A peer-reviewed version of this preprint was published in PeerJ on 2 May 2016.

View the peer-reviewed version (peerj.com/articles/1989), which is the preferred citable publication unless you specifically need to cite this preprint.

Giroux M, Ditlecadet D, Martin LJ, Lanctot RB, Lecomte N. 2016. Sexing a sex-role-reversed species based on plumage: potential challenges in the red phalarope. PeerJ 4:e1989 https://doi.org/10.7717/peerj.1989

Sexing a gender-role-reversed species based on plumage: potential challenges in the red phalarope

Marie-Andrée Giroux, Delphine Ditlecadet, Luc Martin, Richard B. Lanctot, Nicolas Lecomte

Sex-role reversal, in which males care for offspring, can occur when mate competition is stronger between females than males. Secondary sex traits and mate attracting displays in sex-role-reversed species are usually more pronounced in females than in males. The red phalarope is a textbook example of a sex-role-reversed species. It is generally agreed that males are responsible for all incubation and parental care duties, whereas females typically desert males after having completed a clutch and may pair with new males to lay additional clutches. Breeding plumage of female red phalaropes is usually more brightly colored than male plumage, a reversed sexual dichromatism usually associated with sex-role reversal. Here, we confirm with PCR-based sexing that male red phalaropes can exhibit both the red body plumage typical of a female and the incubation behaviour typical of a male in this sex-role-reversed species. Our result, combined with previous observations of brightly coloured red phalaropes incubating nests at the same arctic location (Igloolik Island, Nunavut, Canada), suggests that plumage dichromatism alone may not be sufficient to distinguish males from females in this breeding population of red phalaropes. This stresses the need for more systematic genetic sexing combined with standardized description of intersexual differences in red phalarope plumages. Determining whether such female-like plumage on males is a result of phenotypic plasticity or genetic variation could contribute to further understanding sex-role reversal strategies in the short Arctic summer.

Sexing a gender-role-reversed species based on plumage: potential challenges in the red phalarope

Marie-Andrée Giroux[1,2,3]*, Delphine Ditlecadet[4], Luc Martin[5], Richard B. Lanctot[6], and Nicolas Lecomte[1,3,5,,7]

[1] Canada Research Chair in Polar and Boreal Ecology, Université de Moncton, E1A 3E9 Canada

[2] Canada Research Chair on Northern Biodiversity, Université du Québec à Rimouski, G5L 3A1

[3] Centre d'Études Nordiques, Université du Québec à Rimouski, G5L 3A1

[4] Molecular Biology Unit, Fisheries and Oceans Canada, Moncton, NB, E1C 9B6

[5] Département de Biologie, Université de Moncton, E1A 3E9 Canada

[6] U.S. Fish and Wildlife Service, Migratory Bird Management, Anchorage, Alaska 99503 USA

[7] Québec Center for Biodiversity Science, Université du Québec à Rimouski, G5L 3A1

*Corresponding author:
Marie-Andrée Giroux
Canada Research Chair in on Northern Biodiversity
Départment de biologie
Université du Québec à Rimouski
G5L 3A1 Canada
Email: marie.a.giroux@gmail.com

Abstract

Sex-role reversal, in which males care for offspring, can occur when mate competition is stronger between females than males. Secondary sex traits and mate attracting displays in sex-role-reversed species are usually more pronounced in females than in males. The red phalarope is a textbook example of a sex-role-reversed species. It is generally agreed that males are responsible for all incubation and parental care duties, whereas females typically desert males after having completed a clutch and may pair with new males to lay additional clutches. Breeding plumage of female red phalaropes is usually more brightly colored than male plumage, a reversed sexual dichromatism usually associated with sex-role reversal. Here, we confirm with PCR-based sexing that male red phalaropes can exhibit both the red body plumage typical of a female and the incubation behaviour typical of a male in this sex-role-reversed species. Our result, combined with previous observations of brightly coloured red phalaropes incubating nests at the same arctic location (Igloolik Island, Nunavut, Canada), suggests that plumage dichromatism alone may not be sufficient to distinguish males from females in this breeding population of red phalaropes. This stresses the need for more systematic genetic sexing combined with standardized description of intersexual differences in red phalarope plumages. Determining whether such female-like plumage on males is a result of phenotypic plasticity or genetic variation could contribute to further understanding sex-role reversal strategies in the short Arctic summer.

