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CFO-FUNDED RESEARCH

Collection and Comparison of Natural Ejaculates and Sperm Morphometrics of Greater (*Centrocercus urophasianus*) and Gunnison Sage-Grouse (*C. minimus*)

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Abstract

In spring 2008, we collected four natural ejaculates from strutting male Greater (Galliformes: *Centrocercus urophasianus*) and Gunnison Sage-Grouse (*C. minimus*) in Colorado, USA by placing freeze-dried

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female Greater Sage-Grouse on leks in the soliciting pre-copulatory position and fitting them with false cloacas. We compared between species the sperm concentrations, the percentage of viable sperm, the types and predominance of normal and abnormal sperm in ejaculate samples, and sperm morphometric traits. In addition, we compared sperm concentration and morphometry of both species with other species of Galliformes. Notwithstanding our small sample size, ejaculate characteristics were similar among individuals and between the two grouse species. Total length did not differ substantially between the two species. However, we found that Greater Sage-Grouse possess sperm heads that averaged 10% longer than those of Gunnison Sage-Grouse and Gunnison Sage-Grouse possess sperm tails that averaged 10% longer than those of Greater Sage-Grouse. Total sperm length in both species is among the smallest known for Galliformes. Compared to other Galliformes, sperm concentration was low for Gunnison Sage-Grouse and average for Greater Sage-Grouse.

Introduction

Greater Sage-Grouse (*Centrocercus urophasianus*) and Gunnison Sage-Grouse (*C. minimus*) are two closely related species of Galliformes that occur in western North America. The two species occur in similar habitats and have similar life histories, exhibiting polygynous lek mating systems in which multiple males gather at a lek and perform strut displays that attract females (Young et al. 2000). The ranges of the two species do not overlap. Greater Sage-Grouse is more geographically widespread while Gunnison Sage-Grouse is restricted to southwest Colorado and extreme southeast Utah (Schroeder et al. 1999, Young et al. 2000, Oyler-McCance et al. 2001, Schroeder et al. 2004).

The two species differ in plumage, morphology, behavior, voice, and genetics (Young et al. 2000, Oyler-McCance et al. 2005). However, molecular differences between them are among the smallest documented between vertebrate species, suggesting that speciation is recent (Avise and Walker 1999, Young et al. 2000). The gene pool of the Gunnison Sage-Grouse is depauperate due to limited gene flow among the remaining subpopulations (Oyler-McCance et al. 2005). The listing of both species under the Endangered Species Act has been ruled "warranted but precluded" (Western Colorado Ecological Services Field Office 2010, Wyoming, Montana, Idaho, Nevada, and Oregon Ecological Services Offices 2010).

Ejaculate characteristics, including sperm concentration and percentage of viable, motile and normally-shaped sperm, are widely used as indices of fertility (Froman et al. 1991, Guzick et al. 2001, Malo et

al. 2005, Waldoch et al. 2007). Low sperm counts and sperm velocity, as well as large proportions of non-viable and morphologically abnormal sperm, have been implicated as indicators of reduced fertility in red deer (*Cervus elaphus hispanicus*, Malo et al. 2005) and Rockhopper Penguins (*Eudyptes chrysocome chrysocome*, Waldoch et al. 2007). In addition, sperm morphometric traits, including head size, tail (flagellum) length, and total length, have been shown to be related to sperm longevity and the intensity of competition in birds (Briskie and Montgomerie 1992, Johnson and Briskie 1999, Halfenstein 2008).

Pellat and Birkhead (1994) cite several techniques for collecting ejaculates from birds, but many techniques require specialized training and handling of birds, and can be costly in terms of personnel time and training, as well as stress and potential injury to birds. In addition, these techniques produce ejaculates that are not likely to reflect natural ejaculates in their qualities and characteristics (Pellat and Birkhead 1994). However, natural ejaculates have been obtained from African Ostriches (*Struthio camelus*) and Japanese Quail (*Coturnix japonica*) through the use of live dummy teaser females and from Zebra Finches (*Taeniopygia guttata*), Red-winged Blackbirds (*Agelaius phoeniceus*), and Bank Swallows (*Riparia riparia*) with the use of taxidermically mounted females fitted with a false cloaca (e.g., Pellat and Birkhead 1994, Westneat et al. 1998, Nicholls et al. 2001, Rybnik et al. 2007, Chelmonska 2008). Here we present data on ejaculate characteristics and sperm morphometrics from natural ejaculates collected from Greater Sage-Grouse and Gunnison Sage-Grouse using taxidermically mounted female Greater Sage-Grouse.

