

**Seasonal and diurnal variation in the proportions of female morphs of
the damselfly *Enallagma cyathigerum***

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Abstract – In many damselfly species a female-limited colour polymorphism is encountered which is assumed to be the result of sexual conflict. Typically, one morph resembles the male's body colouration (andromorph), while the other is dissimilar (heteromorph). Little is known about the extent of temporal variation in female morph proportions at the water, where mating occurs. Knowledge on such variation should help to identify the factors that affect female morph proportion and the scales at which these factors operate. Here, we assess the occurrence of diurnal and seasonal variation in female morph proportions at the water for the damselfly *Enallagma cyathigerum*. Diurnal variation was evaluated at six locally nearby populations; while seasonal variation was examined at one of these populations. Further we considered temporal variation in female morph proportion in relation to proxies of male harassment (i.e. male density and sex ratio). Our findings indicate female morph proportion to vary throughout a day, but to be constant on a seasonal scale. Variation in female morph proportions could not be explained by concomitant variation in male density or sex ratio. We suggest future study of male mate choice may consider temporal variation in female morph proportions at the water.

Keywords: polymorphism, morph proportion, damselfly, temporal variation

Running title: Temporal variation in female morph proportions

INTRODUCTION

Female-limited polychromatism occurs frequently in species of damselflies (Odonata: Zygoptera) (Corbet 1999) and has been found genetically determined (reviewed in Andrés and Cordero 1999; Sánchez-Guillèn et al. 2005). Typically, two adult female phenotypes coexist in natural populations: one morph is coloured as the conspecific male (further referred to as andromorph) while the other morph(s) is dissimilar in body colouration (heteromorph) (Corbet 1999). Most contemporary explanations concerning the maintenance of this polymorphism point to sexual conflict as the main selective force (e.g. Andrés et al. 2000). While males pursue maximisation of the copulation rate, females evolved traits to avoid excessive male harassment (e.g. Arnqvist and Nilsson 2000). One such adaptive trait could be a female polymorphism whereby certain phenotypes gain by receiving less harassment from mate searching males that do not readily recognise them as female (e.g. Robertson 1985). Indeed, almost all recent theories on this polymorphism proposed some form of frequency-dependent advantage, and all assume that the primary selective force is differential harassment of morphs by males (e.g. Miller and Fincke 1999; Sherratt 2001; Fincke 2004). Male harassment is considered to affect female fitness because it can reduce individual time and energy budgets, and may increase physical damage (e.g. Robertson 1985; Forbes et al. 1997; Sirot and Brockmann 2001).

Damselflies are obligate aquatic organisms in that their immature (larval) stage is spent in freshwater habitats (Corbet 1999). Their life cycle consequently requires that

adult females return to the water to lay eggs. In many species (including our study species), mating also takes place at the water, so males aggregate there with the aim to intercept females that are ready to mate and lay eggs. Males will compete for access to females appearing at the water. Females who are unreceptive to mating are likely to find the excessive male attention at the water harassing, and thus tend to stay away to avoid such harassment. As such, sex-ratios at the water, i.e. operational sex ratios (OSR), for damselflies are generally male-biased (e.g. Corbet 1999 Table A.11.19; Stoks 2001a; 2001b; Conrad et al. 2002). Higher male densities and more male-biased operational sex ratios are thought to be associated with more intense male harassment (e.g. Rowe et al. 1994), and as such thought to affect female morph proportions at the water, that, similar to sex ratios, can be seen as operational female morph proportions (see also discussion and Forbes et al. 1995; Andrés et al. 2000).

Despite agreement on the function of frequency-dependent selection, surprisingly little work has documented the range of spatial and temporal variation in female morph proportions (operational and at the population level) that exists within species. Knowledge on variation in female morph proportions and the scales at which factors operate is of prime importance to understand the maintenance of sex-limited polymorphism. The few studies (some with an operational, some with a population focus) propose that female morph proportions remain relatively constant over several weeks to several years at individual sites, but vary to some extent among nearby populations (Forbes et al. 1995; Andrés et al. 2000, 2002; Sirot et al. 2003; Wong et al. 2003). More recent work exploring the degree of spatial variation in andromorph proportions within

and among species, however, has revealed far higher variability in female morph proportions (in at least some species) than previously appreciated (Fincke et al. 2005; Sánchez-Guillén et al. 2005). For example Sánchez-Guillén et al. (2005) found andromorph proportions of *Ischnura elegans* to range from 4 to 91% for nearby populations in Northwest-Spain. On spatial and temporal scales also, Svensson et al. (2005) observed significant variation (36 to 100%) in andromorph proportions in *Ischnura elegans* during their four year study and provided support that frequency- and density-dependent harassment drives changes in morph proportions between generations.

