Hormone mediated dispersal and sexual maturation in males of the social paper wasp *Polistes lanio*

Robin J. Southon[1,2], Andrew N. Radford[1], Seirian Sumner[1,2]

[1]School of Biological Sciences, University of Bristol, Bristol BS8 1TQ, UK
[2]Centre for Biodiversity and Environmental Research, Department of Genetics, Evolution and Environment, University College London, London WC1E 6BT, UK

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Corresponding author: Robin J. Southon; rjsouthon@gmail.com

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Summary statement

Delayed dispersal from the natal nest by male paper wasps is regulated by juvenile hormone and associated with sexual maturation.

ABSTRACT

Sex-biased dispersal is common in social species, though the dispersing sex may delay emigration if associated benefits are not immediately attainable. In the social Hymenoptera (ants, some bees and wasps), newly emerged males typically disperse from the natal nest whilst most females remain as philopatric helpers. The mechanisms regulating male dispersal, whether male dispersal is directly linked to sexual maturation and if such mechanisms are conserved across the Hymenoptera is relatively unknown. Through field observations and mark–recapture, we observed that males of the social paper wasp *Polistes lanio* emerge from pupation sexually immature, and delay dispersal from their natal nest for up to seven days whilst undergoing sexual maturation. Delayed dispersal may benefit males by allowing them to mature in the safety of the nest and thus be more competitive in mating. We also demonstrate that both male dispersal and maturation is associated with juvenile hormone (JH), a key regulator of insect reproductive physiology and behaviour, which also has derived functions regulating social organisation in female Hymenoptera. Males treated with methoprene (a JH analogue) dispersed earlier and possessed significantly larger accessory glands than their age-matched controls. These results highlight the wide role of JH in social hymenopteran behaviour, with parallel ancestral functions in males and females, and raise new questions on the nature of selection for sex-biased dispersal.

INTRODUCTION

Patterns of dispersal vary considerably within and between species in response to internal physiological or external environmental changes (Bowler & Benton, 2005; Clobert et al., 2009). Dispersal from the natal birth site allows for gene flow across populations and reduces the risk of inbreeding, whilst facilitating the acquisition of alternative breeding or resource opportunities (Keller & Waller, 2002; Ronce, 2007; Matthysen, 2012; Hansson & Åkesson, 2014). Natal dispersal is often sex-biased within species or populations, arising from different selection pressures on the two sexes and inbreeding avoidance (Pusey, 1987; Lawson Handley & Perrin, 2007; Gros et al., 2008). Sex-biased dispersal may also have consequences on social evolution, as philopatry promotes social interactions among members of the non-dispersing sex (Lehmann & Boesch, 2008; Johnstone et al., 2012; Nagy & Knörnschild, 2016). In the social Hymenoptera (ants, some bees and wasps), dispersal from the natal nest is typically male-biased (Hamilton, 1972; Johnstone et al., 2012). Despite the limited role of newly emerged males within natal nests and social groups, male dispersal is often delayed (Cameron, 1986; Poidatz et al., 2018). What regulates male dispersal in social Hymenoptera, and what function delayed dispersal plays, is poorly understood because research on these organisms is almost exclusively focused on females. We address this knowledge gap by examining male dispersal from the natal nest

in a social wasp, by explaining natal dispersal patterns in terms of reproductive physiology and regulatory processes.

Social Hymenoptera lineages have ancestrally solitary life-histories (Hunt, 1999; Hughes et al., 2008). Philopatry to a nest or area in female offspring was likely a key evolutionary innovation for sociality, allowing females to remain with relatives and help raise related brood via developed traits such as adult–larval trophallaxis (Hunt, 1999). Ancestrally, and in many extant species, female helpers retain the ability to reproduce and so may inherit the position of reproductive on the natal nest or disperse to found new nests (Tibbetts, 2007). Therefore, dispersal patterns in female social Hymenoptera reflect diverse reproductive strategies, allowing for both indirect and direct fitness, and have been studied extensively (Queller & Strassmann 1998). By contrast, male behaviour in hymenopteran societies is poorly studied. Male Hymenoptera are thought to be evolutionary limited to direct fitness investment and, with the exception of some complex ant societies where males have limited dispersal, typically depart from the natal nest soon after emergence in search of mating opportunities (Hamilton, 1964, 1972; Johnstone et al., 2012; Heinze, 2016; Hakala et al., 2019). Males of social Hymenoptera therefore appear to have mostly retained ancestral behaviours and life-histories (e.g. male sex-biased dispersal, rarity of brood care and selection towards increased mating success), which are likely to be regulated by similar mechanisms and processes as those of solitary ancestors.

Research on male dispersal and reproductive strategies has been neglected because males are often regarded as little more than “flying sperm” within Hymenoptera societies (Wilson, 1971; O’Donnell, 1999; Beani et al., 2014). Males have limited direct contributions to the natal group, possessing relatively fast-paced life history characteristics in comparison to females (Heinze, 2016). However, studies indicate that male life-histories are not so simple, and that newly emerged males may delay dispersal from the natal nest whilst maturing sexually, or to synchronize with nuptial flights (Hamilton, 1972; Moors et al., 2009; Poidatz et al., 2018; Hakala et al., 2019). Of benefit to males, post-pupation sexual maturation on the natal nest is likely to enhance fitness if the added protection and nutritional resources of the society allows sexual maturity to reach optimal reproductive potential in a protected environment (Litte, 1977; Hunt et al., 1982; O’Donnell & Jeanne, 1992; Leatemia et al., 1995; Yuval et al., 2002; Costamagna & Landis, 2004; Hunt, 2007). Though males that remain in the natal nest may be detrimental to the society, as males drain resources and contribute little or nothing in terms of resource gathering, brood care or nest-defence behaviour (though see Cameron, 1986; Southon, 2018). Female nestmates may tolerate males on the natal nest for a short period of time, if it boosts their indirect fitness via the mating success of their brothers. Thus, selection for delayed dispersal by males may occur if it were coupled with maximising male mating success.