Methods

Field Sites—Male Greater Sage-Grouse ejaculates were collected on a lek in Moffat County, Colorado, USA. The lek is located on Bureau of Land Management (BLM) property in the Axial Basin approximately 40 km southwest of Craig, Colorado. Gunnison Sage-Grouse ejaculates were collected on a lek in northern Saguache County, Colorado. The lek is located in the upper Gunnison Basin on BLM lands, approximately 40 km southeast of Gunnison, Colorado. Both lek sites were in relatively open habitats dominated by big sagebrush (*Artemesia tridentata*). Sampling took place in the spring of 2008.

Ejaculate Collection—Natural ejaculate samples were collected using freeze-dried dummy Greater Sage-Grouse hens (Kulis Inc., Bedford, OH, USA) taxidermically mounted in the pre-copulatory position (Fig. 1) and placed on the lek. Dummy hens were mounted

with a false cloaca made from 1.9 cm diameter vinyl tubing similar to those used in other studies (Pellat and Birkhead 1994, Fig. 2). False cloacas were filled with 2 ml of Beltsville Turkey Semen Extender II (Beltsville Inc, MD, USA). The use of semen extender significantly prolongs the mobility of avian sperm collected in the field (Sexton 1977, Penfold et al. 2001). In the event that the male ejaculated on the female rather than into the false cloaca during copulation, we pipetted it off the feathers and placed it in 2 ml of semen extender.

The dummy hens were placed on leks by several methods, including a stationary mount, a non-motorized wheeled mount, a motorized wheeled mount, and a sliding track mount. Methods of dummy placement were tested to determine male response to the movements of the dummy hens. We chose dummy placement methods that caused the least amount of disturbance and elicited male mating response. We measured disturbance by dummy avoidance, reduction in strutting, and flushing away from the dummy.

Camouflage blinds (Trekker T-200, Foundton Co. Ltd., Hong Kong, China) were erected on leks during afternoon hours when no strutting males were present. Blinds were placed either within or on the edges of leks. Track systems or rope pulley systems, used to mobilize dummy hens, ran outward from the front of the blinds and were camouflaged. Field researchers entered the blind a minimum of 45

*Fig. 1. A female Greater Sage-Grouse (*Centrocercus urophasianus*) dummy hen used to collect natural ejaculates.*

minutes before sunrise and did not leave until all birds had departed the lek. Detailed notes on the plumage characteristics (broken rectrices, tail patterns) of birds that mated with the dummy hens were recorded to ensure identification of individual birds. In addition, copulations were video-recorded, if sufficient light was available, to aid in individual identification. All ejaculate samples were collected from males who had not been observed copulating earlier on the morning of collection. Ejaculate samples were collected from mobile dummy hens once the dummy hen was pulled back to the blind via track/pulley system or, when stationary mount dummy hens were used, after birds had vacated the lek.

Laboratory Methods—Extended semen samples were transported to laboratories at Western State College in Gunnison, Colorado or a Colorado Division of Wildlife office in Craig, Colorado, approximately 45 minutes from sampling locations. All samples were processed 1-3 hours after collection. First, dilution factors were determined by dividing the volume of ejaculate collected by the volume of the extender. Next, 1 µl of a dilute (0.3%) solution of gluteraldehyde was added to a 5-µl aliquot of extended semen to immobilize the spermatozoa. The immobilized sperm aliquot was placed in a hemacytometer and counted to estimate sperm concentration, accounting for dilution factors. To test viability, we placed a separate 2-µl aliquot of extended semen in 20 µl of eosin-nigrosin solution and incubated it at room temperature for two minutes. Afterwards this solution was smeared on a microscope slide and air-dried. Viable sperm with intact plasma membranes stained yellow, whereas non-viable sperm stained purple (Blom 1950). The percentage of viable sperm was calculated by dividing the number of viable sperm by the total number of sperm counted (~100). In addition, we calculated the percentage of structurally normal sperm versus the type and percentage of abnormal sperm. The most commonly encountered types of abnormalities in the ejaculate samples included headless sperm, tailless sperm, sperm with distal and proximal droplets, and multi-tailed sperm.