Variation on shorter time scales, i.e. seasonal or diurnal, specifically in operational female morph proportions, may have profound effects on the maintenance of sex-limited polymorphism in damselflies. Indeed, it has been shown that female morph proportions at the site of reproduction influence male mate choice (Miller and Fincke 1999; Van Gossum et al. 1999; Van Gossum et al. 2001a,b). Male mate preference is a plastic trait that tracks changes in female morph proportion, with males predominantly preferring the most common morph at the site of reproduction (Van Gossum et al. 2001b). Earlier work (Fincke 1994; Cordero and Andrés 2001) indicated female morph proportions may differ over the time span of about a month. However, we are not aware of any published work that reports on exploring the variation in female morph proportion over an entire reproductive season, which typically lasts several months. Also, we are not aware of any study evaluating daily variation in operational female morph proportions. Interestingly, Miller and Fincke (2004) recently studied two syntopic species of *Enallagma* and showed changes in male mate preference for con- and heterospecific

females throughout a day. More in detail, early in the day when females of both species were scarce at the water, males apparently cued to female-specific traits, later on, when females became more abundant, males switched to morph-specific traits reducing the amount of mistakes with unfavourable partners.

Here, we assess daily and seasonal variation in operational female morph proportions in *Enallagma cyathigerum*. The main aim of our paper was not to explain the consequences of variation in female morph proportion for the maintenance of female-limited polymorphism. Rather, we address whether seasonal and daily variation should be considered to understand this phenomenon. As male and female densities at the water and operational sex ratios may change over time (Corbet 1999), and give indication on the amount of male harassment that takes place, we also evaluated whether these parameters could explain any variation in female morph proportions at the water (see also Forbes et al. 1995; Cordero and Egidio 1998; Andrés et al. 2002).

MATERIAL AND METHODS

Study species

The damselfly *Enallagma cyathigerum* (Charpentier 1840) is a common European damselfly. Males show conspicuous light blue body coloration with black markings (Askew 2004). Females come in two distinct morphs. Andromorph females closely

resemble the conspecific males, but have a more expressed black patterning on the abdomen. Heteromorph's body parts range from brown to green-brown. The study species is found in a wide variety of habitats including eutrophic waters. They are especially abundant at oligotrophic acid pools and sandy soils (Mostert and Dingemanse 2002). Adults emerge from aquatic larvae continuously from mid-May to mid-August in the study area. The period in which adults can be observed is referred to as the reproductive season.

Monitoring of diurnal and seasonal variation

For our study of diurnal and seasonal variation, we assessed operational female morph proportions (number of andromorphs/total number of females at the water), densities (number of individuals encountered in a transect - see further) and operational sex ratios (OSR: number of males/total number of individuals at the water). To determine numbers of males and female morphs we made observations with the naked eye and by using binoculars (Opticron 10x50 during 1999; Kite Pallas 8x32 during 2003), while slowly walking along a marked transect. The observed numbers include all individuals present (both single and mating). Males and female morphs differ in colour, and potentially also in behaviour and micro-habitat use (e.g. Van Gossum et al. 2001a). Therefore, it may be argued that the sexes and the female morphs differ in the likelihood on being detected in their natural setting. For an experienced researcher, however, we think this to be unlikely, as an efficient search image for damselflies (rather than colour) is quickly developed.