If delayed male dispersal is associated with sexual maturation, a robust mechanism for triggering departure would be one that co-regulates reproductive physiology and behaviour. One such candidate mechanism is the family of juvenile hormones (JHs). JHs play an important role in a wide range of developmental and behavioural processes in insects, with pleiotropic functions that regulate

metamorphosis, diapause, polyphenism and reproduction (Dingle & Winchell, 1997; Hartfelder, 2000; Zera & Cisper 2001). Generally, after emergence JH regulates sexual characteristics in both sexes, such as inducing propensity to disperse and mate (Wyatt & Davey, 1996; Goodman & Cusson, 2012). JH (specifically JH3) is strongly associated with the onset of dispersal flight behaviour in hymenopteran females, though it has also been co-opted to regulate nonreproductive behaviours in sterile female castes (Hartfelder, 2000).

The ancestral function of JH in pre-social Hymenoptera lineages was likely a regulator of female reproductive physiology and associated behaviours (Giray et al., 2005). This ancestral function is conserved in many social Hymenoptera, in which upregulation of JH in females (or application of JH analogues) promotes dominance behaviour, ovarian development and sexual receptivity (Bloch et al., 2000; Giray et al., 2005; Tibbetts & Izzo, 2009; Smith et al., 2013; Walton et al., 2020). For other social Hymenoptera, the ancestral once-reproductive role of JH has been modified. In the honeybee *Apis mellifera*, JH regulates reproductive caste determination during development; in adult females, JH serves a limited function in reproductive queen physiology but is involved in regulating sterile female caste age polyethism (Robinson et al., 1991; Hartfelder, 2000). Such new functions of JH as a regulator of nonreproductive female worker behaviour have evolved in a wide range of social Hymenoptera (e.g. *Polybia occidentalis* – O'Donnell & Jeanne, 1993; *Polistes canadensis* – Giray et al., 2005; *Polistes dominula* – Shorter & Tibbetts, 2009). Given the functional diversity of JH in hymenopteran females, an outstanding question is how conserved JH is as regulator of reproduction and behaviour in the male counterparts.

JH has an important role in adult male sexual maturation and behaviour of non-social insects; for example, accessory gland protein synthesis and courtship behaviour in *Drosophila* (Wilson et al., 2003; Wijesekera et al., 2016). The role of JH in regulating reproductive physiology and behaviour of hymenopteran males has received relatively little attention. What is mostly known comes from studies of Apidae bees, specifically the solitary carpenter bee *Xylocopa appendiculata* and the social *A. mellifera*. In these bees, JH appears to have retained a similar ancestral and conserved function found in some female Hymenoptera and other insects, regulating sexual maturation and subsequent dispersal behaviour (Giray & Robinson, 1996; Oliveira Tozetto et al., 1997; Harano et al., 2008; Sasaki et al., 2012; Sasaki & Nagao, 2013). It is likely that JH is a key regulator of male dispersal across the social Hymenoptera. JH's dual role of regulating sexual maturation and dispersal provides a robust mechanism that may maximise the fitness of both males and their female nestmates.

Here we examine patterns of male natal dispersal in the Neotropical social paper wasp *Polistes lanio*, and test the hypothesis that JH co-regulates dispersal and sexual maturation. Males of *P. lanio* make an ideal model for determining dispersal processes because nesting cycles continue throughout the year, with males and females being produced concurrently in most nests, such that dispersal by males is not seasonal or mate-limited (Giannotti & Machado, 1994; Lucas & Field, 2013). Using observations of natural dispersal patterns of males in wild populations, examination of sexual maturity and

experimental applications of the JH analog methoprene, we determine the timing of adult male dispersal from natal nests and the potential co-regulatory role of JH. Specifically, we address three questions: Q1) Is male dispersal age-dependent? Q2) Is delayed dispersal associated with sexual maturation? Q3) Does JH co-regulate dispersal and sexual maturation?

MATERIALS AND METHODS

Study sites

Post-emergence nests (established nests with emerged natal offspring) of *P. lanio* were studied during the tropical wet season in Trinidad, Trinidad & Tobago, June to August 2014 and July to September 2015. Nests were among natural populations in semi-rural areas, with a total of 37 nests used across four sites (of approximately two hectares each): 22 nests at Verdant Vale (site VV) 2014–2015 (10°41'5.44"N, 61°17'24.95"W); nine nests at Eastern Main Rd (EM) 2014–2015 (10°39'1.21"N, 61°15'9.63"W); four nests at Cumuto Tamana Rd (CT) 2015 (10°34'48.01"N, 61°14'38.06"W); and two nests at the University Field Station of the University of the West Indies (UWI) 2015 (10°38'16.04"N, 61°25'37.94"W).

Q1) Is male dispersal age-dependent?

We recorded the age at which 154 natal males dispersed from 27 nests at sites VV, EM and CT. On each nest, all wasps were recorded via daily censuses; after each census, males were removed from the nest with forceps and given a four-coloured spot combination on the dorsal thorax using extra fine tip Uni POSCA markers. During marking, wing length was measured as a straight line between the intersection of a tegula and wing to the furthest apex tip in millimetres, to 2 decimal places (d.p.). A newly emerged adult male was identified by black glossy eyes, absence of wing wear (Garcia & Noll, 2013) and its appearance coinciding with a hatched pupal cell (i.e. a wasp had recently eclosed from the cell). After marking, individuals were immediately returned to the natal nest comb using forceps. The response of nestmates to males that had been marked and returned was observed for 5 min; if a recently marked male was attacked and ejected from the nest by nestmates or flew off the nest within 5 min, it was excluded from analysis as this would indicate that marking had likely interfered with natural dispersal (n = nine exclusions from 163 marked males). The age of dispersal was determined as the first day that an individual was observed absent from the nest during daily census. Between census days, no males returned to natal nests after putative dispersal. If a marked male was observed on another nest (as observed in 19 males), and departed again from these new non-natal nests, the date from the natal nest was used as the true dispersal event.