Finally, we compared the morphometry of sperm from each species, including head length, tail length, and total length. Ten normal and viable sperm were randomly selected from each of the four individual males and photographed using an Olympus DP12 digital camera mounted on a light microscope at 300× magnification and an image size of 2048 × 1536 pixels. Using the sperm images, we measured the head and tail length (µm) of each sperm using ImageJ 1.4 software (NIH, Bethesda, MD, USA). We included the midpiece in the head measurement due to difficulty in determining where the head ended and midpiece began. Total length of each sperm was de-

termined by adding head and tail length. Means ± SD were calculated for each variable.

Results

We collected a total of four natural ejaculate samples: one from an adult male Greater Sage-Grouse using the non-motorized wheeled dummy mount; one from an adult male Greater Sage-Grouse using a stationary mount; one from an adult Gunnison Sage-Grouse using a stationary mount; and one from a sub-adult Gunnison Sage-Grouse using a stationary mount. Each sample originated from one copulation and from a unique male identified by plumage characteristics, primarily tail feather breakages and markings.

Both Greater Sage-Grouse and Gunnison Sage-Grouse males avoided the motorized dummy hen. Greater Sage-Grouse males responded positively to the wheeled non-motorized dummy mount. However, Gunnison Sage-Grouse males markedly avoided the wheeled non-motorized mount, keeping 2-3 meters away from it. Males of both species responded positively to the stationary mounts, especially when the mounts were placed near the center of the lek. The sliding track mount was problematic due to snowfall throughout the sampling period.

Fig. 2. A caudal view of the dummy hen showing the artificial cloaca.

The shapes of sperm from both Gunnison Sage-Grouse and Greater Sage-Grouse were typical of Galliformes and similar to that of the domestic chicken (*Gallus gallus domesticus*). Sperm concentrations in Gunnison Sage-Grouse were 1.33×10^9 sperm/ml for the adult bird and 1.66×10^9 sperm/ml for the sub-adult bird. Sperm concentrations in Greater Sage-Grouse were 1.33×10^9 sperm/ml for one adult male and $5.05 \times$

10^9 sperm/ml for the other. Percentages of viable sperm were similar among the two species, with values of 84% in the adult Gunnison Sage-Grouse and 88% in the sub-adult, and values of 85% and 88% in the two Greater Sage-Grouse males.

The percentage of structurally normal sperm was 70% and 72% in the sub-adult and adult Gunnison Sage-Grouse, respectively, and 71% and 74% in the two adult Greater Sage-Grouse males. The two most frequently encountered sperm abnormalities in both species were tailless sperm and proximal droplets. The percentage of tailless sperm were 16% and 21% in the two Gunnison Sage-Grouse samples and 13% and 23% in the two Greater Sage-Grouse samples. The percentage of sperm with proximal droplets was 6% in both Gunnison Sage-Grouse samples and 3% and 11% in the two Greater Sage-Grouse samples. Other forms of sperm abnormalities together totaled less than 6% of each sample, including headless sperm, sperm with distal droplets, and two-tailed sperm, in order of increasing rarity.

Greater Sage-Grouse sperm head length, at 22.69 ± 1.77 μm, was 10% longer than Gunnison Sage-Grouse sperm head length, at 20.67 ± 1.65 μm. Sperm tail length in Gunnison Sage-Grouse, at 54.59 ± 4.40 μm, averaged nearly 10% longer than Greater Sage-Grouse sperm tail length, at 49.76 ± 4.12 μm. The mean total length of Gunnison Sage-Grouse sperm was 75.26 ± 5.02 μm compared to 72.45 ± 4.60 μm in the Greater Sage-Grouse.

Discussion

The difference in behavioral responses of Greater Sage-Grouse and Gunnison Sage-Grouse to varied presentations of the dummy hen was not unexpected. Gunnison Sage-Grouse are known to be more sensitive to disturbance (Jessica Young, pers. comm.). The difference in willingness to mate with the non-motorized dummy hen supports assertions that the two species differ behaviorally and that they likely respond to disturbance in different ways.

