tardigrades: results and problems. *Zool. Anz*. **240**, 217-221.
55. Suzuki, A. C. 2003 Life history of *Milnesium tardigradum* Doyére (Tardigrada) under a rearing environment. Zool. Sci. 20, 49-57.
56. Pilato, G. & Binda, M. G. 2001 Biogeography and limno-terrestrial tardigrades: are they truly incompatible? *Zool. Anz*. **240**, 511-516.
57. Sohlenius, B. & Boström, S. 2008 Species diversity and random distribution of microfauna in extremely isolated habitable patches on Antarctic nunataks. *Polar Biol*. **31**, 817-825.
58. Cáceres, C. E. & Soluk, D. A. 2002 Blowing in the wind: a field test of overland dispersal and colonization by aquatic invertebrates. *Oecologia* **131**, 402-408.

59. Nkem, J. N., Wall, D. H., Virginia, R. A., Barrett, J. E., Broos, E. J., Porazinska, D. L., Adams, B. J. 2006 Wind dispersal of soil invertebrates in the McMurdo dry valleys, Antarctica. *Polar Biol*. **29**, 346-352.
60. Sohlenius, B. & Boström, S. 2005 The geographic distribution of metazoan microfauna on East Antarctic nunataks. *Polar Biol*. **28**, 439-448.

CHAPTER 4

MALE GENITAL MUTILATION: A CULTURAL ADAPTATION TO SEXUAL CONFLICT[2]

Abstract

Male genital mutilation (MGM) takes several forms and occurs in about 25% of societies. This behavior has puzzled anthropologists, doctors and theologians for centuries, and presents an evolutionary challenge since it involves dangerous and costly surgery. I suggest that MGM is likely to reduce insemination efficiency, reducing a man's capacity for extra-pair fertilizations by impairing sperm competition. MGM may therefore represent a hard-to-fake signal of a man's reduced ability to challenge the paternity of older men who are already married. Men who display this signal of sexual obedience may gain social benefits if married men are selected to offer social trust and investment preferentially to peers who are less threatening to their paternity. Clitoridectomy and vaginal infibulation serve a parallel signaling function in women, increasing a husband's paternity certainty and garnering his increased investment. Especially in societies where paternity uncertainty and reproductive conflict are high, the social benefits of MGM as a signal may outweigh its costs. This 'sexual conflict' hypothesis predicts that MGM should be associated with polygyny, particularly when co-wives reside far apart, and that MGM should reduce the frequency of extramarital sex. MGM rituals should facilitate access to social benefits; they should be highly public, watched mainly by men, and performed by a nonrelative. I found support for these six predictions in two cross-cultural

[2] Reprinted from *Evolution and Human Behavior*, Vol. 29, Wilson, C. G. Male genital mutilation: an adaptation to sexual conflict, pp. 149-164, © 2008 Elsevier Inc. Reprinted by permission of Elsevier Inc.

samples. I also examined an alternative hypothesis suggesting that MGM signals group commitment for collective action, particularly inter-societal warfare. Although other forms of male scarification fit this model, the distribution of MGM is not predicted by frequency of inter-societal warfare.

Introduction

Male genital mutilation (MGM) is any permanent modification of the external genitalia that involves the ablation of tissue and is normative for all males within a society (Murdock, 1967). MGM is present in a substantial minority of pre-industrial human societies and predates recorded history (Dunsmuir and Gordon, 1999). The form of the prescribed mutilation varies among societies. The least extreme is *superincision*: a longitudinal bisection of the dorsal foreskin. Superincision occurs in south-east Asia and the insular Pacific (e.g. Shapiro, 1930). The most widespread mutilation is *circumcision*: the ablation of the entire foreskin. Circumcision occurs in societies throughout Africa; Australia; the Middle East and the Insular Pacific (e.g. Beckett, 1967; Kennedy, 1970; Dunsmuir and Gordon, 1999). A more extensive operation is *subincision,* which creates a longitudinal slit exposing the ventral urethra, and is practiced in several Australian societies (Ashley-Montagu, 1937). The most extreme mutilation is *testicular ablation*: extirpation or crushing of one testis. This mutilation is documented historically in the Sidama, Beja and Khoisan cultures of Africa (Lagercrantz, 1938; al Adawi, 1954; Raven-Hart, 1967), and the Ponapeans of Micronesia (Finsch, 1880). These diverse mutilations seem to represent a behavioral syndrome rather than miscellaneous curiosities: they overlap in geographic distribution, and often share other features including a highly public rite; observance primarily at adolescence (Schlegel & Barry, 1979); the presence of sanctions against the unmutilated; and social benefits contingent on mutilation. Some societies

explicitly equate different forms of MGM. According to Guma (1965), the Sotho of southern Africa view testicular ablation as the original 'method' of MGM, and circumcision is held to be a recent adoption from other societies. The Sotho consider ancestral testicular ablation and the more recent practice of circumcision as variants of the *'lebollo'* ritual, whose declared purpose by either 'method' is to make the boy "strong, fearless, valorous and respectful" (p.241). Shapiro (1930) describes an interchangeable mosaic of superincision and circumcision in certain Polynesian societies, and native Australians who subincise perform circumcision as a prerequisite (Ashley-Montagu, 1937). The interlinked forms of MGM are well documented, but their function remains unresolved despite discourse in several disciplines. Here, I develop an evolutionary hypothesis suggesting a common function for the various mutilations, and test it using comparative ethnographic data.

An evolutionary approach to MGM may complement efforts in other fields. Medical doctors have given considerable attention to circumcision, debating the ethics and effects of performing this mutilation neonatally, but have tended to overlook the other forms of MGM (e.g. Hutson, 2004; Short, 2004). Several doctors have suggested that circumcision arose to improve hygiene by removing skin in which dirt or sand could accumulate (e.g. Winberg et al., 1999; Hutson, 2004). Darby (2005) offered the most recent challenge to this 'hygiene hypothesis', but his conclusion that "health had nothing to do with it" had been noted at least seventy years earlier by anthropologists such as Bryk (1934), who observed that imagined health complications of sand or dirt under the foreskin could hardly match the often fatal risks of hemorrhage and sepsis that arise when boys are universally mutilated under non-sterile conditions by individuals with little or no training, using crude tools or even fingernails. Forty-five men arrived at an African hospital with sepsis following ritual circumcision in December 1988 alone, resulting in a 9% mortality rate (Crowley

& Kesner, 1990). Between 1995 and 2004, 243 deaths and 216 genital amputations occurred at traditional 'circumcision schools' in a single province of South Africa (Sidley, 2006). Several societies have specific customs governing death during initiation rites, suggesting this has not been historically uncommon (e.g. Guma, 1965). The hygiene hypothesis also fails to explain why circumcision is limited to a minority of societies despite the universality of dirt and sand, and it is unclear why a supposedly protective mutilation is almost always delayed until adolescence. Finally, evolutionary theory does not predict fitness benefits from extirpating normal tissue. Genital anatomy is extremely variable, and if the mammalian prepuce were detrimental to overall fitness, selection would presumably have reduced it over evolutionary time. If sand or dirt represented a selective pressure to expose the glans, we would surely observe this outcome frequently in desert-dwelling mammals, yet we do not. As one example, the Arabian camel (*Camelus dromedarius*) retains a "voluminous" prepuce (Mobarak et al., 1972).

Recent medical studies have confirmed a protective effect of circumcision against HIV infection for adult males in high seroprevalence regions of Africa (Auvert et al., 2005; Gray et al., 2007). However, a 'prophylactic' hypothesis is unlikely to represent the adaptive function of MGM behavior, as it shares many weaknesses of 'hygienic' explanations. Circumcision obviously pre-dates HIV itself, and it is unclear whether the prophylaxis generalizes to other STDs. The currently suggested mechanism of protection is relatively specific, involving a reduction in the preputial mucosa with its CD4 and CCR5 HIV cell-surface receptors (Szabo and Short, 2000). If the result did generalize to other sexual infections, it would remain unclear under a prophylactic hypothesis why natural selection should have retained the foreskin despite millennia of selection in populations suffering from STDs. If exposing and keratinizing the glans by reducing sexual mucosa brought overall fitness benefits

through prophylaxis, we would expect selection to have produced this outcome not only in humans, but especially in promiscuous primates with the highest STD loads (Nunn et al., 2000). Contrary to this prediction, promiscuous taxa actually have the most elaborate penises, including a well developed prepuce and other structures with high mucosal surface areas (Dixson, 1987). This suggests that primate STDs may not have been a sufficiently important selective pressure to drive evolutionary or cultural ablation of otherwise adaptive sexual tissue, although prophylaxis may be an incidental effect of reductions undertaken for other reasons. If circumcision functions to reduce STDs, it is also curious that it should be followed by subincision in Australian societies: subincision permanently exposes the internal urethral mucosa and is associated with recurrent penile bleeding (Ashley-Montagu, 1937), which would certainly tend to counteract any prophylactic benefits of circumcision. Testicular ablation is similarly inexplicable as STD prophylaxis.

Anthropologists have also given considerable attention to MGM, with a similar focus on circumcision. Silverman (2004) stated that circumcision "dramatizes unease over separation-individuation through a symbolism that affirms yet blurs the normative boundaries between masculinity and motherhood" (p. 423); Paige and Paige (1981) suggested that it is "a ceremonial solution to the dilemma of fission in strong fraternal interest group societies" (p. 166); Whiting, Kluckhohn and Anthony (1958) suggested that it resolves a gender-identity conflict caused by a boy's underexposure to males and "excessively strong dependence upon the mother" that would otherwise manifest as "open rivalry with his father [and] incestuous approaches to his mother" (p.370). The validity of cross-cultural evidence for this Oedipal interpretation (e.g. Strauss and Orans, 1975) is disputed by Korotayev & de Munck (2003). These psychodynamic hypotheses have value as proximate explanations of the psychology that may drive MGM. However, it is vital to address the selective

pressures that ultimately underpin such psychology itself. At the functional level of analysis, we must seek complementary hypotheses that share the predictions of existing proximate explanations, but whose premises are supported by evolutionary theory in addition to psychodynamic thought.