Keywords: Charadriiformes, *Phalaropus fulicarius*, sexual dichromatism, shorebirds, secondary sexual traits

Introduction

Sex-role reversal, in which males care for offspring, can occur when mate competition is stronger between females than males (Eens and Pinxten 2000; Kvarnemo and Ahnesjo 1996). Biases in the intensity of mating competition can result from differences in operational sex ratios (the ratio of males to females ready to mate), which can in turn be associated with biases in potential reproductive rates (Kvarnemo and Ahnesjo 1996). Theory predicts that, as a result of biases in the intensity of mating competition, secondary sex traits and mate attracting displays in sex-role-reversed species will be more pronounced in females than in males (Andersson 1994; Eens and Pinxten 2000; Trivers 1985).

The red phalarope is a textbook example of a sex-role-reversed species (Alcock 2013). It is generally agreed that males are responsible for all incubation and parental care duties, whereas females typically desert males after having completed a clutch and may pair with new males to lay additional clutches (sequential polyandry; Dale et al. 1999; Schamel and Tracy 1977). The mating system of the red phalarope has been described as *female access polyandry*, a system in which females do not defend resources, but rather limit access to males by converging at feeding areas to mate (Emlen and Oring 1977). Breeding plumage of female red phalaropes is usually more brightly colored than male plumage (Tracy et al. 2002; Figure 1), a reversed sexual dichromatism usually associated with sex-role reversal (Heinsohn et al. 2005). It is also recognized that there is considerably more plumage variations between males, and that the most brightly colored males can approach female levels of coloration (Pyle 208; Tracy et al. 2002). However, mottled crowns has been identified as the characteric that was diagnostic of males (Tracy et al. 2002). Such overlap in plumage of male and female red phalarope (Tracy et al.

2002) might explain why previous studies have reported incidental observations of red phalaropes showing typical female plumage either incubating eggs (3 out of 17 nests; Forbes et al. 1992) or brooding chicks (Sutton 1932).

Here, we describe the observation of a red phalarope exhibiting both the red body plumage, the plain black crown, and the incubation behaviour typical of a female on Igloolik Island (Nunavut, Canada), during summer 2014. Our objective was to genetically sex this individual (hereafter referred as the "ambiguous" individual) to determine whether it was a brightly colored male or a female. We determined the sex of the ambiguous bird by using a DNA marker universaly used for sexing birds (Fridolfsson and Ellegren 1999), comparing the band patterns of the ambiguous bird with those obtained with samples of red phalaropes sexed by dissection.

Methods

Study area

We conducted fieldwork on Igloolik Island (Nunavut, Canada; 69°24'N, 81°32'W) between early June and early August in 2014 (Lecomte and Giroux 2015). This island is located in northwest Foxe Basin next to the Melville Peninsula and south from the northern part of Baffin Island. The study area is located in a mosaic of wet (sedge/grass moss wetland), mesic (non-tussock sedge, dwarf-shrub, moss tundra), and dry (prostrate dwarf-shrub, herb tundra) habitat patches interspersed by ponds and lakes. Habitats were identified as per the Circumpolar Arctic Vegetation map (CAVM-Team 2003).

Nest monitoring

We located red phalarope nests by following birds on incubation recesses back to their nests or by flushing nests when walking or dragging a 30-m rope (9-mm-diameter). We searched for nests intensively within a 36-ha and a 24-ha nest plots, and also recorded the presence of nests found opportunistically outside of the nest plots. The location of each nest was recorded with a Global Positioning System (Garmin eTrex), and three nest markers were placed at 1-m, 5-m and 10-m north of the nest to allow nest relocation. We monitored nests according to a 5-day visitation schedule.