Diurnal variation was studied in six populations (fens) in two heath lands in the north of Belgium in 1999 (distance between fens ranged from 0.61 to 12.00km; see table I). Movements between nearby ponds occur commonly in damselflies but very seldom over distances of more than 0.5km (Conrad et al. 1999). Therefore, we consider it very unlikely that our results are affected by individuals moving between our sites of study. Three populations were located in the nature reserve 'Grenspark De Zoom - Kalmthoutse Heide' and three in the military domain 'Groot Schietveld'. For each population two transects (minimum 30m apart) were demarcated with bamboo sticks. A transect (length 20m) consisted of a 3.5m strip of shore-line vegetation and an adjacent strip of 2m open water. Each population was sampled 3 times on one day, respectively at 11am (morning), 2pm (early afternoon) and 5pm (late afternoon) between 16 and 26 July 1999. The two transects within the same populations were always sampled with a maximal intervening time of half an hour to avoid counting the same individual twice. Within transects, observations were made while slowly walking from one end to the other keeping a close eye on the damselflies present. Therefore, also within transects we consider double counts of individuals unlikely. Monitoring was only carried out on sunny days when animals were sexually active (i.e. males were attempting to mate females). To evaluate effects of density we aimed at including populations with a large range of densities.

Seasonal variation in operational female morph proportions was assessed in 2003 for one of the populations ('Parkingven' situated in 'Grenspark De Zoom - Kalmthoutse Heide') sampled also for diurnal variation. Sampling was exerted every two weeks, from

the end of May till the end of August, resulting in seven observation days, and covered the entire reproductive season. Since the average lifespan of the study species (e.g. Parr 1976) is approximately two weeks, it is most likely that each individual was only observed during one sampling occasion. We divided the margin of this fen into seven interconnected transects, each 20m in length and 3m width (2m land and 1m water), marked with bamboo sticks. To control for potential diurnal variation, monitoring was restricted to the time between 10am and 1pm. Further, ambient temperature was measured every sample day at each transect to test the effects of weather on the observed numbers (note that this was not done when monitoring daily variation in proportions).

Statistical analysis

Seasonal and diurnal variability in morph density, operational andromorph proportion and operational sex ratio (OSR) was assessed using linear mixed model analysis (Littell et al. 1996; Verbeke and Molenberghs 1997). Explanatory variables (fixed effects) were observation day (for season) or moment (for diurnal), morph (male, andromorph, heteromorph) and their interaction. In the analysis of diurnal variation, observation day (and the interaction) was added as an additional explanatory variable. Observation day and moment were treated as class variables. Since temperature differed among observation days ($F_{6, 154}=76.0$, $p<0.001$), we reran the analyses with observation day replaced by temperature. Finally, we also tested whether male density and OSR influenced andromorph proportions. Since data collected on the same transect or fen are

not spatially independent, we added transect (for temporal effects) or transect nested in fen (for testing the relationship between andromorph proportions and OSR or male density) as random effects in the model to avoid pseudoreplication and to account for inter-transect differences.

All linear mixed model analyses were done in SAS 9.1 (SAS Institute Inc. 2005) using the proc MIXED module in the case of normal error distribution (densities, log (n+1) transformed), and with proc GLIMMIX in the case of binomial errors (morph proportions and OSR). Degrees of freedom of the fixed effects F-tests were adjusted for statistical dependence using the Kenward-Roger method (Kenward and Roger 1997). Variance components were estimated by the restricted maximum likelihood method (REML) (Littell et al. 1996). Model selection was started with the full model, including all interactions (Verbeke and Molenberghs 1997). Non significant terms were eliminated in a backward selection procedure until only significant terms remained in the model.

RESULTS

Diurnal variation

The analysis of diurnal density variation revealed a significant moment x fen interaction (fig. 1: $F_{10, 77} = 2.36$, $p = 0.017$). Overall densities evolved differently among fens. Densities gradually decreased during the day in fen 2 and increased in fens 1 and 3, while

densities were highest in early afternoon (2 pm) in fens 4 and 5. In addition, densities of males and female morphs differed significantly among fens (fig. 1: fen x morph interaction $F_{10, 77} = 3.19$, $p = 0.002$). Population 2 showed the lowest density and population 5 the highest (fig. 1). Males always outnumbered females ($F_{1, 77} = 384.75$, $p < 0.001$). Differences were highest in fen 3 and 5 and smallest in fen 2. Among the females, heteromorphs were the most common morph in all studied populations ($F_{1, 77} = 216.31$, $p < 0.001$).