Hypothesis

The signalling theory of ritual (Rappaport, 1999; Irons, 2001; Sosis, 2004) was developed as an evolutionary explanation for ritual behavior that is physically or financially costly. Irons (2001) noted that the considerable costs incurred by many ritual behaviors may allow them to function as honest signals of commitment to a social group. Only truly committed individuals are prepared to pay the costs, which can be recouped through the increased willingness of group members to trust and cooperate with the signaller. Cheating is prevented because the benefits of trust and cooperation extend only to those who have conformed with the ritual. Sosis and colleagues have provided empirical support for this model (Sosis, 2000; Sosis & Ruffle, 2003; Sosis, Kress & Boster, 2007). I propose a hypothesis that integrates the signalling theory of ritual with principles of sexual selection.

Genital morphology in primates is adapted for sperm competition

Natural selection shapes male genital morphology to increase the probability of fertilization (Eberhard, 1985). Dixson (1987) and Verrell (1992) linked interspecific variation in primate genital morphology to differences in mating system and level of sperm competition. In taxa where females mate monogamously, males have small testes relative to body weight and a short penis with simple morphology. Coitus is brief with few intromissions. In taxa where females are promiscuous, males have large testes relative to body weight; a long penis with elaborations including spines,

plungers, labile scoops, flexible ridges and other distal structures; and multiple or protracted intromissions during copulation. These adaptations increase the probability that a male's sperm will achieve fertilization, and decrease this probability for rival sperm. Sperm competition has also played a role in human evolution, driven by moderate rates of female extra-pair copulations (EPCs) even though the marriage system is either polygynous or monogamous (Goetz & Shackelford, 2006). Sperm competition is in evidence because (1) human testes are relatively large for a monogamous primate, although smaller than in primates with truly promiscuous mating systems (Gomendio et al., 1998); (2) human coitus involves multiple intromissions; and (3) the penis is long and wide relative to stature, compared to other primates (Short, 1979; Smith, 1984). Human penile elaboration consists of a uniquely developed prepuce that is anchored close to the glans, rather than at the proximal base of the penis as in *Gorilla* and *Pongo* (Hill & Matthews, 1948; Dahl, 1994; Taves, 2002). The distal attachment of this 'foreskin' makes it more than a protective cover: the entire structure deeply enters the female during copulation, and in common with the labile distal elaborations of more promiscuous primates, it has dynamic interactions both mechanical and neuronal with the glans, vagina and clitoris during intercourse (Immerman & Mackey, 1997; Cold & Taylor, 1999).

Sperm competition causes paternity uncertainty, suspicion and conflict

The consequence of sperm competition is paternity uncertainty: a source of inter- and intrasexual conflict in human societies. Even populations presumed to be highly genetically monogamous show evidence of paternity uncertainty, as matrilateral kin seem to invest significantly more in offspring than do patrilateral kin (Gaulin et al., 1997; McBurney et al., 2002). To attempt to prevent EPCs and increase paternity certainty, men practice a suite of behaviors including mate-guarding, sexual

coercion, biased parental investment and aggression toward suspected sexual rivals or unfaithful spouses (Goetz & Shackelford, 2006). Daly and Wilson (1988) reviewed cross-cultural evidence indicating that men respond with "extreme violence" to extra-pair activity, including murder of adulterous wives or sexual rivals. Sexual rivalry is typically ranked among the top three motives for homicide, accounting for up to 25% of murders. In some traditional societies, violent revenge against adulterers is not only legally tolerated but mandated by custom, and in at least 15 societies "adulterous conception was offered as grounds for infanticide" (Daly and Wilson 1988: 47). There are clearly strong advantages if a man can reduce uncertainty about the paternity of his own offspring, and ameliorate suspicions of other males that he is mounting challenges to their paternity.

MGM is likely to impair sperm competition

Male genitalia vary extensively among closely related primate taxa, and represent a critical target of sexual selection for fertilization efficiency and sperm competition (Eberhard, 1985). It is improbable that ablating substantial amounts of this mechanically, neurally and endocrinologically specialized sexual tissue can be neutral with respect to its evolved function. MGM is likely to impair the capacity for sperm competition and fertilization. *Testicular ablation* has the most obvious effect, since loss of a testicle causes a significant reduction in sperm count (Woodhead et al., 1973). *Subincision* results in the emission of semen at the base of the penis instead of the glans, with greatly reduced pressure. Although ejaculate still enters the vagina (Ashley-Montagu, 1937), it will fall short of the cervix, reducing the probability of conception as well as the potential to dilute or displace viable prior sperm, which are stored higher in the reproductive tract (Suarez & Pacey, 2006). *Circumcision* permanently denudes the glans by ablating between 20% and 51% of the penile skin

and mucosa, including the highly innervated and vascularized 'ridged band' and often the frenulum (Taylor et al., 1996). From a cross-taxonomic perspective, monogamous primates with low capacities for sperm competition typically possess a naked glans with no labile distal accessory structures, whereas most species with high capacities for sperm competition possess well-developed distal structures which, like the foreskin, interact mechanically with the glans and vagina during copulation (Dixson, 1987: his Fig. 2-4). Some promiscuous primates have such highly developed accessory distal structures that the glans itself is barely distinguishable except during the deepest penetration (Ib. Fig. 2). Ablating the uniquely distal foreskin of the human penis destroys its labile mechanics and increases its morphological and mechanical resemblance to the simple penis of monandrous primates. I suggest that a circumcised man's efficacy at sperm competition is correspondingly reduced toward the lower level seen in such monandrous primate taxa. The same argument applies to a lesser extent for the related procedure of *superincision*.

Although the effect of circumcision on self-reported sexual function in industrial societies has frequently been investigated, no consensus has been reached, perhaps due to limitations of this methodology (O'Hara & O'Hara, 1999; Fink et al., 2002; Senkul et al., 2004; Richters et al. 2006; Sorrells et al. 2007). In any case, self-report cannot reliably assess an impact on sperm competition, since this phenomenon is subtle and inaccessible to the reporting individual. Miscellaneous findings can be used to suggest potential mechanisms by which circumcision may impact competition for fertilizations. These include increased effort required to overcome friction during intromission (Taves, 2002); desensitization of the penis, raising the threshold for sexual arousal (Immerman & Mackey, 1997; Sorrells et al., 2007); increased latency to ejaculation (Senkul et al., 2004), which would especially hinder hasty covert copulations; decreased stimulation of the female, potentially impacting cryptic choice

(O'Hara and O'Hara, 1999); an 8 mm decrease in mean length of the erect penis caused by lack of sufficient residual skin (Richters et al. 1995); elimination of volatile sex pheromones secreted by the preputial mucosa (Immerman & Mackey 1997); and disruption of neural feedback involved in adaptive modulation of copulation dynamics (Cold & Taylor, 1999). These mechanisms are wholly speculative, based on limited observations. The primary effect could be due to any one of them, or the interaction of several. However, isolation of mechanism is not necessary for the further development of this hypothesis, as comparative evidence from the morphology of other primates is sufficient to suggest an association of circumcision with lower sperm competition. The idea that substantial amounts of male sexual tissue can be ablated without effect is hard to reconcile with our understanding of selection for genitalia, and any such argument must bear the burden of proof. Gallup and Burch (2004) suggest that circumcision improves "semen displacement" by the coronal ridge. This idea is not derived from biological observation, but from experiments with a plastic vagina and latex phalluses, commercially manufactured as sex toys. It must be doubted for several reasons: (1) circumcision has no effect on the height of the coronal ridge, which is fully exposed during deep penetrations in any case; (2) the vagina is a highly hostile acidic environment: viable prior spermatozoa can survive only in the cervical mucus, uterus and Fallopian tubes, well beyond the reach of the coronal ridge (Suarez & Pacey, 2006); (3) the coronal ridge actually is more pronounced in monandrous primates; promiscuous species favor a weakly differentiated glans with labile accessory structures (Dixson, 1987).

MGM may be a costly signal of reduced ability to challenge paternity

If, as I have argued, MGM impairs a man's sperm competition ability and effectiveness in mounting paternity challenges, it could represent a hard-to-fake signal

of his trustworthiness (Irons, 2001; Sosis, 2004). MGM would be an especially reliable signal with regard to sexual conduct, because once the mutilation is complete, cuckoldry will necessarily be less successful even if a man does try to copulate with married women. MGM could therefore function as a physically mediated signal of compliance with the social assignment of reproduction. Married men would be selectively favored to trust and preferentially invest in a young male who undergoes MGM, because he poses a decreased threat to their paternity. It is important to note that no knowledge of reproductive biology is required on the part of the males: the hypothesis posits that selection will favor men who demand and reward genital mutilations when paternity uncertainty is high, though they will lack psychological access to the ultimate reason. Older men are certainly well placed to offer access to resources, status and socially sanctioned reproductive opportunities in exchange for a signal of sexual compliance. Once young men gain wives of their own, they will be favored to protect their paternity in turn by offering similar benefits to the next generation contingent upon the same costly signal. In this system, it would be adaptive for all men to regard unmutilated males with suspicion and mistrust, as they represent a greater continuing threat to paternity. The decreased suspicion and increased cooperation experienced by a mutilated individual may be sufficient to outweigh the cost of MGM itself, particularly in societies where the risks of cuckoldry, and therefore the levels of reproductive conflict and suspicion, are high. In addition to these individual-level benefits, societies and groups suffer significant internal conflict and violence arising from sexual jealousy and paternity uncertainty. Behavior that limits the success and frequency of EPCs is likely to reduce this internal conflict, and may spread as a group-beneficial norm (Boyd & Richerson, 2002).