Capture

We captured the ambiguous individual using a bownet placed on its nest on June 16th 2014 (1 day before hatching). We marked the bird with a metal band, a unique individual combination of three coloured darvic bands, and a unique site-specific combination of two coloured bands. We measured and recorded its bill length (exposed culmen) using a caliper (±0.1 mm precision), wing length using a ruler (±1 mm), and body mass using a hanging Pesola scale (±1g). We collected blood from the basilic vein using a small gauge (27.5) needle to puncture the vein before drawing the blood into a capillary. Blood was preserved in 95% ethanol. Finally, we took pictures of the general appearance of the bird.

Control individuals

Red phalaropes were opportunistically collected after being found dead during the breeding season at Barrow, Alaska in 2011 (male) and 2012 (female). Sex of those control carcasses was confirmed by visual inspection of their reproductive system. Samples of muscles were collected during dissection, preserved in tissue preservation buffer (240.24g Urea, 100mls 1M Tris HCl

105 pH 8.0, 11.69g NaCl, 3.72g EDTA, 5 g N-Lauroyl-sarcosine, npH2O to 1 Liter). These samples
106 were used as controls for PCR-based sex determination of the ambiguous individual.

107 **PCR-based sex determination**

108 Three birds were sexed by PCR: one control male, one control female, and the ambiguous bird.
109 A small piece of tissue (close to 1 mm^3) of the control birds was washed with 50 µl sterile water
110 and centrifuged for 3 min at 10,000 rpm. Water was removed and the tissue washed a second
111 time to remove any remaining salts from the preservation buffer that could have interfered with
112 the PCR reaction. The tissue was then broken down using the point of a sterile tip in 50 µl of
113 sterile water. Blood samples of the ambiguous bird were properly mixed and 50 µl were
114 transferred to a new tube and centrifuged for 3 min at 10,000 rpm. Ethanol was removed and
115 pelleted red blood cells were re-suspended in 50 µl DEPC water. The mixtures produced for each
116 bird were incubated for 20 min at 55□C with constant shaking and 5 µl was directly used as
117 DNA template for the PCR reactions.
118 Sex determination was made according to Fridolfsson and Ellegren (1999), with minor
119 modifications. Reactions were done in 25 µl reactions containing 12.5 µl Amresco Hot Start Taq
120 Master Mix, 2x (Amresco LLC., Solon, Ohio, US), 0.5 µM each primer and 5 µl of the DNA
121 template. Sequences of the primers used were 2550F:5'-GTTACTGATTCGTCTACGAGA–3'
122 and 2718R: 5'-ATTGAAATGATCCAGTGCTTG-3'. PCR conditions were as follows: 94°C for
123 1 min of initial denaturation, 35 cycles at 94°C for 30 sec, 50°C for 30 sec and 72°C for 1 min,
124 followed by a final extension at 72°C for 5 min. PCR products were finally separated using 1.2%
125 agarose gel electrophoresis with GelRed™ nucleic acid stain (Biotium, Inc., Hayward,

California, US). The sex of the ambiguous bird was determined by comparing the pattern displayed by the control male and female on the electrophoresis.

Permits

Capture techniques and immobilization procedures were approved by the Université de Moncton Animal Care Committee (permit # 14-05) and by Environment Canada (Scientific permit to capture and band migratory birds, #10872). Red phalarope collections in Alaska were done under federal and state permits issued to R. Lanctot. Field research was approved by the Department of Environment – Government of Nunavut (permit # WL-2014-039) and the Canadian Wildlife Service (permits #NUN-SCI-14-04).

Results

Nest density

In summer 2014, the density of red phalarope nests on our study plots averaged 25 nests/km^2 (SD=11, n=12 and 4 nests in the 36 and 24-ha plots, respectively). The ambiguous individual incubated a nest located approximately 0.6 km outside both nest plots.

Nesting behaviour

The nest incubated by the ambiguous individual (Figure 1) was found by flushing the bird on 27 June 2014. The nest was revisited on July 2, July 7, and July 12 when we saw signs of hatching on the eggs. We then visited the nest every 1-2 days until it hatched on July 17. The brightly colored individual was observed incubating the nest at every visit except on June 13 when the nest was not being attended.