Behavioral avoidance of the motorized dummy hen by males of both species was likely an artifact of either the "unnatural" rotational motion of wheels, the noise generated by the electric motor, or both. Placement of the immobile dummy hen on the lek prior to strutting caused the least amount of disturbance to males of both species and elicited positive responses.

The use of dummy hens to collect ejaculate from male *Centrocercus* grouse is feasible and preferable to manual collection methods for several reasons. First, utilizing dummy hens does not require capture of adult males and avoids stress and the potential injuries and deaths associated with handling. Second, it involves fewer disturbances of

lekking birds than capturing males on the lek. Lastly, it provides an efficient and non-invasive means for collecting natural ejaculates that are more likely to resemble ejaculates in natural mating attempts (Pellat and Birkhead 1994).

Ejaculate characteristics were generally similar between individuals and species with the exception of sperm concentration. Sperm concentrations were similar among the two male Gunnison Sage-Grouse. Sperm concentrations varied more substantially between the two Greater Sage-Grouse but overlapped among species. One possible explanation for the observed differences in sperm concentration is related to differences in spatial dynamics on the lek, where reduced distances between lekking male Greater Sage-Grouse resulted in greater defense and guarding behavior of the dummy female by Greater Sage-Grouse than by Gunnison Sage-Grouse. Allocation of ejaculate, in the form of sperm concentration, has been observed in Adelie Penguins (*Pygoscelis adeliae*) and in Bank Swallows (*Riparia riparia*) when males partook in copulations while facing potentially increased sperm competition (Hunter et al. 2000, Nicholls et al. 2001). However, Red Junglefowl (*Gallus gallus*) males allocate ejaculate according to the size of female sexual ornaments, female promiscuity, and familiarity with female (Pizzari 2003). Our sample sizes are small and limit the ability to confirm ejaculate allocation in grouse. However, our results do suggest that further investigation of potential ejaculate allocation in *Centrocercus* grouse is warranted.

Gunnison and Greater Sage-Grouse sperm concentrations are moderate to low compared to other Galliformes, but are similar to the more closely related Black Grouse (*Tetrao tetrix*; Table 1). High sperm concentrations are typically associated with high levels of sperm competition (Møller 1988). It is currently believed that most female sage-grouse copulate only once or twice per clutch, suggesting that sperm competition is low in *Centrocerus* grouse. However, recent molecular work on Greater Sage-Grouse has documented multiple-paternity broods, suggesting that some level of sperm competition risk may exist (Semple et al. 2001). How male sage-grouse allocate sperm on the lek and, potentially, off the lek is worthy of further study.

Other unstudied factors associated with lek mating systems may account for the variation observed, including individual male dominance, health, location within the lek, and age. We found no substantial difference in sperm concentration between sub-adult and adult male Gunnison Sage-Grouse, but our sample size was limited. Eng (1963) found that during late April and early May, sub-adult Greater Sage-Grouse testes volume was slightly more than half that

of adult Greater Sage-Grouse. However, he hypothesized that sub-adult male Greater Sage-Grouse were capable of fathering offspring. Our data suggest that sub-adult males in the closely related Gunnison Sage-Grouse have fertility potential similar to that of adults, supporting Eng's hypothesis and agreeing with previous findings (Eng 1963, Wiley 1973, 1974, Hartzler and Jenni 1988). Due to the low survivorship of male *Centrocercus* grouse, it is likely that fertility of sub-adult males would be adaptive (Zablan 1987).

We found that Gunnison Sage-Grouse had 10% longer sperm tails than Greater Sage-Grouse. However, Greater Sage-Grouse sperm heads were 10% longer than Gunnison Sage-Grouse. In a comparison with 28 other species of Galliformes, *Centrocercus* grouse have large sperm heads (Table 2). However, both species of sage-grouse possess among the shortest sperm tail length and total sperm length of the 30 documented species (Table 2).

A number of factors can contribute to sperm shape and length, including phylogeny, sperm competition risk, shape of female sperm

Table 1. A comparison of Greater Sage-Grouse and Gunnison Sage-Grouse sperm concentration with 23 species of Galliformes (sorted from highest to lowest). Note that Greater Sage-Grouse sperm concentration contrasted sharply between the two sampled individuals.