Since MGM is universal and uniform within societies, one might initially imagine that the competitive ability of every male is reduced to the same extent. At

first glance, it seems that the fertilization impairment is not specific to extra-pair activity, but simply raises the 'playing field' for all men. However, closer examination reveals that MGM disproportionately reduces EPCs. This follows from the observation that there is a higher marginal cost for additional EPCs than additional within-pair copulations. Thus, if MGM increases the ratio of copulations to fertilizations, married men can readily compensate for the fertility impairment by increasing their copulation frequency. This option is much costlier for would-be-cuckolders because additional EPCs are more risky than additional within-pair copulations, so the payoff to EPCs is disproportionately lowered. It is also possible that MGM primarily affects sperm *competition* rather than overall fertility. This scenario also entails disproportionately large costs for cuckolders, as they depend heavily on physical adaptations for sperm competition to compensate for socially restricted sexual access to married women. By contrast, a married man can copulate regularly and simply flood a wife with his sperm, rendering more subtle physical adaptations for sperm competition relatively less important. In either scenario, MGM decreases the relative payoff of risky EPC tactics, with little impact on the fitness of married males who have unrestricted sexual access to their wives. Following this cost-benefit analysis, MGM is expected to decrease the frequency of EPCs for two reasons. First, individual men would be favored to reduce their use of cuckoldry tactics if the benefits are curtailed by genital mutilation while the risks remain unchanged. Such adaptive modulation could be mediated by several potential cues, and does not require conscious reasoning about reproductive morphology. Second, even if individuals show no adaptive modulation, cuckolds in societies practicing MGM will contribute fewer offspring to the next generation due to the physically reduced efficacy of EPCs in achieving fertilizations. Over time, this will reduce the representation of genes predisposing males to EPC-seeking behavior.

Under this 'sexual conflict' hypothesis, MGM functions in a parallel context to female genital mutilation (FGM). Women who undergo vaginal infibulation or clitoridectomy have a reduced capacity to experience sexual pleasure, which may reduce the frequency of EPCs. This reduces the paternity uncertainty of a husband, increasing the trust and investment he is selected to offer. These benefits to a woman and her children seem to outweigh the heavy cost of the mutilation itself in societies with high paternity uncertainty (Mackie, 1996; Reason, 2004). In sum, conflict over paternity may favor men who invest preferentially in spouses with FGM, and cooperate preferentially with peers who submit to MGM. This hypothesis frames MGM and FGM jointly as costly, hard-to-fake signals of sexual compliance that counteract paternity uncertainty, reducing reproductive conflict and increasing social trust and investment in the mutilated individual.

Predictions

Sexual conflict hypothesis

Suspicion and conflict caused by paternity uncertainty are likely to be higher in polygynous societies than monogamous societies. This is because some men have many wives, but others have either one wife or no wife. In particular, young men may experience considerable delay in finding a wife, due to sexual monopoly by older men (Ember, 1984). Copulating with married women may sometimes be the only reproductive option available, and successful polygamists will find it difficult to guard all their wives against such cuckoldry. Therefore, the trust gained by signalling sexual compliance should be greater in more polygynous societies, and such societies should be more likely to practice MGM. The distribution of FGM should follow the same pattern. Whiting, Kluckhohn and Anthony (1958) and Strauss and Orans (1975) found associations between polygyny and MGM while testing the proximate oedipal gender-

identity hypothesis for MGM. This result is encouraging, but the authors were only able to consider polygyny as 'present' or 'absent', and they did not use one of the standard cultural samples. Their conclusions have recently been criticised by Korotayev & de Munck (2003). A well-resolved codification of incremental polygyny frequency is now available (White, 1988); it is thus appropriate to revisit this equivocal relationship in more detail using an ordinal measure of polygyny in a standard cultural sample. This not only offers a test of the novel sexual conflict hypothesis, but also a potential rebuttal to recent criticism of a complementary proximate hypothesis.

In some polygynous societies, co-wives occupy one dwelling. This makes it relatively easy for their husband to guard them against extra-pair activity, and they can also police one another. In other polygynous societies, co-wives live in separate dwellings and can neither be guarded nor watch each other so easily. Polygynous societies in which co-wives live further apart may thus be more prone to EPCs, paternity uncertainty and reproductive conflict, and they should be especially likely to practice MGM and FGM.

Counterintuitively, the sexual conflict hypothesis does not predict that societies practicing MGM will have fewer EPCs on average than societies in which MGM is not practiced. Although MGM is indeed predicted to *reduce* the frequency of EPCs by reducing their payoff and desirability, it is also predicted to arise disproportionately in societies with an inherently *high underlying expectation* of EPCs due to frequent polygyny and distantly residing co-wives. It is likely that these two opposed predictions will be mutually negating, resulting in no overall relationship between MGM and EPCs across cultures. However, if the underlying risk of EPCs can be held constant by considering societies with comparable polygyny and marital

residence patterns, the hypothesis does indeed predict that MGM should be associated with reduced extramarital activity.

Finally, under a costly signalling hypothesis it would benefit a man to gain the increased trust and status associated with MGM without actually having to be mutilated. He could 'cheat' either by falsely claiming to have undergone the mutilation, or by securing a less severe mutilation for himself than for his peers. Other men would be unlikely to discover deceit subsequently, since the genitals are almost always concealed by clothing. One way to police such a system is to conduct the mutilation publicly, ensuring that the costly signal is directly observed. The sexual conflict hypothesis predicts that public onlookers should be predominantly male, since the costly signal is directed at other men. To reduce the chance of collusion in arranging biased or selective mutilations, the MGM operator should be impartial and unrelated to the initiate. Unmutilated males should be subject to suspicion, sanctions and restrictions until the operation is complete, at which point they should gain social trust and status. By contrast, FGM cannot easily be cheated, because the signal is aimed at the woman's social mate, who will always be intimately aware of her true mutilation status (Paige & Paige, 1981). FGM is thus expected to require less publicity and less social enforcement than MGM, and I use it as a control, specifically predicting greater publicity for male than for female genital mutilation. Hypotheses that propose direct individual benefits to MGM (e.g. health benefits) might not predict extensive social oversight and sanctions, because a non-conformist would primarily be harming his own health rather than breaking a social contract. There would be no need for the community to observe and police the procedure, no need for additional social incentives, no reason to waste effort punishing the unmutilated, and especially no social enforcement of kinship impartiality on the surgeon's part, because kin would have no incentive to 'cheat' by performing a more lenient mutilation.

In summary, the sexual conflict hypothesis makes six predictions:

1. More polygynous societies should be more likely to practice MGM than more monogamous societies.
2. MGM should increase with increasing distance that co-wives reside apart.
3. MGM should be associated with reduced extramarital sex, if we compare societies with the same underlying level of EPC risk due to frequency of polygyny and co-wife residential distance.
4. MGM should be performed more publicly than FGM, and public MGM audiences should be more frequently male-biased than public FGM audiences are female-biased.
5. Unmutilated men should be subject to social and sexual sanctions and mistrust, whereas mutilated men should gain increased trust, including social and sexual privileges.
6. The operator should be unrelated to the initiate.

'Scars for war' hypothesis

Recently, Sosis, Kress and Boster (2007) proposed and tested the hypothesis that costly male rituals signal increased cooperation and solidarity among men who must organize for warfare. They reported a cross-cultural association between the costliness of male ritual in a society and the overall frequency of warfare. MGM, scarification and piercing were classified as 'rituals that leave permanent markers', and the authors noted their particular association with inter-societal (*external*) warfare. Sosis et al. suggested that the high cost and visibility of these 'scars for war' might be especially effective at preventing males from defecting to another group when inter-societal conflict is high. However, the association they found with external warfare

was not as strong for MGM as for piercing and scarification. Moreover, MGM may not be a very good identifier because the permanent mark is neither specific to one group within a locality, nor obviously displayed. Nevertheless, in addition to testing the predictions of the sexual conflict hypothesis, I considered the alternative hypothesis that MGM may provide a costly signal of cooperation and identification for inter-group warfare, as do scarification and piercing. This hypothesis predicts that MGM should be more likely in societies with a higher frequency of external warfare.

Methods

To test Predictions 1-4, I used the Standard Cross-Cultural Sample (SCCS) of Murdock and White (1969): a global sample of 186 pre-industrial societies selected for cultural independence. MGM codes were originally published by Murdock (1967) for societies in his Ethnographic Atlas, and I obtained them for the SCCS from the *World Cultures* electronic journal (Divale, 2007), as SCCS Variable 241: "Male genital mutilations". Three societies (Abkhaz, Ajie, Bogo) had no data on MGM and were excluded from all analyses. Although MGM is coded absent for the Kwoma and Mbundu in the *World Cultures* database, Murdock (1967) noted for the Kwoma that "slashing of the boy's penis" occurs at 11-15 years of age, and Mbundu circumcision is well described in multiple cultural reports (e.g. Hambly, 1934). I therefore followed Schlegel and Barry (1979) in considering MGM present in both societies. In total, MGM was considered present in 49 societies (27%) and absent in 134. For female genital mutilation, Schlegel & Barry (1979) and Ericksen (1989) provided surveys of SCCS societies. Unless FGM was coded as present by one of these authors (15 societies), I conservatively coded it as 'no data/absent' (171 societies). Two ordinal polygyny codes for the SCCS were taken from White (1988): SCCS Variables 860: "Frequency of polygny" and 863: "Distance between co-wives". For the latter, I

collapsed codes 5 and 6 (only two societies are coded as 6), and codes 3 and 4. To test Prediction 3, I required a single measure of underlying EPC risk due to marital conditions, in order to control for these factors. I therefore combined "Frequency of polygyny" (4 point scale) with "Distance between co-wives" (5 point scale) by taking the mean of the two. I operationalized this mean by coding it back into three ordinal categories: (0 - 1.5 = 1; 1.51 - 3.0 = 2; 3.1 - 4.5 = 3). I called this new 3-point measure the Polygyny Risk Factor (PRF). Extramarital activity has been coded for the SCCS by Broude and Green (1976) as SCCS Variables 170: "Frequency of Extramarital Sex- Male" and 171: "Frequency of Extramarital Sex- Female". I inverted their counterintuitive scale so that a higher value now indicates higher frequency of EPCs, and then took the mean of the male and female codes, calling this continuous variable "Extramarital sex frequency (SCCS)". I was then able to construct an interaction plot of extramarital sex frequency (SCCS) versus MGM, grouped by polygyny risk factor in order to control for underlying EPC risk. This permitted a test of Prediction 3 at three constant levels of PRF.