A repeated-measures generalized linear mixed model (GZLMM1) (binomial distribution, logit model), with the binary response variable of a dispersal event occurring or not per census date, was used to test whether natal dispersal could be explained by age (fixed effect); natal nest of origin was included as a random effect. To visualise dispersal events in the population, a Kaplan-Meier estimation was used, reporting the dispersal probability with age as a survival plot. A Cox model (Cox1, Efron method

for ties, describing hazard ratio as dispersal rate) was used test possible alternative influences of body size and nest characteristics on age of male dispersal (Table S1). First, dimensional reduction was performed on nest characteristics using a principle component analysis (PCA). A correlation coefficient matrix of nest characteristic variables was scored, being number of eggs, larvae, pupae, empty cells, parasitised cells, mean females and mean males per nest. Number of empty and parasitized cells was removed from the PCA, having coefficients $r < 0.3$. Included PCA variables were centred and scaled, with Bartlett's sphericity test ($p < 0.001$) and Kaiser-Meyer-Olkin statistic (KMO > 0.5) being satisfied. The PCA generated a single component with an eigenvalue > 1 (explaining 77% of variance), in which a regression factor score was calculated – henceforth described as "nest/group size" (see Table S2 for PCA results). The final Cox model (Cox1) tested whether age of dispersal differed by wing length (an indicator of body size, Figure S1), nest/group size, number of empty and parasitised cells; clustered by nest. Proportional hazards were assumed, as dispersal was only recorded from the natal nest.

Q2) Is delayed dispersal associated with sexual maturation?

To assess sexual maturation, we measured the sizes of multiple reproductive organs in males of different ages and states of dispersal. Typical indicators of sexual maturation in male *Polistes* include reduction in the size of the testes area (which become degraded as spermatozoa production ceases) and an increase in the size of seminal vesicles and accessory glands from fluid swelling (Moors et al., 2009).

A total of 166 males were collected for dissection at sites VV, EM and CT. We defined three dispersal states: 'natal-nesting' males on their natal nest; 'dispersed-nesting' males that had dispersed and were caught on another nest (i.e. not their natal nest); and 'dispersed-flight' male caught around the site but not on any nest (although not mutually exclusive with dispersed-nesting, as males may be in transit). Data were gathered for 141 natal-nesting males from 11 nests: 42 newly emerged (zero-day-old males collected on day of emergence); 38 one-day-old; 35 two-days-old; 21 three-days-old; three four-days-old; and two five-days-old. We collected 13 dispersed-nesting males on six non-natal nests, and 12 dispersed-flight males found off nests. All males were preserved in 70% ethanol and stored at -20°C.

We conducted dissections in phosphate-buffered saline using a Leica M165 C stereo microscope with a Leica IC80 HD digital camera attachment. Dissections were conducted by cutting a rectangular section into the ventral side of the abdomen and peeling back tergites, removing the testes and paired seminal vesicles with accessory glands. Removed organs were prepared on a slide. Using ImageJ 1.51j8, two-dimension measurements were taken of the area around the outer layer of the testes, seminal vesicles and accessory glands separately (mm^2 to 2 d.p.) (Figure 1). Area of the seminal vesicles and accessory glands is reported as the mean area between paired organs respectively, as the simultaneous function of ejaculation is unknown. In analyses, area is referred in relation to mSV (mean seminal vesicle) and mAG (mean accessory gland) size. Measurements were taken blind to the age or dispersal state of the individual.

First, to determine whether body size was a potential cofounding variable (i.e. if larger individuals had a head-start over smaller conspecifics), we assessed if wing length was correlated with larger initial reproductive organ size (testes, mSV and mAG size) in zero-day-old males using Pearson's correlation (Pearson1–3). Second, to investigate if sexual maturation (i.e. smaller testes and larger mSV and mAG organs) was related to age in natal-nesting males, we ran three separate general linear mixed models (GLMM1/GLM1–2) with the response variables testes, mSV or mAG size. Fixed effects for GLMMs consisted of wasp age, site location, wing length and year; natal nest origin as a random effect. Finally, to investigate differences in sexual maturation between natal-nesting, dispersed-nesting and dispersed-flight males, we ran three separate general linear models (GLM3–5) with the response variables testes, mSV or mAG size. The fixed effects for these models consisted of dispersal state, site location, wing length and year. We performed a post-hoc Tukey test on the multilevel variable of dispersal state (single-step method for *p* value adjustment).

Q3) Does JH co-regulate dispersal and sexual maturation

Methoprene is a proven JH analog in *Polistes* females (Giray et al., 2005; Shorter & Tibbets, 2009). To find a suitable non-lethal dose in males, 72 natal males (of approximately one to seven-days-old) were collected from seven nests at sites VV, CT and UWI. Topical applications of 1 µl methoprene/acetone treatments were made to the central dorsal side of the thorax using a micro-syringe. Treatments consisted of methoprene (µg) in acetone (µl)—0 µg/µl, 250 µg/µl and 500 µg/µl—or a blank control (18 males per treatment, randomly assigned with respect to nest of origin). Dose range was based on a previous study in *A. mellifera* males (Sasaki et al., 2012), and its natural titre increase in males prior to flight behaviour was also confirmed in *A. mellifera* (Giray & Robinson, 1996). Males were marked as above. Males from the same nest were kept together in 25×15×15 cm ventilated plastic enclosures, which were exposed to field-realistic conditions, but sheltered from direct sunlight, rain and ant predation. All males had ad libitum access to food, in the form of a cut piece of starch mango *Mangifera indica*, and water. Mortality rates were recorded every 24 hours for 10 days post-treatment. A Cox model (Cox2, Efron method for ties) was used to determine whether mortality rates between treatments, clustered by nest, significantly differed from that of the blank control group. Proportional hazards were assumed, as housing conditions did not change throughout the trial.

To observe the effect of methoprene on dispersal behaviour, 61 newly emerged males (zero-day-old individuals) from seven nests at sites VV, EM and CT were selected. Each male was marked and treated as above during daylight hours, with a 1 µl topical application of either 0 µg/µl (30 males) or 250 µg/µl (31 males) methoprene in acetone. Treated males were returned to their natal nests and observed for 5 min (there were no instances of immediate departure). Nests were then censused 24 and 48 hours after treatment to determine timing of early dispersal. To test whether methoprene application induced departure, a Cox model (Cox3, Efron method for ties, describing hazard ratio as dispersal rate) was used to determine whether dispersal rate of males treated with methoprene differed from that of the control treatment, clustered by nest. Proportional hazards were assumed, as dispersal was recorded from only the natal nest.