Species		Concentration ($\times 10^9$ sperm/ml) mean ± S.D.			Reference
Common Name	*Latin Name*				
Domestic Turkey	*Meleagris gallopavo gallopavo*	11.1	±	0.2	Noirault and Brillard 1999
Mikado Pheasant	*Syrmaticus mikado*	6.6	±	3.0	Jalme et al. 2003
Temminick's Tragopan	*Tragopan temminckii*	5.4	±	3.4	Jalme et al. 2003
Lady Amherst's Pheasant	*Chrysolophus amherstiae*	4.8	±	2.6	Jalme et al. 2003
Cabot's Tragopan	*Tragopan cabotii*	4.7	±	1.6	Jalme et al. 2003
Swinhoe's Pheasant	*Lophura swinhoii*	4.1	±	1.8	Jalme et al. 2003
Berlioz's Silver Pheasant	*Lophura nyctemera berliozi*	4	±	1.8	Jalme et al. 2003
Blyth's Tragopan	*Tragopan blythii*	3.8	±	2.2	Jalme et al. 2003
Himalayan Monal	*Lophophorus impeyanus*	3.8	±	2.2	Jalme et al. 2003
Satyr Tragopan	*Tragopan satyra*	3.3	±	1.8	Jalme et al. 2003
Red Junglefowl	*Gallus gallus*	3.1	±	1.6	Jalme et al. 2003
Greater Sage-Grouse	***Centrocercus urophasianus***	**3.1**	**±**	**2.0**	**Present study**
Palawan Peacock Pheasant	*Polyplectron emphanum*	2.7	±	1.8	Jalme et al. 2003
Cheer Pheasant	*Catreus wallichi*	2.5	±	1.0	Jalme et al. 2003
Grey Peacock Pheasant	*Polyplectron b. bicalcaratum*	2.5	±	1.0	Jalme et al. 2003
Elliots's Pheasant	*Syrmaticus ellioti*	2.4	±	1.9	Jalme et al. 2003
Edward's Pheasant	*Lophura edwardsi*	2.4	±	2.0	Jalme et al. 2003
Blue-eared Pheasant	*Crossoptilon auritum*	2.3	±	1.5	Jalme et al. 2003
Cabot's Tragopan	*Tragopan cabotii*	2.3	±	0.2	Zhang 2006
Capercaillie	*Tetrao urogallus*	1.6	±	-	Ciereszko et al. 2010
Gunnison Sage-Grouse	***Centrocercus minimus***	**1.5**	**±**	**0.2**	**Present study**
Black Grouse	*Tetrao tetrix*	1.3	±	-	Ciereszko et al. 2009
Common Koklass	*Pucrasia macrolopha*	1.2	±	0.8	Jalme et al. 2003
Japanese Quail	*Coturnix japonica*	0.7	±	0.2	Chelmonska et al. 2008
Common Piping Guan	*Pipile cumanensis cumanensis*	0.4	±	-	DeMatteo et al. 2004

storage tubules, and female reproductive phenology (Birkhead and Møller 1992, Briskie and Montgomerie 1992, Briskie and Montgomerie 1993, Briskie et al. 1997, Gage 1998, Johnson and Briskie 1999, Immler et al. 2007). Determining what factors best explain the differences in sperm morphometry observed between Greater Sage-Grouse and Gunnison Sage-Grouse is difficult given our limited sample size and lack of understanding of the interspecific range of variation in sperm morphometry within sage-grouse. Length of sperm storage and the relationships between sperm longevity and morphometry are still debated (Cardullo and Baltz 1991, Immler and Birkhead 2007, Immler et al. 2007). Both species possess similar breeding life histories that would suggest that length of sperm storage does not explain the observed differences (Petersen 1980, Young 1994, Schroeder et al. 2004). However, the shape of female sperm storage tubules in *Centrocercus* grouse has yet to be described.

Data describing ejaculate characteristics and sperm morphometry traits of North American Galliformes is lacking. Better documentation of galliform ejaculate and sperm morphometry traits could prove useful in understanding sperm competition theory. In addi-

Table 2. A comparison of Greater Sage-Grouse and Gunnison Sage-Grouse sperm head, tail, and total length (μm) with 28 species of Galliformes (sorted by total length from longest to shortest).