Schlegel & Barry (1979) coded MGM and FGM rituals in SCCS societies for publicity and gender of participants, providing sufficient data to test Prediction 4 across 27 societies. I used SCCS Variables 537/538: "Number of participants", combining codes 3 and 4 ("Local group"; "Large group"); and SCCS Variable 539/540: "Sex of participants." Predictions 5 and 6 involve variables that are usually beyond the resolution of available ethnographic reports, and would be impossible to code in a satisfactory manner for the SCCS. Instead, I searched materials in the Human Relations Area Files (eHRAF) for evidence of rights and privileges associated with MGM; social sanctions against the unmutilated; and any rules regarding the involvement of relatives in MGM rituals. I considered only eHRAF materials from societies in the Probability Sample File (PSF): a commonly used cross-cultural sample

of 60 societies (Ember and Ember, 1998), of which 17 practice MGM. I located textual evidence from these societies and either quoted directly or paraphrased from eHRAF documents. Considerable parsing effort was expended, a broad selection of societies was represented, and it is unlikely that a significant source of potentially contradictory data was overlooked.

I also tested the hypothesis of Sosis, Kress and Boster (2007), who reported an association between costly male rites and warfare in the PSF. I used the larger SCCS to re-examine the relationship between frequency of warfare and MGM, attempting to replicate their coding schema as exactly as possible. They rated warfare following Ember and Ember (1992), so I took my SCCS warfare codes from that publication (SCCS Variable 1650: "Frequency of external warfare"). I also examined the effect of external warfare on male scarification in the SCCS as a control to ensure that the SCCS and PSF produce consistent results. If MGM were indeed a 'scar for war', then both scarification and MGM would be expected to increase with frequency of external warfare in the SCCS. If neither scarification nor MGM increased with frequency of external warfare, then the decision to use the SCCS might be suspected of obscuring the 'scars for war' effect previously identified in the PSF. However, if scarification were to increase with frequency of external warfare whereas MGM did not, then sample choice could not be blamed and MGM might not be a 'scar for war' after all. Scarification has been coded for the SCCS by Ludvico and Kurland (1995) as Variable 1692: "Scarification: Males" (Divale, 2007). I scored scarification as either present or absent by combining categories 2 and 3 ("tattooing and cicatrisation"; "scarification includes removal of skin").

In addition to replicating the methodology of Sosis et al. to test the warfare hypothesis in the SCCS, I tested the sexual conflict hypothesis in their chosen sample, the PSF, to check that my results were robust across the slightly different samples. To

ensure that I changed only the sample and not the coding scheme, I limited my analyses to societies that the PSF shares with the SCCS. Fortunately, 36 of the 60 PSF groups are SCCS societies or their synonyms. For each of the remaining 24 societies in the PSF, I searched the Ethnographic Atlas (Murdock, 1967) to see whether a closely related society existed in the SCCS that could serve as their representative. I found that eight PSF societies had an SCCS society in the same culture cluster, which I used in their place (Blackfoot: Gros Ventre; Highland Scots: Irish; Hopi: Zuni; Iroquois: Huron; Lau Fijians: Mbau Fijians; Lybian Bedouins: Rwala Bedouins; Shluh: Riffians; Tlingit: Haida). The remaining 16 PSF societies were excluded because they are neither present in the SCCS, nor does any SCCS society occur in their culture cluster. I refer to the matched 44 societies as the reduced PSF (rPSF). I validated this sample by replicating the main result of Sosis et al. with respect to external warfare and male scarification, and then examined the explanatory power of polygyny, co-residence and external warfare with respect to MGM, just as I had for the SCCS. Finally, I was able to test the robustness of Prediction 3 in the full PSF (rather than the rPSF), because Huber, Linhartova & Cope (2004) recently coded these societies for frequency of extramarital sex. Just as described above for the SCCS, I constructed an operational polygyny risk factor (PRF) for PSF societies by taking the mean of polygyny level and distance between co-wives and recoding it to a 3-point scale. I then produced an interaction plot of Huber et al's extramarital sex frequency versus MGM, grouped by PRF in order to control for underlying EPC risk.

Statistical analyses were performed in MINITAB® v.14. Most data were categorical but ordinal, so Spearman's *rho* was appropriate. Warfare frequency was treated as a continuous variable and analysed using the Mann-Whitney-Wilcoxon statistic. The SCCS and PSF are each carefully designed to maximize cultural independence and limit internal pseudoreplication. Full data tables listing all relevant

coded variables for SCCS, PSF and rPSF societies are provided as supplementary material online.

Results

Polygyny, co-wife residence and genital mutilation

For 183 SCCS societies, frequency of polygyny significantly predicts incidence of MGM (Fig. 4.1a; Spearman's *rho* = 0.297, n=183, $p < 0.001$). Each stepwise increase in frequency of polygyny is matched by a rise in MGM. Societies with higher levels of polygyny are also much more likely to practice female genital mutilation (Spearman's *rho* = 0.188, n=184, p = 0.011; data not shown), although this is much rarer overall. When all SCCS societies are considered together, MGM and FGM have an overall incidence of 27% and 8% respectively. However, in that subset of societies where polygyny is prevalent and preferred by most men of sufficient age or wealth, MGM and FGM have incidences of 48%, and 20% respectively. Figure 4.1b shows that increasing distance between co-wives is also associated with a rise in MGM (Spearman's *rho* = 0.388, n=156, $p < 0.001$). MGM is present in 48% of all highly polygynous societies, but in that subset where every wife has separate housing, the incidence increases to 63%. FGM occurs in only 15 societies, and in 12 of these (77%), every wife has a separate residence. Separate housing for co-wives may be the strongest predictor of MGM and FGM. The relationship between polygyny and MGM was also evident for societies of the rPSF (Fig. 4.2a: Spearman's *rho* = 0.375, n=44, $p < 0.012$), as was the effect of distance between co-wives (Fig. 4.2b: Spearman's *rho* = 0.308, n=42, p = 0.047). Predictions 1 and 2 are supported in both samples.

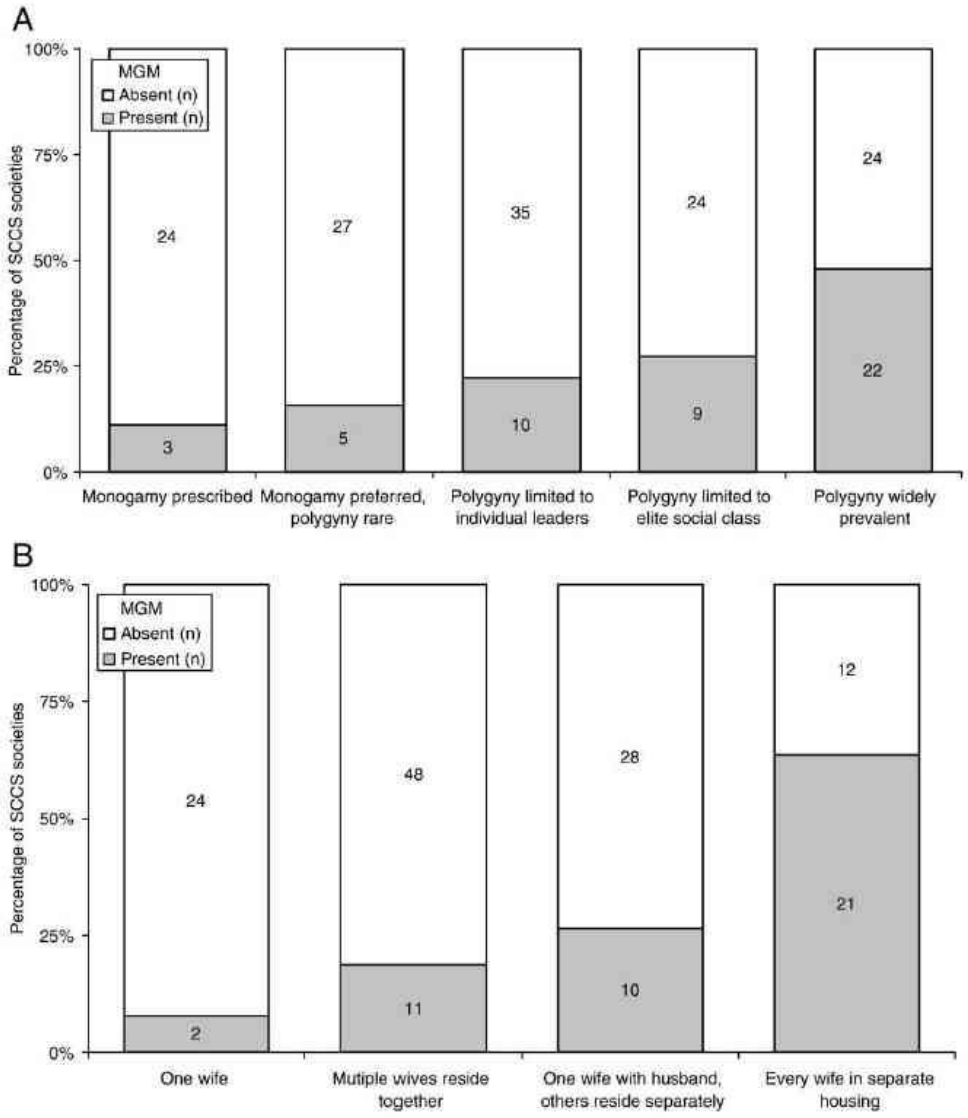


Figure 4.1 (**A**) Incidence of MGM in SCCS societies is predicted by frequency of polygyny (Spearman's rho=0.297, n=183, *P*<.001). (**B**) Incidence of MGM in SCCS societies increases with greater residential distance between co-wives (Spearman's rho=0.388, n=156, *P*<.001).