To ascertain whether methoprene accelerates sexual maturation, 19 males were collected from six nests at sites VV, CT and UWI. Each male was marked and treated as above with either 0 µg/µl (11 males) or 250 µg/µl (8 males) methoprene in acetone. Males were then housed in plastic enclosures as above. After 48 hours, males were collected, and bodies stored in 70% ethanol at -20°C. Reproductive organ measurements were taken as described above. There was no male mortality observed in enclosures within the 48 hour period. To test whether methoprene applications accelerated maturation, we used three separate GLMMs (GLMM2/GLM6–7) with response variables of testes, mSV or mAG size. As above, maturity is indicated by smaller testes, larger accessory glands and seminal vesicles. The fixed effect for models was methoprene treatment, with natal nest of origin as a random effect.

Statistical analyses

Analyses were performed in R 3.3.3 (R Core Team, 2017), using packages 'car' (Fox & Weisberg, 2011), 'ggpubr' (Kassambara, 2017), 'Hmisc' (Harrell Jr et al. 2017), 'lme4' (Bates et al., 2015), 'lmerTest' (Kuznetsova et al., 2016), 'multcomp' (Hothorn et al., 2008), 'RLRsim' (Scheipl et al., 2008) and 'survival' (Therneau & Grambsch, 2000). Histograms and Q-Q plots assessed deviations from normal distribution. When appropriate, model fit was assessed by checking the residual deviance against the degrees of freedom or residual vs fitted value plot. Variance of the random effect in models is reported with standard deviation (SD). If the random effect had a variance and SD of 0.00, the effect was dropped, and a subsequent non-mixed model used. All analyses were tested at $\alpha = 0.05$, with averages reported as median with interquartile range (IQR), mean ± standard error (SD) or standard error (SE).

RESULTS

Q1) Dispersal is age-dependant

All males eventually dispersed from natal nests. Age at dispersal ranged from one to seven-days-old, with the median age of dispersal being three-days-old (IQR = 2.0). Male age had a significant positive effect on the likelihood of dispersal occurring (GZLMM1: $\chi^2_1 = 53.21$, $p < 0.001$; random effect of natal nest origin variance ± SD = 0.38 ± 0.62). Dispersal probability increased between each age category (Kaplan-Meier), ranging from 19% in one-day-old males to 100% in seven-days-old males (Figure 2). Body size and recorded nest characteristics did not significantly alter male dispersal rates from the natal nest (Cox1: wing length, Wald $\chi^2 = -0.013$, $p = 0.990$, mean ± SE wing length = 18.24 ± 0.09 mm; nest/group size, Wald $\chi^2 = 0.663$, $p = 0.507$; number of empty cells, Wald $\chi^2 = -0.764$, $p = 0.445$; number of parasitised cells, Wald $\chi^2 = 0.471$, $p = 0.638$).

In 12% of natal dispersal events (n = 19), males appeared soon after on non-natal foundress and post-emergence nests. These non-natal nests ranged from 0.5 to 16.5 metres away from natal origin nests (skewed towards lower dispersal distances). Males appeared either on the same day or up to two days after initial natal dispersal. Dispersed-nesting males were a median age of two-days-old (IQR = 1.5)

and stayed on non-natal nests for one to two days. Males always eventually left non-natal nests, and in only two instances did a male appear on a second non-natal nest (both post-emergence nests, 5.0 and 10.0 m away from the first non-natal nest), where the they remained for a day each before departure.

Q2) Delayed dispersal is associated with sexual maturity

On emergence, body size did not appear to influence initial reproductive organ size (10 nests, 41 males). Wing length was not significantly correlated with testes (Pearson1: $r = 0.09$, $df = 39$, $p = 0.585$), mSV (Pearson2: $r = 0.12$, $df = 39$, $p = 0.441$) or mAG size (Pearson3: $r = 0.04$, $df = 39$, $p = 0.814$) in zero-day-old males (mean ± SE: wing length, 18.05 ± 0.13 mm; testes size, 1.84 ± 0.07 mm^2; mSV size, 0.06 ± < 0.01 mm^2; mAG size, 0.09 ± < 0.01 mm^2).

Aging on natal nests was associated with sexual maturation, with older males showing signs of being more sexually mature than younger males (11 nests, 141 males), indicated by a decrease in testes and increase in seminal vesicle and accessory gland size (Figure 3). There was a significant negative effect of age on testes size (GLMM1: $\chi^2_1 = 37.92$, $p < 0.001$). No significant variation in testes size was detected between the three sites ($\chi^2_1 = 0.52$, $p = 0.771$), with wing length ($\chi^2_1 = 1.41$, $p = 0.234$, mean ± SE wing length = 17.98 ± 0.07 mm) or between the two years ($\chi^2_1 = 0.10$, $p = 0.754$); natal nest origin variance ± SD = 0.02 ± 0.13. There was a significant positive effect of age on mSV size (GLM1: $F_{1,133} = 29.68$, $p < 0.001$). No significant effect with mSV size was detected between the three sites ($F_{2,133} = 0.15$, $p = 0.929$), with wing length ($F_{1,133} = 2.21$, $p = 0.137$) or between the two years ($F_{1,133} = 0.33$, $p = 0.569$); natal nest origin random effect was dropped as there was no detectable variance. There was a significant positive effect of age on mAG size (GLM2: $F_{1,133} = 33.83$, $df = 133$, $p < 0.001$). No significant effect of mAG size was detected between the three sites ($F_{2,133} = 2.61$, $p = 0.271$), with wing length ($F_{1,133} = 2.00$, $p = 0.157$) or between the two years ($F_{1,133} = 1.03$, $p = 0.310$); natal nest origin random effect was dropped as there was no detectable variance.

Signs of greater sexual maturity were detected in dispersed males found in flight, compared to males found on nests (both natal-nesting and dispersed-nesting) (Figure 4). Dispersal state had a significant effect on testes size (GLM3: $F_{2,157} = 13.49$, $p < 0.001$). Dispersed-flight males had significantly smaller testes than natal-nesting (Tukey: $p < 0.001$) and dispersed-nesting ($p = 0.015$) males; testes size was not significantly different between natal-nesting and dispersed-nesting males ($p = 0.290$). No significant effect on testes size was detected between the three sites ($F_{2,157} = 0.21$, $p = 0.811$), with wing length ($F_{1,157} = 0.92$, $p = 0.338$, mean ± SE wing length = 18.06 ± 0.07 mm) or between the two years ($F_{1,157} = 0.12$, $p = 0.728$). Dispersal state had a significant effect on mSV size (GLM4: $F_{2,156} = 37.25$, $p < 0.001$). Dispersed-flight males had significantly larger mSV sizes than natal-nesting (Tukey: $p < 0.001$) and dispersed-nesting ($p < 0.001$) males; mSV size was not significantly different between natal-nesting and dispersed-nesting males ($p = 0.201$). No significant effect of mSV size was detected between the three sites ($F_{2,156} = < 0.01$, $p = 0.995$), with wing length ($F_{1,156} = 1.97$, $p = 0.163$) or between the two years ($F_{1,156} = 0.19$, $p = 0.661$). Dispersal state had a significant effect on mAG size (GLM5: $F_{2,157} = 24.69$, $p < 0.001$). Dispersed-flight males had significantly larger mAG sizes than natal-nesting (Tukey:

$p < 0.001$) and dispersed-nesting ($p < 0.001$) males; mAG size was not significantly different between natal-nesting and dispersed-nesting males ($p = 0.347$). No significant effect on mAG size was detected between the three sites ($F_{2,157} = 0.57$, $p = 0.565$), wing length ($F_{1,157} = 1.20$, $p = 0.276$) or between the two years of study ($F_{1,157} = 0.71$, $p = 0.401$).

Q3) JH co-regulates dispersal and sexual maturation

In determining appropriate levels of methoprene (72 males), males treated with 500 µg/µl methoprene had significantly higher mortality rates compared to blank controls (Cox2: Wald $\chi^2 = 3.54$, HR = 6.91, lower 95% CI = 2.37, upper 95% CI = 20.14, $p < 0.001$), confirming that the treatment had been successfully absorbed. Males treated with 500 µg/µl methoprene were seven times more at risk of mortality than blank control treated males. Survivorship of males treated with 0 µg/µl (Wald $\chi^2 = 1.51$, HR = 1.87, lower 95% CI = 0.83, upper 95% CI = 4.31, $p = 0.132$) and 250 µg/µl methoprene (Wald $\chi^2 = -0.60$, HR = 0.66, lower 95% CI = 0.17, upper 95% CI = 2.61, $p = 0.552$) was not significantly different from blank control treated males. Treatment with 250 µg/µl methoprene was considered appropriate for use in further methoprene experiments (Figure S2).

Treatment of newly emerged zero-day-old males with methoprene on natal nests (7 nests, 61 males) resulted in faster rates of dispersal (Figure 5). Males treated with 250 µg/µl methoprene were three times more likely to disperse earlier than 0 µg/µl control males (Cox3: Wald $\chi^2 = 3.82$, HR = 3.16, lower 95% CI = 1.76, upper 95% CI = 5.80, $p < 0.001$).

Males treated with 250 µg/µl methoprene at zero-day of age showed some signs of accelerated sexual maturity relative to 0 µg/µl control males of the same age 48 hours after application from emergence (19 males) (Figure 6). mAG size of males treated with 250 µg/µl methoprene was significantly larger than 0 µg/µl control males (GLM6: $F_{1,17} = 4.60$, $p = 0.047$; random effect dropped). However, there was no significant difference in mSV size (GLM7: $F_{1,17} = 0.47$, $p = 0.502$; random effect dropped) and testes size (GLMM2: $\chi^2_1 = 0.04$, $p = 0.851$; natal nest origin variance ± SD = 0.01 ± 0.08).

DISCUSSION

In the social Hymenoptera, delayed male dispersal from the natal nest is likely correlated with a period of sexual maturation post-pupation. Here we provide evidence that males of the social paper wasp *P. lanio* delay dispersal from the natal nest, and that dispersed males found in flight tend to be more mature compared to males who are still on the nest. We show through experimental manipulation that the JH analog methoprene induces precocious dispersal in males and can accelerate parts of the sexual maturation process within 48 hours of application. These results suggest that JH plays a key mechanistic role in regulating dispersal and sexual maturation in males of this species, co-regulating physiology and behaviour, and potentially helping to maximise fitness. These findings support the theory that the JH family is crucial in regulating insect behaviour and life-histories.

Male dispersal was predicted by age. A pattern of age-determined dispersal of males indicates that dispersal is strongly regulated internally by chronology in *P. lanio*. Animals typically disperse from natal groups within a fixed age range, correlating with sexual maturation or first breeding attempts (Martín & Bucher, 1993; González et al., 2006; Fernandez-Duque, 2009). Males of *P. lanio* dispersed from the natal nest at one to seven-days of age, similar to ages found in other *Polistes* and closely related wasps in both temperate and tropical climates (*Polistes ferreri* zero to five-days-old; *Polistes jokahamae* zero to six-days-old; *Polistes major* zero to thirteen-days-old; *Ropalidia marginata* zero to eight-days-old; *Ropalidia cyathiformis* mean dispersal 19 days-old – Gadagkar & Joshi, 1984; Cameron, 1986; Makino, 1993; Sinzato et al., 2003; Sen & Gadagkar, 2006; Sen & Gadagkar, 2011). Males that leave the nest early may be individuals that later appear on non-natal nests, but early dispersal is likely to be sub-optimal as males will not be fully mature. Of the dispersed males caught in flight around the field sites, a single male was known to be one-day-old; that male showed evidence of sexual immaturity with larger testes along with smaller seminal vesicles and accessory glands than other likely older dispersed males caught in flight. Overall, delayed dispersal appears to allow *P. lanio* males the opportunity to mature further on the nest (O'Donnell, 1999). It is likely that delayed dispersal for sexual maturation is a general strategy in wasp species.

Dispersed males caught in flight showed significant signs of sexual maturation in comparison to natal-nesting males. Completion of male sexual maturation varies across hymenopteran species but is usually greater than seven days-old in social bees and wasps (see Table S3), whilst *P. lanio* males disperse on average before seven days of age. Male *Polistes* have a range of mating strategies both within and between species (Beani & Turillazzi, 1988; Beani & Turillazzi, 1990; Lee & Starr, 2007; Molina & O'Donnell, 2009), and sexual maturation or fully mature reproductive organ size may vary subtly among dispersed males. Though no correlation has been found so far to traits such as body size and initial reproductive organ size (*P. lanio* – this study) or sperm length (*Polistes simillimus* – De Souza et al., 2018). There may be selection for males to disperse just before reaching completed maturation in order to reduce chances of inbreeding (Pusey & Wolf, 1996). Genetic studies have detected no significant levels of inbreeding in the sister species *P. canadensis* (Lengronne et al., 2012; Southon et al., 2019).