The observed andromorph proportions per transect varied between 0 and 39 % (mean = 17%, median = 16%, 25-75% quartile range 8% - 29%) and differed among study populations ($F_{5, 27} = 3.19$, $p = 0.022$). Although sample sizes were low in some instances (i.e. in populations where densities were low), on average estimates of female morph proportions per transect were based on suitable sample sizes (Table II; see also Cordero & Andrés 2001). Andromorph proportions changed during the day (fig. 2a: $F_{2, 27} = 5.75$, $p = 0.008$) and patterns did not differ significantly among fens (fen x moment interaction: $F_{10, 17} = 0.89$, $p = 0.561$). According to the model estimates, average andromorph proportion is less than 20% during morning and late afternoon and raises to about 27% in the early afternoon (2 pm). Also, OSR changed during the day, but did so differently among fens (fig. 2b, interaction fen x moment: $F_{10, 18} = 3.05$, $p = 0.019$). OSR ranged from 73 to 100 % (mean = 87%, median = 90%, 25-75% quartile range = 81% - 93%). Male proportion increased during the day for fens 2 and 3. For the other fens, male proportions decreased from the morning to early afternoon. Later during the day, proportions increased again (e.g. fens 5, 6) or further decreased (e.g. fen 3).

Neither male density ($F_{1, 11.27} = 0.02$, $p = 0.900$) nor OSR ($F_{1, 25.05} = 2.14$, $p = 0.156$) could explain variation in andromorph proportions.

Seasonal variation

Male and female morph densities varied differently among observation days (fig. 3; interaction morph x day: $F_{12, 120} = 2.25$, $p = 0.013$). Phenology in male and heteromorph density appeared very similar, both steadily increasing until a peak value was reached on July 28th, where after densities decreased again. Andromorph densities were always very low and showed no clear pattern. In general, males outnumbered females ($F_{1, 120} = 694.38$, $p < 0.001$) and heteromorphs were the most common female morph ($F_{1, 120} = 82.39$, $p < 0.001$) throughout the entire study period (mean andromorph proportion = 17%, median = 0%, 25-75% quartile range 0% – 25%). Date of appearance (Julian date) did not differ among the female morphs and males ($F_{2, 102} = 0.11$, $p = 0.897$).

OSR ranged from 62 to 100 % (mean = 90%, median = 90%, 25-75% quartile range = 86% - 96%). Andromorph proportion ($F_{6, 27.66} = 0.87$, $p = 0.53$) nor OSR ($F_{6, 42} = 1.35$, $p = 0.256$) changed significantly over the season. The proportion of andromorphs among the females increased with increasing OSR (fig. 4: $F_{1, 28.31} = 5.47$, $p = 0.027$), but not with male density ($F_{1, 14.91} = 0.76$, $p = 0.397$).

Separate analyses for temperature revealed a significant morph x temperature effect on densities ($F_{2, 94} = 4.63$, $p = 0.012$). Whereas densities of males and heteromorphs increased when temperature raised, the densities of andromorphs did not change. As a consequence, the proportion of andromorphs tended to decrease when temperature rose ($F_{1, 33.72} = 3.34$, $p = 0.077$). Temperature did not affect the OSR ($F_{1, 47} = 0.96$, $p = 0.331$).

DISCUSSION

Temporal variation in observed andromorph proportions for *E. cyathigerum* at the water lay between 0% and 40% of females being andromorphic, which is consistent with the variability found for many species of damselflies (McKee et al. 2005). However, Fincke et al. (2005) in their review noted variation in andromorph proportion for *E. cyathigerum* ranging from 17 to 76% for different countries in Europe. Our results, further indicate that female morph proportions not only vary among populations (Sánchez-Guillèn et al. 2005) or years (Svensson et al. 2005), but also on shorter temporal scales. For our study species, andromorph proportions fluctuated on a daily level, but appeared stable throughout the season. However, seasonal variation may be present in other populations or species as indicated by recent work on the damselfly *Nehalonia irene* for which andromorph proportions at a site could range from 0% to 36% over the course of a reproductive season (Van Gossum et al. submitted). Together, short-term temporal variation in andromorph proportions may have relevance for understanding maintenance of female-limited polymorphism.