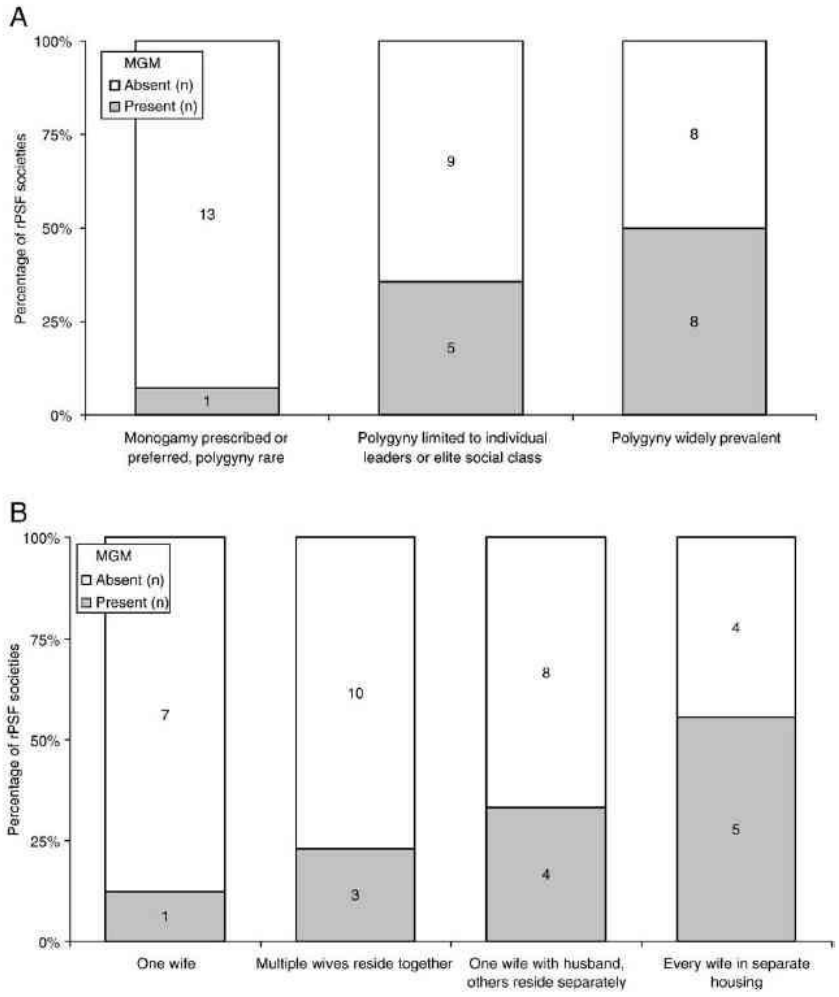


Figure 4.2 (**A**) Incidence of MGM in rPSF societies is predicted by frequency of polygyny (Spearman's rho=0.375, n=44, *P*<.012). (**B**) Incidence of MGM in rPSF societies increases with greater residential distance between co-wives (Spearman's rho=0.308, n=42, *P*=.047).

Extramarital sex frequency versus MGM by polygyny risk factor (PRF)

Data were available for 60 SCCS societies (Fig. 4.3a) and 43 PSF societies (Fig. 4.3b). Extramarital sex increases in frequency significantly with increasing polygyny risk factor [Fig. 4.3, right panel: ordinal logistic regression coefficient (+/- SE) for SCCS = 0.83 (0.39), p= 0.033; for PSF = 0.85 (0.38), p=0.023], supporting the assertion that polygyny frequency and co-residence are reasonable proxies for cuckoldry risk. However, for societies with comparable levels of PRF (Fig. 4.3, left

panel), MGM seems to be associated with a *reduction* in extramarital sex. It would be inappropriate to add MGM to the ordinal logistic regression model because the small sample sizes of MGM cells would violate its asymptotic assumptions, and collapsing categories does not help (Murad et al. 2003). Therefore, I have not attempted to offer a statistical interpretation of the effect of MGM, and this result is qualitative. However, it does hold at all three levels of PRF, and is stable in both the SCCS and the PSF samples, so it may be considered reasonable evidence consistent with Prediction 3. More extensive codification of extramarital sex in SCCS societies (68% remain uncoded) would be necessary to test this finding more rigorously.

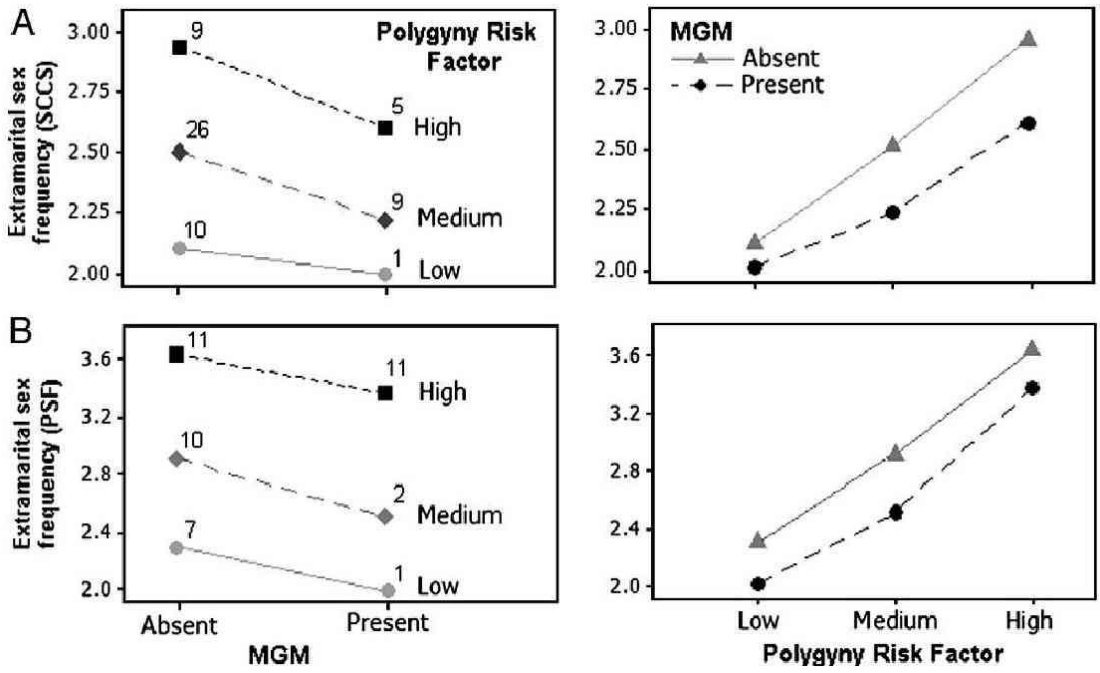


Figure 4.3 Polygyny risk factor is a three-level measure of cuckoldry risk that takes both frequency of polygyny and distance between co-wives into account. Increasing PRF is associated with increasing frequency of extramarital sex in both the SCCS [ordinal logistic regression coefficient=0.83 (S.E.=0.39); p=.033] and PSF [regression coefficient=0.85 (S.E.=0.38), p=.023]. At all three levels of PRF, mean frequency of extramarital sex appears to be lower in societies where MGM is present. This relationship holds for societies of the SCCS (A: n=60) and the PSF (B: n=42). The number of societies composing each data point is indicated on the left panel.

Publicity, social benefits and kinship

For 19 societies with available data, MGM always is performed in public. It does not seem to be the case that all genital mutilations simply attract public interest, because FGM is not public in three of the seven practicing societies for which there are data (43%), which is a significant departure from the male result (Fisher's exact test, $n = 26$, $p = 0.013$). Public MGM onlookers are exclusively male in 9 of 19 societies (47%) for which appropriate data are available. By contrast, public onlookers to FGM are exclusively female in one of four societies (25%). In the remaining cases of MGM and FGM, the onlookers are predominantly, but not exclusively of the same sex as the initiate. These results suggest that it is important for many males to publicly observe MGM, but this does not seem to apply so strongly for females observing FGM. Prediction 4 is supported.

A sufficiently detailed account of MGM customs was available to examine Prediction 5 for 14 of the 17 PSF societies practicing MGM, and Table 4.1 summarizes the relevant eHRAF materials. In 13 of 14 societies (93%), MGM brings immediate social benefits that may include respect, status, and access to weapons, shelter or tribal lore. In the remaining society, MGM is performed at birth. In nine of 12 societies (75%), MGM is required before marriage is permitted. In ten of 14 societies (71%), any male remaining intact is treated with derision or suspicion, placing social pressure on him to submit to MGM. In the remaining four societies, either no males remain unmutilated, or there are no data concerning their treatment. Prediction 5 is supported in the societies examined.

Table 4.1 Evidence from eHRAF materials on societies practicing MGM suggests that (1) MGM is often a prerequisite for marriage; (2) MGM brings social status and resources; and (3) the unmutilated may suffer social and sexual sanctions.