Physical characteristics

147 The ambiguous individual was observed walking with an apparent handicap and upon capture,
148 we noted that two digits of its right foot were missing second phalanges. Its bill and wing lengths
149 overlapped values reported for male and female red phalaropes trapped in Igloolik in a prior
150 study (J. Dale, unpubl. data, in Tracy et al. 2002), while body mass was on average 7.9g and
151 12.2g lower than those males and females, respectively (Table 1). Plumage patterns indicated the
152 ambiguous bird had red body feathers and plain black crown indicative of a female, but wing
153 feathers possibly resembling a male (Fig. 1).

154 **PCR-based sex determination**

155 The male and female positive controls exhibited the expected discriminative band pattern. The
156 band of the female was smaller than the male's, with sizes close to 300 bp and 550 bp,
157 respectively (Figure 2). The pattern of the ambiguous bird was identical to the one of the control
158 male pattern, namely with a single band around 550 bp (Figure 2).

159 ## Discussion

160 According to the PCR test, the brightly coloured, ambiguous red phalarope was a male. Our
161 result, combined with previous observations of brightly coloured red phalaropes males (Forbes et
162 al. 1992; Tracy et al. 2002), stresses the need for conducting more systematic genetic sexing
163 combined with standardized description of red phalarope plumages. This is especially needed
164 because the characteristic that is considered diagnostic of even the bright males, namely the
165 mottled crown, was not observed in the male described in this study.

166 There are a variety of reasons why bright male plumages might occur in this species.
167 Johns (1964) showed that the red nuptial feathers in phalaropes could experimentally be induced
168 by an injection of testosterone. Hence, a higher testosterone level during spring moult (April;

169 Tracy et al. 2002) could cause the brighter plumage observed in the male red phalarope in our
170 study. However, there is little information on hormone levels in red phalaropes so the variability
171 of testosterone levels during the molting season remains to be quantified. It is also unknown
172 whether the physiological mechanism (higher testosterone level or another mechanism) behind
173 this feather coloration is a result of phenotypic plasticity, genetic variation or both. It is
174 interesting that our ambiguous male had cryptic wing feathers possibly like his male counterparts
175 (Figure 1); such wing feather coloration would provide the necessary camouflage to avoid
176 predation while incubating a nest. Further studies are required to sex individuals displaying such
177 wing coloration and other potential distinguishing criteria betweeen males and females currently
178 discussed among shorebird biologist but yet unpublished (e.g. tawny stripes on the back).This is
179 especially needed on Igloolik Island as occasional observations conducted during summer 2015
180 on Igloolik Island point to the possibility that male feather coloration is highly variable (Lecomte
181 & Giroux, unpublish. data), suggesting that our ambiguous male is not a singularity.

182 Although redder males are thought to be of higher quality in a species characterized by
183 typical sex roles such as the bar-tailed godwit *Limosa lapponica* (Piersma and Jukema 1993),
184 determining whether a female-like coloration would be associated with any variations in
185 reproductive traits for males remains to be studied in this species (see an equivalent study in
186 ruffs: Küpper et al. 2016). To better understand the mechanisms inducing bright feather
187 coloration in males, further studies are needed to compare physiological parameters and
188 hormonal levels in bright individuals compared to typical bright females and dull males, and
189 mate selection and breeding success of various patterned males.

190 The PCR method used to sex these three individuals is regularly used to sex birds from

different species, and has been shown to be reliable (Fridolfsson and Ellegren 1999). Males of many species tested with this method always have a one-band pattern, characteristic of their ZZ sexual chromosomes. Females, that have ZW sexual chromosomes, usually have two bands, with the biggest band corresponding to the Z-chromosome, as in males. However, females from some species can also display a single band pattern because the W chromosome can sometimes be preferentially amplified against the Z chromosome. In all cases, the single female band is always smaller than the single male band, still allowing the robust discrimination of both sexes (Fridolfsson and Ellegren 1999). As such, the pattern exhibited by the control female in our study is not unexpected and still allows differentiating males from females. Thus we are confident of the sexual assignment of our ambiguous bird as a male.