Species		$Head_a$ (μm)	Tail (μm)	Total (μm)	Reference
Common name	**Latin Name**				
Japanese Quail	*Coturnix japonica*	191.40	50.00	240.00	Korn et al. 2000
Germain's Peacock Pheasant	*Polyplectron germaini*	33.75	108.41	130.03	Immler et al. 2007
Grey Peacock Pheasant	*Polyplectron bicalcaratum*	22.86	101.16	113.93	Immler et al. 2007
Palawan Peacock Pheasant	*Polyplectron emphanum*	26.14	97.46	111.32	Immler et al. 2007
Red Junglefowl	*Gallus gallus*	19.30	90.00	109.30	Lake et al. 1968
Indian Peafowl	*Pavo cristatus*	25.65	78.62	97.50	Immler et al. 2007
Vietnamese Pheasant	*Lophura hatinhensis*	26.96	73.50	95.26	Immler et al. 2007
Elliots's Pheasant	*Syrmaticus ellioti*	21.76	72.81	90.62	Immler et al. 2007
Red Junglefowl	*Gallus gallus*	17.28	76.89	89.80	Immler et al. 2007
Temminick's Tragopan	*Tragopan temminckii*	16.56	75.97	88.68	Immler et al. 2007
Blyth's Tragopan	*Tragopan blythii*	16.25	76.17	88.41	Immler et al. 2007
Cabot's Tragopan	*Tragopan caboti*	20.13	71.66	87.67	Immler et al. 2007
Himalayan Monal	*Lophophorus impeyanus*	15.28	75.47	87.37	Immler et al. 2007
Satyr Tragopan	*Tragopan satyra*	16.09	75.13	87.15	Immler et al. 2007
Silver Pheasant	*Lophura nycthemera*	17.50	73.62	85.75	Immler et al. 2007
Brown-eared Pheasant	*Crossoptilon mantchuricum*	20.58	66.66	83.09	Immler et al. 2007
Black Grouse	*Tetrao tetrix*	17.44	64.12	81.56	Ciereszko et al. 2009
Mikado Pheasant	*Syrmaticus mikado*	15.50	69.14	80.19	Immler et al. 2007
Swinhoe's Pheasant	*Lophura swinhoii*	17.04	68.45	79.43	Immler et al. 2007
Green Pheasant	*Phasianus versicolor*	14.75	67.61	78.33	Immler et al. 2007
Hume's Pheasant	*Syrmaticus humiae*	22.84	59.67	78.11	Immler et al. 2007
Guinea Fowl	*Numida meleagris*	18.50	59.00	77.50	Thurston et al. 1982
Cheer Pheasant	*Catreus wallichi*	14.40	66.95	76.80	Immler et al. 2007
Lady Amherst's Pheasant	*Chrysolophus amherstiae*	16.31	65.02	76.76	Immler et al. 2007
Domestic Turkey	*Meleagris gallopavo gallopavo*	15.70	61.00	76.70	Marquez and Ogasawara 1975
Gunnison Sage-grouse	***Centrocercus minimus***	**20.67**	**54.59**	**75.26**	**Present study**
Capercaillie	*Tetrao urogallus*	15.65	57.62	73.27	Ciereszko et al. 2009
Blue-eared Pheasant	*Crossoptilon auritum*	14.81	62.50	73.20	Immler et al. 2007
Greater Sage-grouse	***Centrocercus urophasianus***	**22.69**	**49.76**	**72.46**	**Present study**
Edward's Pheasant	*Lophura edwardsi*	16.36	59.87	71.39	Immler et al. 2007

$Head_a$ measurement includes sperm midpiece.

tion, grouse worldwide are experiencing dramatic population declines (Storch 2007). The collection of wild male grouse ejaculates may be important to captive breeding programs using artificial insemination. Through the use of a dummy hen similar to ours and combined with lek observations, biologists could selectively harvest ejaculate from the males with the most copulations with wild females while minimizing disturbance to wild birds. In this way captive females would be fertilized by males preferred by wild females, perhaps producing more fit offspring for captive propagation and/or release into the wild.

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PEER REVIEW

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