Indeed, it has been shown that female morph proportions (Miller and Fincke 1999; Van Gossum et al. 1999; Van Gossum et al. 2001b) and densities (Miller and Fincke 2004) affect male mate preferences. When female morph proportions fluctuate, males can modify their search image. Such alterations in male preference can occur rapidly on the scale of a few days to a few hours (Van Gossum et al. 2001b; Miller and Fincke 2004). Additionally, changes in male density or OSR may also affect male mate choice (Van Gossum et al. 2001a). Therefore, we think it is necessary to evaluate whether this short-term variation in morph proportion, density, OSR, or a combination of these factors influence mate searching males, i.e. might affect the evolution of female-limited polymorphism in damselflies.

One of the considerations when sampling female morph proportion has been to examine long-term selection. For example, Svensson et al. (2005) pooled data on morph numbers that were collected at different moments, spread as much as possible over an entire season to allow comparison among years. Cordero and Andrés (2001) suggested cumulating numbers of females from several consecutive days to estimate andromorph proportion based on satisfactory sample sizes. Most studies when cumulating data or when comparing data on andromorph proportions between sites do not provide detailed information on time of day or moment in the season when sampling occurred. Here, we indicate that these widely used methodologies may hide an important source of variation.

Phenologies of males and females were comparable. Earlier studies indicated presence of differences between female morphs in phenology, and typically refer to

whether one of the female morphs is present earlier during the reproductive season (Fincke 1994; Abbott and Svensson 2005; Robb et al. 2006, but see Cordero 1994). It has been suggested (Fincke 1994; Abbott and Svensson 2005) that if the genes for body colour and timing of emergence were in linkage disequilibrium, this association could explain one female morph occurring earlier. For *E. cyathigerum* we did not detect any difference in date of appearance between the morphs. Possibly, this was due to the large intervals among sampling dates in our study (every two weeks).

Previous research indicated that andromorph proportions correlate positively with male density and/or OSR (Forbes et al. 1995; Cordero and Egido 1998; Andrés et al. 2002). The idea is that when male density or OSR increases at the reproductive site (the edge of the fen), per capita male harassment becomes more intense (see also Rowe et al. 1994). In addition, it is assumed that male harassment affects female fitness negatively and that male damselflies harass female morphs differentially (e.g. Sirot and Brockmann 2001). Typically, andromorphs are considered to receive least male harassment. Consequently only andromorphs should be able to remain at the reproductive site, while heteromorphs are forced to forage away from males when not receptive (Hinneking 1987; Forbes et al. 1995). During the present study, andromorph proportion was positively correlated with OSR during the seasonal study in 2003. However, this relationship was absent during the analysis of diurnal variation in 1999. An absence of a positive correlation between andromorph proportion and male density or OSR was also observed for some species included in a study by McKee et al. (2005).

Female morph proportions might be variable over time because of differences in the related thermal properties. Indeed, it has been shown that colour characteristics may have important effects on thermal characteristics and ecology of colour morphs (Brakefield and Willmer 1985; De Jong et al. 1996; Forsman 1997; Forsman et al. 2002). As such this may lead to temporal or geographical fluctuations in morph proportions in relation to thermal environmental conditions (e.g. Brakefield 1984). Here, we show for *E. cyathigerum* variation in morph proportion may be affected by the ambient temperature or a correlated factor. We suggest future research may question the selective advantage of colour morph in relation to thermoregulation (see for example Forsman et al. 2002).

In conclusion, recent work (including this study) indicates that female morph proportions and densities are far more variable in magnitude and occur at more temporal and spatial scales than hereunto believed. Appreciation of this variation and questioning its consequences may help while studying the mechanisms involved in the maintenance of female-limited polymorphism.

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Table I: Distances (minimal and maximal) between our study sites and surfaces of these sites. H1 to H3 are situated in the nature reserve ‘Grenspark De Zoom - Kalmthoutse Heide’, while G4 to G6 are in the military domain ‘Groot Schietveld’, both located in the north of Belgium.