PSF Society	MGM required for access to marriage or sexual relations	MGM increases social status, resources or influence	Unmutilated men attract suspicion, punishment or derision
Amhara	No specific data (occurs within days of birth)	No specific data (occurs within days of birth)	Uncircumcised men from other areas called "insulting names"; seen as "almost on a level" with slaves
Aranda	"One of the most important ceremonies which must be passed through before any youth is allowed a wife"	Required for access to tribal secrets and full membership, and to be "worthy of a man's estate"	No specific data
Azande	After MGM, male is "recognized as a person old enough to have sex"	"Marks an increase in status…brings about new sets of social relationships"	"Public opinion…makes it to all intents and purposes obligatory"; 'uncircumcised' is a "scornful epithet"
Dogon	Required for marriage and necessary even to learn the name of a future wife	Makes one "a member of the society of men" with access to the exclusive men's shelter	In a ritual game, men "rush upon the uncircumcised whom they rightly or wrongly suspect of having stolen the fruits of the sa tree"
Eastern Toraja	Not required, but girls "show an aversion for an uncircumcised boy"	Recognized as "brave"; superincision allows "the true spirit" to come over him	Taunted as "weakling, coward"; teased. This leads to "fighting" until he finally submits to the operation
Hausa	Prerequisite "for marriage or any sexual activity"	Boy "receives a hut and field of his own"…MGM "marks his entry into the community"	"An uncircumcised boy who dared court a girl would be laughed at"
Kanuri	Required for betrothal to a young girl; boys may be given a concubine after MGM	Boy is "socially regarded as attractive in a male sense"… "A mark of approaching manhood"	No specific data
Lau Fijians	No specific data	Boy receives a "bachelor's house" and is "called a man"	No specific data
Masai	Required for marriage; required for sexual relations	Required to be regarded as an adult, to carry weapons and to join a male age-set group	Uncircumcised boys were "severely punished by the warriors if they had sexual intercourse"
Mbuti	MGM is an acquisition from Bantu villagers; some uncircumcised Mbuti do marry within their own group	MGM initiates youth into the society of archers or net-hunters; required for social acceptance by Bantu villagers	Following Bantu villager custom, "any uncircumcised youth is a laughing stock"… may even be considered a "source of evil"
Somali	Required for marriage	MGM gives access to the highest social 'grade'	Considered "ritually impure"
Tikopia	Women may "prefer superincised persons", but there is no sexual ban on the unincised: "it is regarded primarily as a social disability not as a sexual one"	Allowed to "participate with the adults freely", sit among men and participate in formal gatherings and rituals	An unincised male is "apt to be taunted openly by boys of his own age" and laughed at.
Tiv	Required for marriage and sexual relations	"A symbol of adult male status" required to be allowed to participate in rituals as a "full member of the community"	May be ordered around by any circumcised person; laughed and jeered until he submits to mutilation
Wolof	Required for marriage	Institutionalized male friendships are "formed in the circumcision shed"; males circumcised together "owe each other aid and assistance"	No specific data

In only ten PSF societies could the kinship status of the MGM 'surgeon' be ascertained with certainty (Table 4.2). In eight of these (80%), it is clear that the operator is not related: either he always comes from a different tribe or ethnic group, or there is an explicit rule that relatives may not operate. This is not necessarily because relatives lack surgical expertise: even specialist practitioners of genital mutilation appear to be restricted from operating on their close kin among the Dogon

and Wolof. Furthermore, although Masai mothers are reported to perform FGM on their own daughters (Bagge, 1904), the surgically less extensive MGM operation may only be performed by unrelated outsiders. Only in the Tikopia is there evidence that MGM is performed by a relative (the senior mother's brother). However, the same source suggests this is a recent change, because the very presence of this close relative was ritually forbidden in prior times (see Table 4.2). Various conflicting accounts of MGM are given for the Eastern Toraja, varying by region and religious context. In one account, an uncle may perform superincision. However, only an uncle who is a highly respected warrior may perform this unusual exception to the standard public ceremony, and the same source also explains that parents must never be present, no matter who performs MGM. Similar restrictions on the very presence of relatives are evident in other PSF societies. For instance, Masai parents must remain within their hut for the duration of their son's circumcision, or they risk being beaten (Bagge, 1904). These results are consistent with Prediction 6.

Table 4.2 Evidence from eHRAF materials on PSF societies suggests that relatives of a boy do not normally perform the MGM operation, and may even be banned from the ceremony. MGM is performed either by a hired professional from an unrelated tribe, or an unrelated professional from his own tribe.

PSF Society	Kinship restrictions on performing or observing MGM
Aranda	"Fathers-in-law are also important figures in the initiation of their sons-in-laws, often being the men who actually perform the operation."
Azande	Among some southern tribes, parents may not be present at the circumcision.
Dogon	MGM is performed by blacksmiths, but "the daughters of blacksmiths…are not themselves excised, their brothers are not circumcised"
Eastern Toraja	Superincision may be performed at a feast by a tribal leader, or in the smithy [*presumably by a blacksmith — CGW)*]. Although "the boy's parents must not watch their son's circumcision", sometimes "older boys who are ashamed to have themselves circumcised before the eyes of many ask an uncle or a blood relative to perform this operation on them; in any case this must be someone who has slain one or more enemies."
Hausa	Operation is performed by "a Mohammedan barber."
Maasai	A member of a different tribe or ethnic group performs the operation, for "no Maasai would normally accept the task." "During the circumcision itself the parents of the boy remain in their hut, for if the latter moans or cries out from the pain, they are punished with abusive words and blows by the assembled warriors."
Mbuti	A Bantu villager performs the operation, for the "old rule" is that "Mbuti must have no part in the actual ritual and hold no ritual office."
Somali	The operation is performed by a *midgan*: a hired member of a low-caste pariah tribe.
Tikopia	Modern operation is performed by the senior maternal uncle. However, "of olden days it was taboo for the *tuatina maori* (senior maternal uncle) to touch the lad. He sat in the house and did not go out; the expert had to be a distant mother's brother" [*it is unclear whether this refers to the uncle of an unrelated boy; a distant cousin; or an uncle of the boy who lives far away — CGW*].
Wolof	"At circumcision, the operator cannot be just any person in a 'servant' position, but must be a smith. Since circumcision, unlike childbirth, involves a group of boys from several families, the operator must be someone in an inferior position to *all* the families — a casted person. For circumcision, someone who was a nephew of just one individual or family would not do; the smith, however, is in a position of client-ship to everyone present."

Warfare and scarification

I replicated the finding of Sosis, Kress and Boster (2007) with respect to scarification and external warfare in the SCCS. For 121 societies with available data, scarification is significantly associated with presence and frequency of external warfare (Fig. 4.4a: Mann-Whitney $W = 5241.5$, n=121, p = 0.0096), readily demonstrating the 'scars for war' effect in the SCCS. However, I found no association between MGM and frequency of external warfare in the SCCS (Fig. 4.4b: Mann-Whitney $W = 3053.0$, n=153, p = 0.58; n.s.). The relationship between scarification and external warfare is clearly present in the rPSF (Fig. 4.4c: Mann-Whitney $W = 117.0$, n=32, p = 0.0013). However, although there is a trend for MGM to increase with external warfare among rPSF societies, it is not significant (Fig.4.4d: Mann-Whitney $W = 464.0$, n=38, p = 0.17; n.s.). Sosis et al. (2007) did find a significant association in the full PSF ($X^2 = 4.12$, n=59, p = 0.043), but the absence of a relationship in the large SCCS raises the possibility that the PSF and rPSF trends may be spurious, perhaps resulting from a confounding association between warfare and polygyny in these smaller samples. Indeed, a further ANOVA for the PSF reveals that the relationship between MGM and external warfare loses significance ($F_{1, 57} = 0.018$, p= 0.89) if polygyny ($F_{1, 57} = 17.166$, $p < 0.001$) is included in the model as a covariate (R. Sosis, pers. comm.). This result brings both samples into agreement: unlike scarification, MGM is not significantly associated with external warfare. The PSF trend is best explained as an artifact of a polygyny confound that disappears in a larger sample.

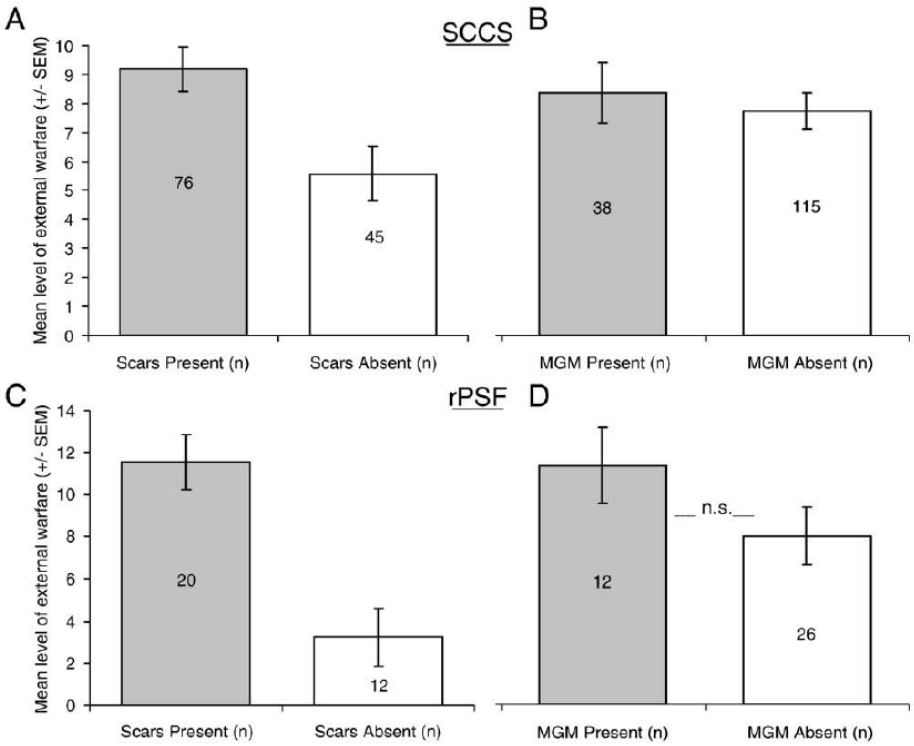


Figure 4.4 (**A**) Frequency of external warfare in SCCS societies significantly predicts the incidence of male scarification rituals (Mann–Whitney W=5241.5, n=121, p=.0096). (**B**) Frequency of external warfare in SCCS societies does not significantly predict the incidence of MGM rituals (Mann–Whitney W=3053.0, n=153, *P*=.58; ns). (**C**) Frequency of external warfare in rPSF societies significantly predicts the incidence of male scarification rituals (Mann–Whitney W=117.0, n=32, *P*=.0013). (**D**) Frequency of external warfare in rPSF societies does not significantly predict the incidence of MGM rituals (Mann–Whitney W=464.0, n=38, *P*=.17; ns). The apparent trend in (D) is explained by a confound between external warfare and polygyny in the small rPSF sample (see text).