JH appeared to co-regulate dispersal and sexual maturation, with methoprene treatments causing earlier departure and increased accessory gland sizes. JH is likely to play a similar role in *P. lanio* males as it does in other male insects, including previously studied bee species (Giray & Robinson, 1996; Oliveira Tozetto et al., 1997; Harano et al., 2008; Sasaki et al., 2012; Sasaki & Nagao, 2013), where JH is thought to play a regulatory role in not only dispersal (e.g. milkweed bugs *Oncopeltus fasciatus* – Caldwell & Rankin, 1972) and courtship behaviour (e.g. *Drosophila melanogaster* – Wijesekera et al., 2016) but also sexual maturation such as in accessory gland activity (e.g. red flour beetles *Tribolium castaneum* & *D. melanogaster* – Wilson et al., 2003; Parthasarathy et al., 2009). Accessory gland fluids facilitate sperm transfer, but in many species also contain a concoction of biochemicals (e.g. sex peptides) that increase mating success and influence post-copulation female behaviour (Gillott, 2003).

There are also likely to be a number of other hormones at play; for example, ecdysteroids and dopamine, the latter of which is expected to be regulated by JH and linked to the behavioural and physiological changes observed in this study (Harano et al., 2008). Because of the apparent coupled effect of JH on dispersal and sexual maturation in our study, we were not able to determine cause and effect; i.e. if JH independently regulates both these processes or if one (e.g. dispersal) is a downstream effect of the sexual maturation process. In *P. canadensis*, JH titres are higher in reproductives than guards, which in turn have higher levels than nonreproductive foragers (Giray et al., 2005). Although the effects of JH in regulating behaviour in *P. lanio* is unknown, it is likely the same dual action of JH will function in this sister species. Whilst JH appears to have a critical influence on male dispersal, there are likely a number of other physiological and environmental factors that could also explain delayed dispersal. Physiologically, flight for newly emerged adult holometabolous insects is severely limited until cuticle sclerotization and waterproofing is complete (Vincent & Wegst, 2004). Environmentally, nests undergoing social turmoil or in the declining phase may provide reduced nutritional resource opportunities; equally, hazardous weather conditions such as prolonged periods of heavy rainfall will stop flight activity. Additionally, dispersal decisions may involve resident females, who could force newly mature males to depart the nest to avoid inbreeding with sisters or to avoid depleting nest resources (Hakala et al., 2019). Further questions arising from this study involve quantifying the natural JH titre changes of aging males, and investigating whether the functionality of JH is specific to reproductive life history, or if it also regulates other male behaviours observed during their brief period on the nest – for example newly emerged male interactions with brood and female nestmates (Cameron, 1986; Beani et al., 2014; Southon, 2018).

Males occasionally dispersed from their natal nest to nearby non-natal nests. It is possible that non-natal males are related to the nests they visit, as neighbouring nests in aggregations of *P. canadensis* form viscous genetic population structures (Lengronne et al., 2012; Southon et al., 2019). Males may be accepted onto neighbouring nests due to recognition errors (by nestmate females) or may provide some non-genetic benefits to females, through defence or low levels of helping (Cameron, 1986; Southon, 2018). Alternatively, males could be seeking opportunistic mating (though mating attempts are rarely observed on the nest – R.J.S. and S.S. pers. obs.) or be cheating non-natal nestmates to obtain forage and protection. Nest drifting is a common behaviour in females of tropical *Polistes*, whereby workers split time between natal and non-natal nests (Sumner et al., 2007). However, non-natal nest visitation of males differs from female drifting in that males were never observed to return to nests (at least between days). It is likely that the mechanisms permitting females to move between nests predisposes males to be able to do the same.

Our study provides insights into the mechanisms regulating male dispersal in tropical *Polistes*. Explaining why delayed male dispersal is tolerated by females, and the role of males (if any) during their brief period on natal and non-natal nests, are outstanding questions for *Polistes* and for males in social hymenopteran societies in general. Future research should address the potential adaptive (or maladaptive) significance of delayed male dispersal in social Hymenoptera societies.

List of Symbols and Abbreviations

JH – juvenile hormone

analog – analogue

d.p. – decimal place

mSV – mean seminal vesicle

mAG – mean accessory gland

IQR – interquartile range

SD – standard deviation

SE – standard error

Supplementary Information

Southonetal2020_Polistesmaledispersal_projectdata.xlsx – Raw Data File

Southonetal2020_Polistesmaledispersal_supplementarytablesfigures.xlsx – Supplementary Tables and Figures

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Competing Interests

The authors declare no known competing interests.

Author Contributions

All authors contributed to the designing the study and writing the manuscript. R.J.S. conducted experiments, dissections and data analyses.

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Table & Figure Legends

Figure 1. Reproductive organs of newly emerged *P. lanio* male. The (A) testes connect to (B) seminal vesicles, seminal vesicles and (C) accessory glands (dashed-line separating) connect into aedeagus. Scale comparison between older dispersed male, reduced (D) testes size and increased (E) seminal vesicle with accessory gland size. White bar 1 mm scale.

Figure 2. Male dispersal rates from natal nests in the population by age in days (Kaplan-Meier estimate, lower & upper 95% CI) (n = 154).

Figure 3. Natal male age in days has a negative relationship with testes size (GLMM1, *** $p < 0.001$), and a positive relationship with mSV and mAG size (GLM1–2, *** $p < 0.001$). Median centre line with IQR (n = 141).

Figure 4. Dispersed males caught in flight have significantly smaller testes, larger mSV and mAG sizes than both natal-nesting and dispersed-nesting males (GLM3–5, Tukey: ** $p < 0.05$; *** $p < 0.001$). Median centre line with IQR (n = 166).

Figure 5. Eariler dispersal rates in natal zero-day-old males treated with methoprene after 48 hours (Cox3, lower & upper 95% CI, $p < 0.001$) (n = 61).

Figure 6. Increase in mAG size (GLM6, ** $p < 0.05$) in zero-day-old males treated with methoprene after 48 hours (not significant: GLMM2 and GLM7, testes and mSV size $p > 0.05$). Median centre line with IQR (n = 19).