Population	Local name	Surface (m ²)	Distance to nearest/most distant study site (km)
H1	Parkingven	38.600	0.61/10.92
H2	Keetslenkven	1.090	0.61/11.34
H3	Drielingven	83.590	0.74/12.00
G4	Kooitjesven	9.440	1.63/11.37
G5	GrootLoodven	47.100	0.72/11.30
G6	Ven 12	8.840	0.72/12.00

Table II: Average sample sizes of total number of females (andromorphs + heteromorphs) per population studied for diurnal variation (mean \pm SE). Population sample size was calculated based on three sample moments during the day for each of two transects (N = 6). While sample sizes were adequate for populations 3 to 6, sample size was very low for population 2. For population 1 sample size was appropriate except in transect 2 at 2pm, when sample size was zero. H1 to H3 are situated in the nature reserve ‘Grenspark De Zoom - Kalmthoutse Heide’, while G4 to G6 are in the military domain ‘Groot Schietveld’, both located in the north of Belgium.

Population	Local name	Mean \pm SE	Minimum N	Maximum N
H1	Parkingven	18.5 \pm 16.9	0	40
H2	Keetslenkven	3.0 \pm 2.1	1	6
H3	Drielingven	33.8 \pm 35.3	8	103
G4	Kooitjesven	19.3 \pm 6.6	10	26
G5	GrootLoodven	82.2 \pm 19.3	59	111
G6	Ven 12	20.8 \pm 1.3	14	30

FIGURE LEGENDS

Figure 1. Diurnal variation in male and female morph densities. Means are given $\pm 1SE$. Fens H1 to H3 are situated in the nature reserve 'Grenspark De Zoom - Kalmthoutse Heide', while G4 to G6 are in the military domain 'Groot Schietveld', both located in the north of Belgium.

Figure 2. Diurnal variation in andromorph proportion (2a) and operational sex ratio (2b). Means are given $\pm 1SE$. Fens H1 to H3 are situated in the nature reserve 'Grenspark De Zoom - Kalmthoutse Heide', while G4 to G6 are in the military domain 'Groot Schietveld', both located in the north of Belgium.

Figure 3. Seasonal density evolution for males and female morphs. Sample sizes refer to the total number of females sampled at the respective dates. Means are given $\pm 1SE$.

Figure 4. Relationship between operational sex ratio and andromorph proportion throughout the season.

Figure 1.