Discussion

There is no evidence that any ablation of genital tissue has direct or intrinsic reproductive benefits that have outweighed its costs over evolutionary time. If this were the case, humans and other mammals would presumably have evolved an exposed glans, a ventral urethral aperture or a solitary testis. The existence of the necessary genetic and developmental variation is apparent in medical conditions such as aposthia, hypospadias and cryptorchism, and genital morphology is the most

responsive of all morphological traits to selection (Eberhard, 1985), yet the observed morphology has persisted. In societies that practice MGM, the operation must be performed by a non-relative in the public view of other men, implying underlying conflict and an incentive to cheat. These observations could be explained if MGM acts as a costly signal rather than conferring a direct personal advantage. I argue that MGM impairs sperm competition, signalling compliance with the social assignment of reproduction by reducing the capacity for extra-pair fertilizations. In societies whose marriage patterns promote paternal uncertainty and reproductive conflict, MGM is a more reliable indicator of trustworthiness than any other costly rite because it physically reduces a man's capacity to mount paternity challenges. Once the man is mutilated under appropriately policed conditions, he is released from various restrictions and gains social status including access to resources, weapons and women. These social benefits may outweigh both the impaired sperm competition and the considerable risks of infection, so that the costly signal improves overall fitness. As predicted by this hypothesis, MGM is practiced most frequently in societies with high levels of polygyny, particularly when multiple wives live far apart and cannot simultaneously be guarded from EPCs. Once these two underlying risk factors are controlled for, societies practicing MGM also seem to have a reduced incidence of extramarital sex, providing reasonably direct evidence that MGM may have the hypothesized effect. I have argued that MGM reduces the payoff to EPCs by impairing either overall fertilization efficiency; sperm competition; or both. However, the sexual conflict hypothesis is silent with respect to precise mechanisms, and is applicable whether the primary effect of MGM is mechanical, neuronal, hormonal, behavioural, psychological or psychosomatic in origin. Some speculative mechanisms were discussed in Section 2.3, but the hypothesis is agnostic and does not fall with the elimination of any given suggestion.

A relationship between MGM and polygyny is not a novel finding: Whiting, Kluckhohn and Anthony (1958) and Strauss and Orans (1975) reported a similar association while testing the proximate psychological gender-identity hypothesis using a binary measure of polygyny. However, their conclusions have recently been criticised by Korotayev and de Munck (2004). My results suggest such criticism may not be well-founded, as I have supported and extended the original findings using for the first time a standard sample and well-resolved incremental polygyny codes that only became available in 1988. The predictions of a proximate psychological hypothesis now appear to interdigitate well with this novel and complementary ultimate hypothesis.

A relationship in both the SCCS and the rPSF between external warfare and male scarification provides further support for the 'scars for war' hypothesis of Sosis, Kress and Boster (2007). However, there is no significant association between external warfare and MGM, suggesting that MGM is neither a 'scar for war', nor an arbitrary maladaptive by-product of belligerent societies. My results cannot be explained by some general trend for polygynous societies to brutalize young men more than monogamous societies, because Sosis et al. found no significant relationship between polygyny and costliness of male ritual after controlling for warfare. Polygyny does not cause increased brutalization of young men in general, but is associated specifically with a tendency to demand mutilation of the genitals rather than other rites of apparently comparable cost. This trend is independent of warfare, but very sensitive to distance between co-wives. These observations are explained by the sexual conflict hypothesis: a direct impediment to sperm competition may decrease the profitability of EPCs, and provides a more reliable indicator of cooperation than any other costly rite in societies where reproduction is highly skewed.

Finally, certain aspects of the global distribution of MGM may be explained by the sexual conflict hypothesis. Genital mutilations are less common in Asia and the Americas than in Africa and Oceania. This may be because polygyny is rarer in Asian and American societies than in African and Oceanian societies, and seldom involves separate residences for co-wives. Nevertheless, the absence of MGM in any coded American society is puzzling. MGM was not 'forgotten' during the migration to the New World: Columbus found circumcision among certain natives (Dunsmuir & Gordon, 1999), and the Totonacs of Central America practiced it aboriginally (Murdock, 1967). Intriguingly, exclusive social cults open only to those with no foreskins are also reported amongst the highly polygynous Pawnee and Assiniboine (Murie, 1914; Denig, 1930). However, MGM was not common in the New World, even in South American societies with very high levels of polygyny. This may be due in part to the cultural influence of Catholic conquistadors (Schendel, 1968), but there may be a more interesting factor. Ucko (1969) reviews the customs of sheathing the penis and binding up the foreskin, which seem to be disproportionately represented in extremely polygynous South American societies. Yanomamo men, for instance, "tie the penis up by means of the hip cord, which is done before donning it by tying a knot and drawing the foreskin through it….it would be the most flagrant offense against good form to walk about without the hip cord" (Becher, 1960: 60). Could binding the foreskin or wearing a penis sheath parallel MGM by providing a costly signal of impaired sperm competition? Ucko (1969) mentions consequences including "a great elongation of the foreskin, sometimes to twice its original length…general swelling of the penis, the impossibility of erection while such a sheath is worn…inflamed penis tips, chaffed skin…" (p. 45). Cultural requirements to jam the penis into a tight sheath or stretch the foreskin to unnatural lengths may be as harmful to sperm competition as MGM. None of the eight South American societies in the SCCS with the most

extreme level of polygyny practices MGM, but Ucko mentions foreskin binding or sheathing in 50% of them (Amahuaca; Jivaro; Warrau; Yanomamo). Intriguingly, this fraction concords with the global incidence of MGM in all 46 SCCS societies with extreme polygyny: 48%. Perhaps polygynous South American societies developed a parallel signalling behavior to serve the same ultimate function.

Conclusion

Kennedy (1970) observed that "attempts to formulate a theory that can account for all customs of genital operations seem doomed to failure" (p. 189). The sexual conflict hypothesis could challenge this view. The hypothesis proposes that MGM and FGM both function as costly signals of compliance with the social assignment of reproduction. Genital mutilations may impair the evolved capacity for extra-pair fertilizations, decreasing paternal uncertainty and reproductive conflict and garnering trust and social investment from powerful married men. As predicted by the sexual conflict hypothesis, MGM is associated with polygynous societies at high risk for extramarital sex, and it appears to reduce this risk. MGM is performed by a non-relative in the public view of other men, and a mutilated male gains immediate access to social and sexual privileges that are suggested to outweigh the cost of the mutilation itself. The sexual conflict hypothesis may have value for anthropologists, doctors and theologians pursuing future studies of this ancient problem.

REFERENCES

al Adawi, I.A. (1954). Description of the Sudan by Muslim geographers and travelers. *Sudan Notes and Records*, *35*, 5-16.

Ashley-Montagu, M.F. (1937). The origin of subincision in Australia. *Oceania*, *8*, 193-207.

Auvert, B., Taljaard, D., Lagarde, E., Sobngwi-Tambekou, J., Sitta, R. & Puren, A. (2005). Randomized, controlled intervention trial of male circumcision for reduction of HIV infection risk: the ANRS 1265 trial. *Public Library of Science: Medicine*, *2*, e298.

Bagge, S. (1904). The circumcision ceremony among the Naivasha Masai. *The Journal of the Anthropological Institute of Great Britain and Ireland*, *34*, 167-169.

Becher, H. (1960). *The Surara and Pakidai, two Yanoama tribes in northwest Brazil*. New Haven, CT: *HRAF*, 1995.

Beckett, J. (1967). Marriage, circumcision and avoidance among the Maljangaba of north-west New South Wales. *Mankind, 6*, 456-464.

Boyd, R. and Richerson, P.J. (2002). Group beneficial norms can spread rapidly in a structured population. *Journal of Theoretical Biology*, *215*, 287-296.

Broude, G. and Greene, S.J. (1976) Cross-cultural codes on twenty sexual attitudes and practices. *Ethnology*, *15*, 409-429.

Bryk, F. (1934). *Circumcision in man and woman* (tr. David Berger). New York, American Ethnological Press.

Cold, C.J. & Taylor, J.R. (1999). The prepuce. *British Journal of Urology, 83*, Supplemental 1, 34-44.

Crowley, I.P. & Kesner, K.M. (1990). Ritual circumcision (*Umkhwetha*) amongst the Xhosa of the Ciskei. *British Journal of Urology, 66*, 318-321.

Dahl, J.F. (1994). Size and form of the penis in orang-utans. *Journal of Mammalogy*, *75*, 1-9.

Daly, M. & Wilson, M. (1988). *Homicide*. New York: Aldine de Gruyter.

Darby, R. (2005). The riddle of the sands: circumcision, history, and myth. *Journal of the New Zealand Medical Association*, *118*, 1-2.

Denig, E.T. (1930). *Indian tribes of the upper Missouri*. New Haven, CT: *HRAF*, 2002.

Divale, W. (2007). Standard Cross-Cultural Sample SPSS file. *World Cultures*.

Dixson, A.F. (1987). Observations on the evolution of the genitalia and copulatory behaviour in male primates. *Journal of Zoology*, *213*, 423-443.

Dixson, A.F. (1998). *Primate sexuality*. Oxford: Oxford University Press.

Dunsmuir, W.D. & Gordon, E.M. (1999). The history of circumcision. *British Journal of Urology*, *83* Supplemental 1, 1-12.

Eberhard, W.G. (1985). *Sexual selection and animal genitalia*. Cambridge, MA: Harvard University Press.

Ember, M. (1984). Alternative predictors of polygyny. *Behavior Science Research, 19*, 1-23.