Table S1. (supplementary file) Characteristics of natal male nests. Locations: VV = Verdant Vale; EM = Eastern Main Rd; CT = Cumuto Tamana Rd. Means to 1 d.p.; nest comb size recorded once during departure/observation dates; mean number of adults between departure/observation dates.

Table S2. (supplementary file) Results for dimensional reduction of nest characteristics with PCA.

Table S3. (supplementary file) Minimum age of maturation in male Hymenptera. Note: 1 = complete sperm transfer into seminal vesicles; 2 = only in 10% of trials did males aged zero to one-day-old mate, comapred to 80% of males older than two-days.

Figure S1. (supplementary file) Wing length correlates with (A) mesoscutum width, (B) gastral tergite length and (C) wet weight of newly emerged zero-day-old males (Pearson's correlations, $\alpha = 0.05$, $p < 0.001$).

Figure S2. (supplementary file) Significant (Cox2) higher morality hazard rates in males treated with 500 µg/µl to blank control males ($p < 0.001$). Treatments 0 µg/µl and 250 µg/µl not significantly different to blank control group. Survival analysis of males treated with 0, 250 or 500 µg/µl of methoprene in acetone, and a blank control, to select a suitable dosage for further testing.

Figures

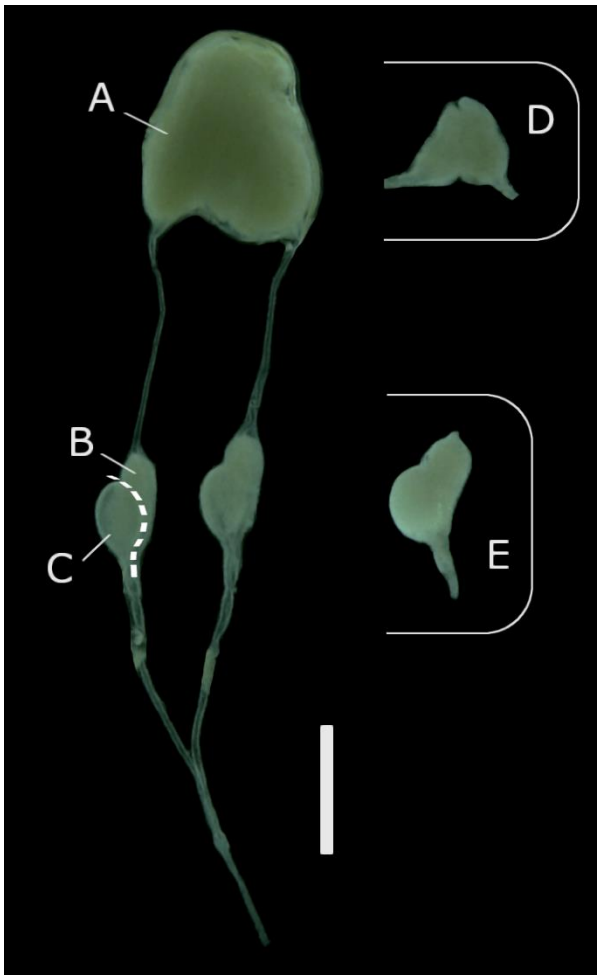

Figure 1.

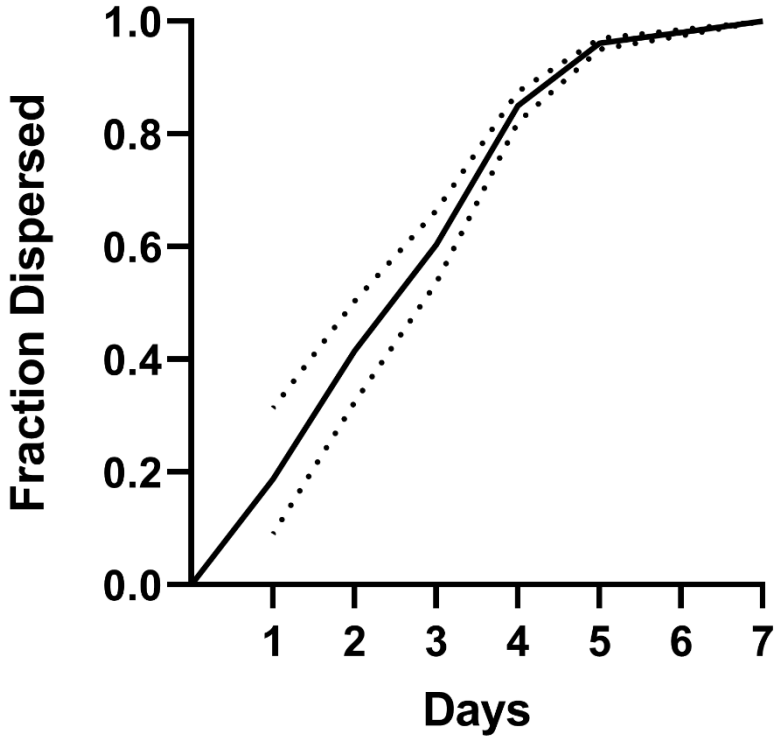

Figure 2.

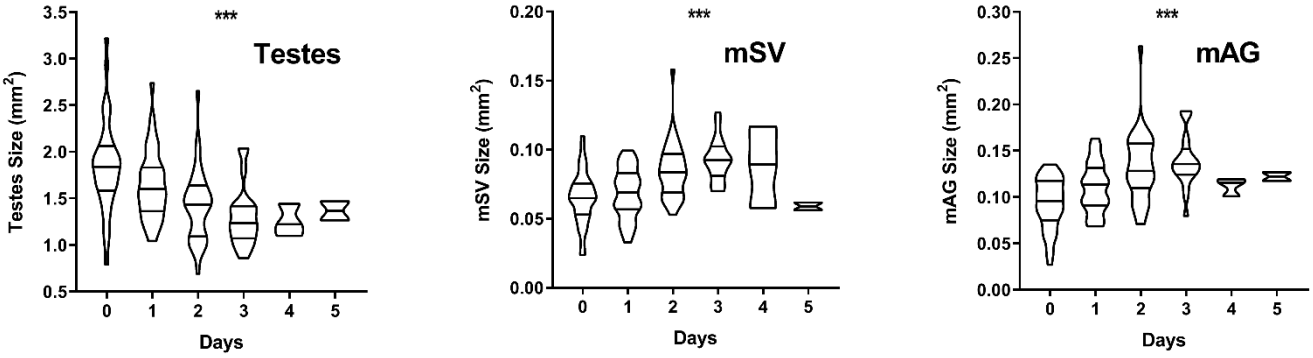
***
Testes
Testes Size (mm2)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0 1 2 3 4 5
Days
***
mSV
mSV Size (mm2)
0.20
0.15
0.10
0.05
0.00
0 1 2 3 4 5
Days
***
mAG
mAG Size (mm2)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0 1 2 3 4 5
Days

Figure 3.