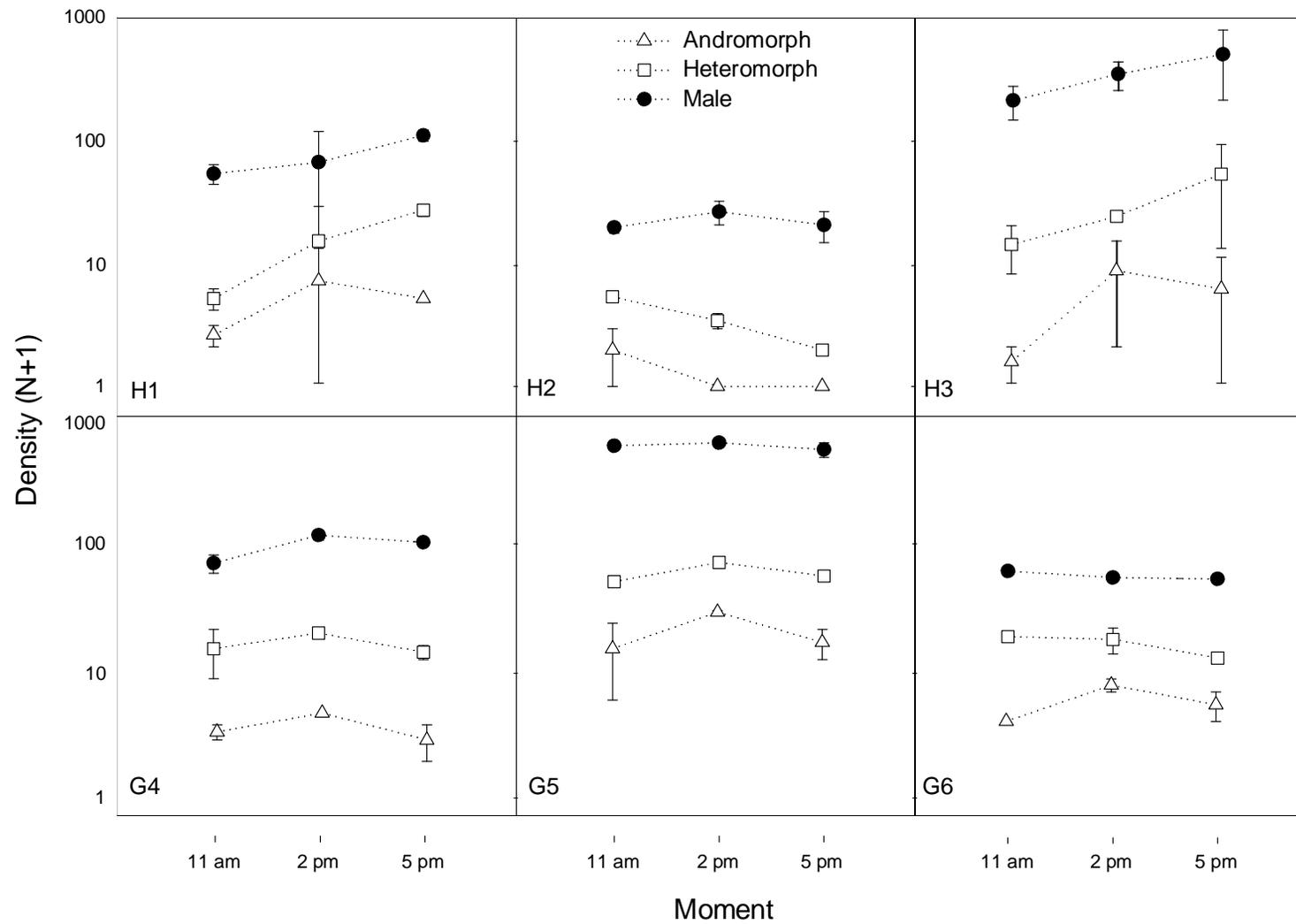


Figure 2.