Ember, C.R. & Ember, M. (1992). Resource unpredictability, mistrust, and war. *Journal of Conflict Resolution*, *36*, 242-262.

Ember, C.R. & Ember, M. (1998). Cross-cultural research. In H.R. Bernard (Ed.) *Handbook of methods in cultural anthropology* (pp. 647-687). Walnut Creek, CA: AltaMira.

Ericksen, K.P. (1989). Female genital mutilations in Africa. *Cross-Cultural Research*, *23*, 182-204.

Finsch, O. (1880). Ueber die Bewohner von Ponape. *Zeitschrift für Ethnologie*, *12*, 301-332.

Gallup, G.G. & Burch, R.L. (2004). Semen displacement as a sperm competition strategy in humans. *Evolutionary Psychology*, *2*, 12-23.

Gaulin, S. J. C., McBurney, D.H. & Brakeman-Wartell, S.L. (1997). Matrilateral biases in the investment of aunts and uncles: a consequence and measure of paternity uncertainty. *Human Nature, 8,* 139-151.

Goetz, A.T. & Shackelford, T.K. (2006). Sperm competition and its evolutionary consequences in humans. In S.M. Platek and T.K. Shackelford (Eds.) *Female infidelity and paternal uncertainty: evolutionary perspectives on male anti-cuckoldry tactics* (pp. 103-128). Cambridge: Cambridge University Press.

Gomendio, M., Harcourt, A.H. & Roldan, R.S. (1998). Sperm competition in mammals. In T.R. Birkhead and A.P. Moller (Eds.*) Sperm competition and sexual selection* (pp. 667-756). San Diego: Academic Press.

Gray, R.H., Kigozi, G., Serwadda, D., Makumvi, F., Watya, S. & Nalugoda, F. et al. (2007). Male circumcision for HIV prevention in men in Rakai, Uganda: a randomized trial. *The Lancet, 369,* 657-666.

Guma, S.M. (1965). Some aspects of circumcision in Basutoland. *African Studies*, *24*, 241-249.

Hambly, W.D. (1934) *The Ovimbundu of Angola*. Chicago: Field Museum of Natural History.

Hill, W.C.O. and Matthews, L.H. (1949). The male external genitalia of the gorilla, with remarks on the os penis of other Hominoidea. *Proceedings of the Zoological Society of London*, *119*, 363-378.

Huber, B.R., Linhartova, V. and Cope, D. (2004) Measuring paternal certainty using cross-cultural data. *World Cultures*, *15*, 48-59.

Hutson, J.M. (2004). Circumcision: a surgeon's perspective. *Journal of Medical Ethics, 30,* 238-240.

Immerman, R.S. & Mackey, W.C. (1997). A biocultural analysis of circumcision. *Social Biology*, *44*, 265-275.

Irons, W. (2001). Religion as a hard-to-fake sign of commitment. In R. Nesse (Ed.) *Evolution and the capacity for commitment* (pp. 292-309). New York: Russell Sage Foundation.

Kennedy, J.G. (1970). Circumcision and excision in Egyptian Nubia. *Man: New Series*, *5,* 175-191.

Korotayev, A. & de Munck, V. Galton's asset and Flower's problem: cultural networks and cultural units in cross-cultural research. *American Anthropologist*, *105*, 353-358.

Lagercrantz, S. (1938). Zur Verbreitung der Monorchie. *Zeitschrift für Ethnologie*, *70*, 199-208.

Ludvico, L.R. & Kurland, J.A. (1995). Symbolic or not-so-symbolic wounds: the behavioral ecology of human scarification. *Ethology and Sociobiology*, *16,* 155-172.

Mackie, G. (1996). Ending footbinding and infibulation: a convention account. *American Sociological Review, 61*, 999-1017.

McBurney, D.H., Simon, J., Gaulin, S.J.C. & Geliebter, A. (2002). Matrilateral biases in the investment of aunts and uncles: replication in a population presumed to have high paternity certainty. *Human Nature, 13*, 391-402.

Mobarak, A.M., ElWishy, A.B. & Samira, M.F. (1972). The penis and prepuce of the one-humped camel (*Camelus dromedarius*) *Zentralblatt für Veterinarmedizin*, *19*, 787-795.

Murad, H., Fleischman, A., Sadetzki, S., Geyer, O. & Freedman, L.S. (2003). Small sample sizes and ordered logistic regression: does it help to collapse categories of outcome? *The American Statistician, 57,* 155-160.

Murdock, G.P. & White, D.R. (1969). Standard Cross-Cultural Sample. *Ethnology, 9,* 329-369.

Murdock, G.P. (1967). *Ethnographic Atlas*. Pittsburgh: University of Pittsburgh Press.

Murie, J.R. (1914). *Pawnee Indian societies*. New Haven, CT: *HRAF*, 2002.

Nunn, C.L., Gittleman, J.L. & Antonovics, J. (2000). Promiscuity and the primate immune system. *Science, 290,* 1168-1170.

O'Hara, K. & O'Hara, J. (1999). The effect of male circumcision on the sexual enjoyment of the female partner. *British Journal of Urology, 83* Supplemental 1, 79-84.

Paige, K. E. & Paige, J.M. (1981). *The politics of reproductive ritual*. Berkeley: University of California Press.

Rappaport, R. (1999). *Ritual and religion in the making of humanity*. Cambridge: Cambridge University Press.

Raven-Hart, R. (1967). *Before van Riebeeck*. Cape Town: C. Struik Ltd.

Reason, L.L. (2004). The behavioral ecology of female genital cutting in northern Ghana. *Research in Economic Anthropology, 23,* 175-202.

Richters, J. Gerofi, J. & Donovan, B. (1995). Are condoms the right sizes? A method for self-measurement of the erect penis. *Venereology, 8,* 77-81.

Richters, J., Smith, A.M.A.,de Visser, R.O., Grulich, A.E. & Rissel, C.E. (2006). Circumcision in Australia: prevalence and effects on sexual health. *International Journal of Sexually Transmitted Diseases and AIDS, 17,* 547–554.

Schendel, G. (1968). *Medicine in Mexico: from Aztec herbs to betatrons*. Austin: University of Texas Press.

Schlegel, A. & Barry, H. (1979). Adolescent initiation ceremonies: a cross-cultural code. *Ethnology 18,* 199-210.

Senkul, T., Iseri, C., Sen, B. Karademir, K., Saracoglu, F. & Erden, D. (2004). Circumcision in adults: effect on sexual function. *Urology*, *63*, 155-158.

Shapiro, H.L. (1930). The practice of incision in the Tuamotus. *Man*, *30*, 140-143.

Short, R.V. (1979). Sexual selection and its component parts, somatic and genital selection, as illustrated by man and the great apes. *Advances in the Study of Behavior*, *9*, 131-158.

Short, R.V. (2004). Male circumcision: a scientific perspective. *Journal of Medical Ethics*, *30*, 241.

Sidley, P. (2006). Botched circumcisions kill 14 boys in a month. *British Medical Journal*, *333*, 62.

Smith, R.L. (1984). Human sperm competition. In R.L. Smith (Ed.) *Sperm Competition and the Evolution of Animal Mating Systems* (pp. 601-659). San Diego: San Diego: Academic Press.

Sorrells, M.L., Snyder, J.L., Reiss, M.D., Eden, C., Milos, M.F., Wilcox, N. & Van Howe, R.S. (2007). Fine touch pressure thresholds in the adult penis. *British Journal of Urology*, *99*, 864-869.

Sosis, R. (2000). Religion and intra-group competition: preliminary results of a comparative analysis of utopian communities. *Cross-Cultural Research*, *34*, 70-87.

Sosis, R. (2004). The adaptive value of religious ritual. *Scientific American*, *92*, 166-172.

Sosis, R. & Ruffle, B. (2003). Religious ritual and cooperation: testing for a relationship on Israeli religious and secular kibbutzim. *Current Anthropology*, *44*, 713-722.

Sosis, R., Kress, H.C. & Boster, J.S. (2007). Scars for war: evaluating alternative signaling explanations for cross-cultural variance in ritual costs. *Evolution and Human Behavior*, *28*, 234-247.

Strauss, D.J. & Orans, M. (1975). Mighty sifts: a critical appraisal of solutions to Galton's problem and a partial solution. *Current Anthropology, 16,* 573-585.

Suarez, S.S. & Pacey, A.A. (2006). Sperm transport in the female reproductive tract. *Human Reproduction Update, 12*, 23-37.

Szabo, R. & Short, R.V. (2000). How does male circumcision protect against HIV infection? *British Medical Journal, 370,* 1592-1594.

Taves, D.R. (2002). The intromission function of the foreskin. *Medical Hypotheses, 59,* 180-182.

Taylor, J.R., Lockwood, A.P. & Taylor, A.J. (1996). The prepuce: specialized mucosa of the penis and its loss to circumcision. *British Journal of Urology, 77,* 291-295.

Ucko, P.J. (1969). Penis sheaths: a comparative study. *Proceedings of the Royal Anthropological Institute of Great Britain and Ireland, 1969,* 27-67.

Verrell, P.A. (1992). Primate penile morphologies and social systems: further evidence for an association. *Folia Primatologica, 59,* 114-120.

White, D.R. (1988). Rethinking polygyny: co-wives, codes and cultural systems. *Current Anthropology, 29,* 529-572.

Whiting, J., Kluckhohn, R. & Anthony, A. (1958). The function of male initiation ceremonies at puberty. In E. Maccoby, T. Newcomb & E. Hartley (Eds.) *Readings in social psychology* (pp. 359-370). New York: Holt, Rinehart and Winston.

Winberg, J., Bollgren, I., Herthelius, M., Gothefors, L. & Tullus, K. (1989). The prepuce: a mistake of nature? *The Lancet, 333,* 598-599.

Woodhead, D.M, Pohl, D.R. & Johnson, D.E. (1973). Fertility of patients with solitary testes. *The Journal of Urology, 109,* 66-67.