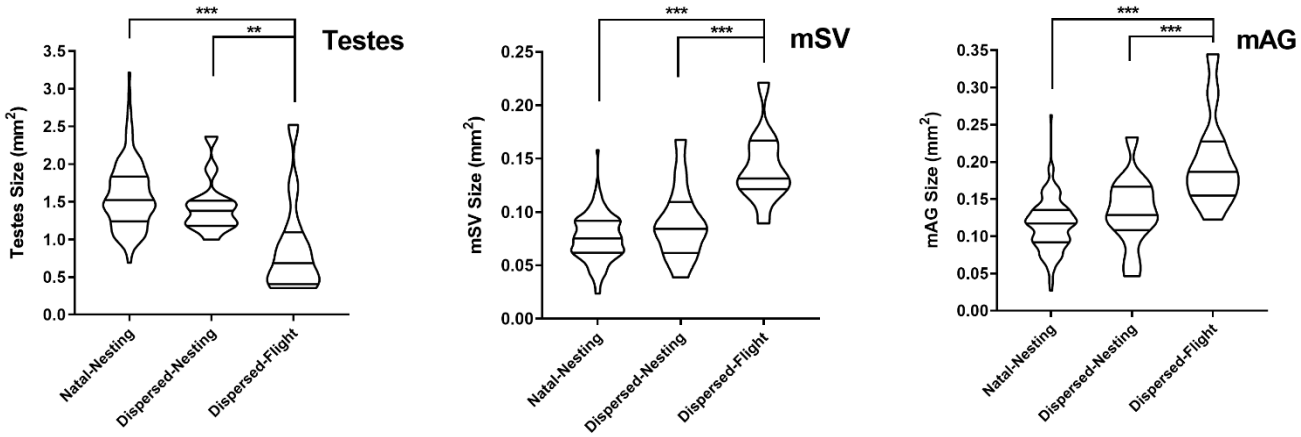
Testes
Testes Size (mm2)
***
**
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Natal-Nesting
Dispersed-Nesting
Dispersed-Flight
mSV
mSV Size (mm2)
***
***
0.25
0.20
0.15
0.10
0.05
0.00
Natal-Nesting
Dispersed-Nesting
Dispersed-Flight
mAG
mAG Size (mm2)
***
***
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Natal-Nesting
Dispersed-Nesting
Dispersed-Flight

Figure 4.

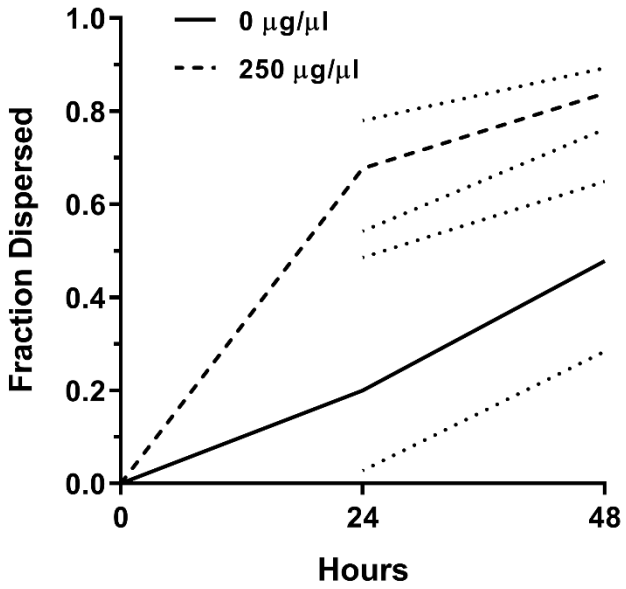
0 μg/μl
250 μg/μl
Fraction Dispersed
1.0
0.8
0.6
0.4
0.2
0.0
0
24
48
Hours

Figure 5.

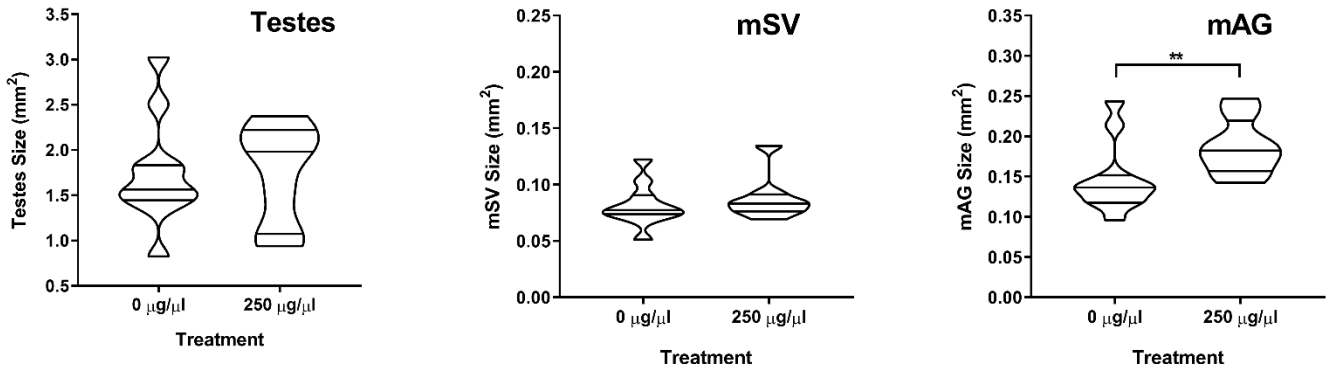
Testes
Testes Size (mm2)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0 μg/μl
250 μg/μl
Treatment
mSV
mSV Size (mm2)
0.25
0.20
0.15
0.10
0.05
0.00
0 μg/μl
250 μg/μl
Treatment
mAG
**
mAG Size (mm2)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0 μg/μl
250 μg/μl
Treatment

Figure 6.