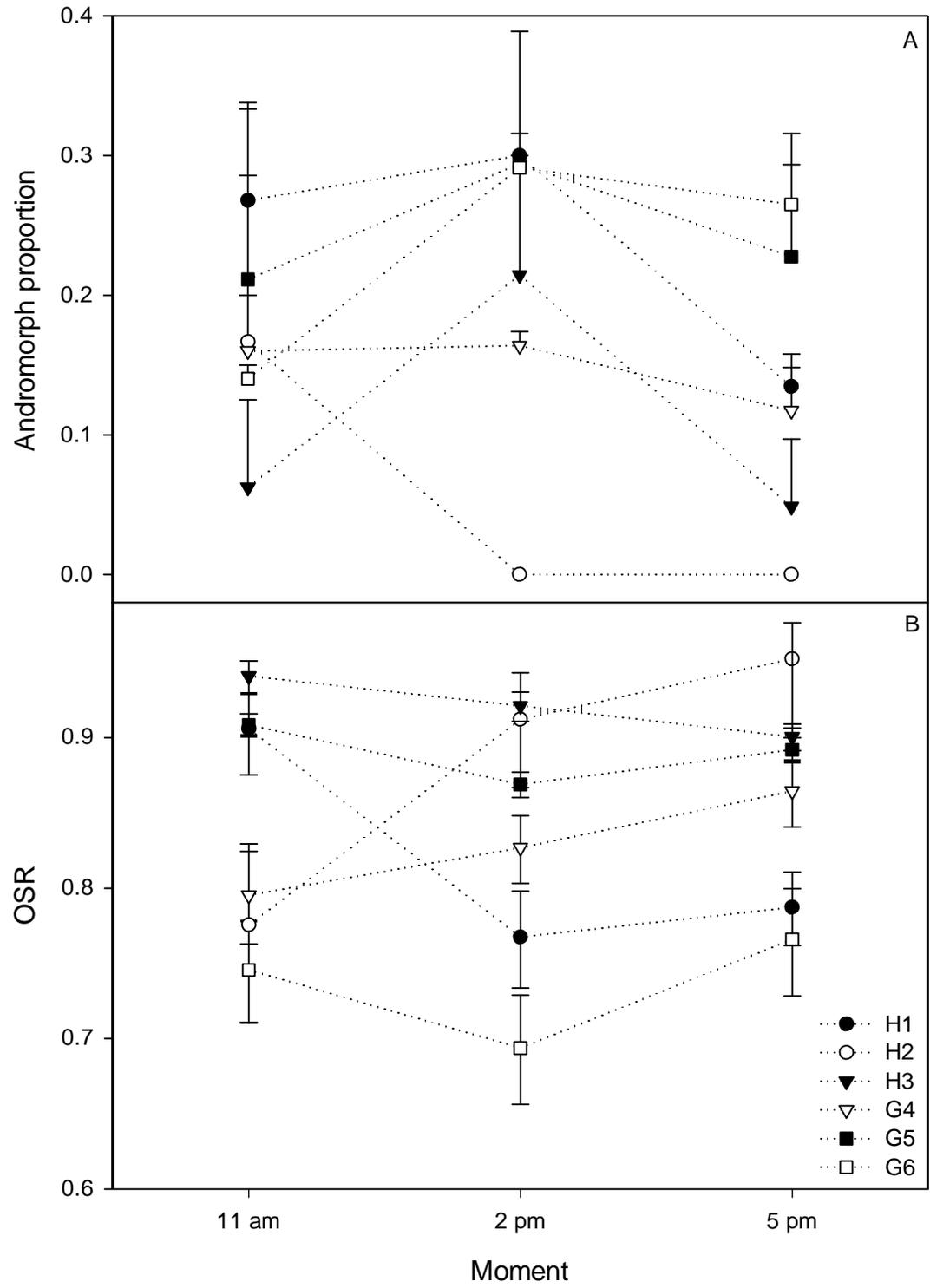


Figure 3.

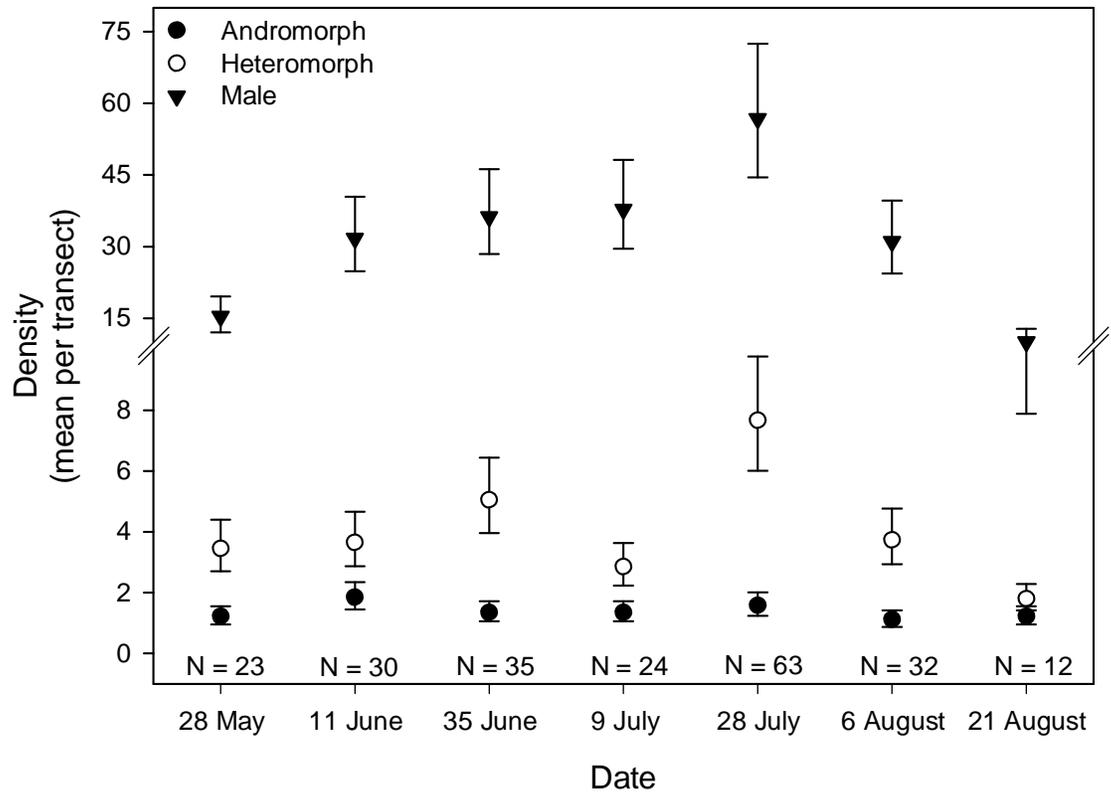


Figure 4.