Our result indicates that in some situations plumage dichromatism alone may not be sufficient to distinguish red phalarope males from females. Identifying diagnostic plumage characteristics of males would require range-wide studies scoring plumage of genetically sexed individuals with standardized protocols (Reynolds 1987; Troscianko and Stevens 2015). We also recommend further work to determine whether these female-like plumages on males are a result of phenotypic plasticity or genetic variation, and brightly colored males derives reproductive benefits from their coloration.

Acknowledgements

We thank M.-C. Frenette, M. Trottier-Paquet for their valuable assistance in the field, the Government of Nunavut for their logistical support, D. Edwards for sharing its thoughts about our observation, as well as two anonymous reviewers that commented a previous version of the

212 manuscript. K. Sage and S. Talbot from the U. S. Geological Service, Alaska Science Center
213 provided samples from archived red phalaropes. This study was supported by (alphabetical
214 order): Canada Foundation for Innovation, Canada Research Chairs, Environment Canada,
215 Government of Nunavut, Indian and Northern Affairs Canada, Igloolik Hunters and Trappers
216 Organization, Natural Sciences and Engineering Research Council of Canada, Polar Continental
217 Shelf Project (PCSP), U. S. Fish and Wildlife Service, Université de Moncton. The findings and
218 conclusions in this article are those of the authors and do not necessarily represent the views of
219 the U. S. Fish and Wildlife Service.

References

Alcock J (2013) Animal Behavior: An evolutionary approach. Sinauer Associates, Inc.,

Andersson M (1994) Sexual selection. Princeton University Press, Princeton, NJ

CAVM-Team (2003) Circumpolar Arctic Vegetation Map. (1:7,500,000 scale). U.S. Fish and Wildlife Service, Anchorage, Alaska

Dale J, Montgomerie R, Michaud D, Boag P (1999) Frequency and timing of extrapair fertilisation in the polyandrous red phalarope (Phalaropus fulicarius) Behav Ecol Sociobiol 46:50-56 doi:10.1007/s002650050591

Eens M, Pinxten R (2000) Sex-role reversal in vertebrates: behavioural and endocrinological accounts Behav Processes 51:135-147 doi:http://dx.doi.org/10.1016/S0376-6357(00)00124-8

Emlen S, Oring L (1977) Ecology, sexual selection, and the evolution of mating systems Science 197:215-223 doi:10.1126/science.327542

Forbes G, Robertson K, Ogilvie C, Seddon L (1992) Breeding Densities, Biogeography, and Nest Depredation of Birds on Igloolik Island, N.W.T Arctic 45:295-303 doi:10.2307/40511461

Fridolfsson A-K, Ellegren H (1999) A Simple and Universal Method for Molecular Sexing of Non-Ratite Birds J Avian Biol 30:116-121 doi:10.2307/3677252

Heinsohn R, Legge S, Endler JA (2005) Extreme Reversed Sexual Dichromatism in a Bird Without Sex Role Reversal Science 309:617-619 doi:10.1126/science.1112774

Johns JE (1964) Testosterone-Induced Nuptial Feathers in Phalaropes The Condor 66:449-455 doi:10.2307/1365222

Küpper C et al. (2016) A supergene determines highly divergent male reproductive morphs in the ruff Nat Genet 48:79-83 doi:10.1038/ng.3443

http://www.nature.com/ng/journal/v48/n1/abs/ng.3443.html - supplementary-information

Kvarnemo C, Ahnesjo I (1996) The dynamics of operational sex ratios and competition for mates Trends Ecol Evol 11:404-408 doi:http://dx.doi.org/10.1016/0169-5347(96)10056-2

Lecomte N, Giroux M-A (2015) New avian breeding records for Igloolik, Nunavut Can Field-Nat 129:194-196

Piersma T, Jukema J (1993) Red Breasts as Honest Signals of Migratory Quality in a Long-Distance Migrant, the Bar-Tailed Godwit The Condor 95:163-177 doi:10.2307/1369398

Pyle P (208) Identification Guide to North American Birds. Part II: Anatidae to Alcidae. Slate Creek Press, USA,

Reynolds JD (1987) Mating system and nesting biology of the Red-necked Phalarope Phalaropus lobatus: what constrains polyandry? Ibis 129:225-242 doi:10.1111/j.1474-919X.1987.tb03203.x

Schamel D, Tracy D (1977) Polyandry, replacement clutches, and site tenacity in the red phalarope (Phalaropus fulicarius) at Barrow, Alaska Bird-Banding 48:314-324 doi:10.2307/20699123

Tracy DM, Schamel D, Dale J (2002) Red Phalarope (Phalaropus fulicarius). Ithaca: Cornell Lab of Ornithology. Retrieved from the Birds of North America Online: http://bna.birds.cornell.edu/bna/species/698.

Trivers RL (1985) Social evolution. The Benjamin Cummings Publishing Company, Menlo Park, California

264 Troscianko J, Stevens M (2015) Image calibration and analysis toolbox – a free software suite
265 for objectively measuring reflectance, colour and pattern Methods in Ecology and
266 Evolution:n/a-n/a doi:10.1111/2041-210X.12439

267

268

269 **Table 1.** Morphometric measurements of red phalaropes captured in Igloolik in a previous study
270 (Tracy et al. 2002) compared to those of the ambiguous individual measured in 2014. Samples
271 sizes are within brackets.

	Previous study	Ambiguous individual
Bill length		
Male	22.2 ± 1.5 (48)	23.5
Female	22.7 ± 1.2 (14)	
Wing length		
Male	128.4 ± 2.3 (48)	130
Female	134.9 ± 2.9 (14)	
Body mass		
Male	52.9 ± 3.8 (45)	45
Female	57.2 ± 4.7 (13)	

272

273

274 **Figure captions**

275 **Fig. 1.** Comparison between the breeding plumage of three red phalaropes: 1) typical male, 2)
276 ambiguous bird (brightly coloured individual incubating), and 3) typical female. All pictures
277 were taken in Igloolik, Nunavut, Canada. Photos: N. Lecomte.

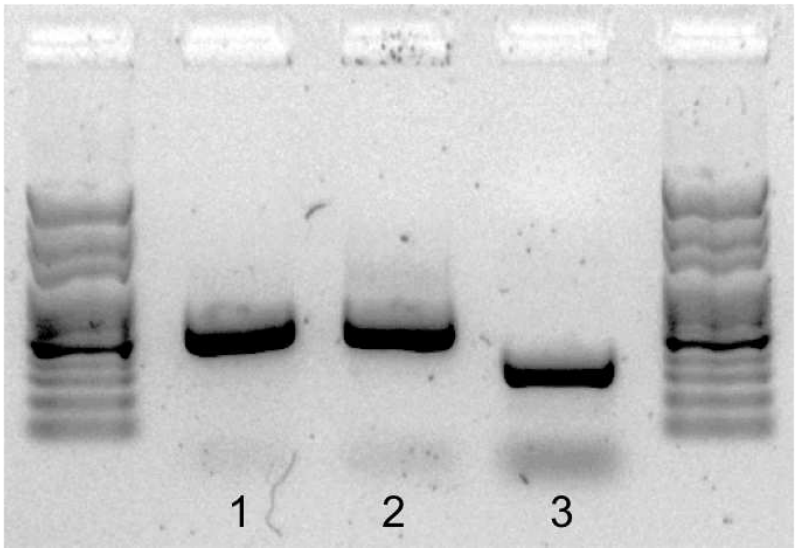
278 **Fig. 2.** PCR sex determination for red phalaropes at Igloolik, Nunavut, Canada and Barrow,
279 Alaska. PCR products were separated with agarose gel electrophoresis and stained with
280 GelRed™ nucleic acid (Biotium, Inc., Hayward, California, US; see methods) using sexing
281 primers specific to birds (2550F/2718R; Fridolfsson & Ellegren 1999). Lanes on sides are for the
282 molecular weight marker while 1) is for a Barrow male (550bp), 2) for the ambiguous bird
283 (550bp), and 3) for typical Barrow female (330 bp).

284

285 **Figure 1**

286 

287 **Figure 2**



288