

RESEARCH ARTICLE

The relative cost of using luminescence for sex and defense: light budgets in cypridinid ostracods

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SUMMARY

Luminescent signals can be used by animals for a number of purposes, including courtship and defense, sometimes by the same individual. However, the relative costs of producing these different behaviors are largely unknown. In the marine ostracod *Photeros annecohenae*, males utilize extracellular luminescence for complex courtship displays, and both males and females luminesce as a predation defense. We compared the relative luminescent output of courtship with that of defensive displays and also with respect to their total luminescent stores. Courtship displays are relatively inexpensive compared with defensive displays, with an average defensive display releasing 50 times more luminescence than the average courtship display. Furthermore, in order to completely exhaust its stores, a male would have to produce 450 typical courtship displays or approximately 10 average defensive displays. Both courtship pulses and defensive displays show first-order decay kinetics, yet courtship pulses decay three times faster than defensive displays, suggesting that there is differential release of the luciferin, luciferase and mucus in order to control the reaction kinetics.

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INTRODUCTION

For many communication systems little is known about either relative or absolute energetic costs of producing the signal. The information that is available suggests that sometimes these costs can be high, as in the acoustic signals produced by insects and anurans involved in courtship and mate attraction (Prestwich, 1994; Grafe and Thein, 2001; Ophir et al., 2010), whereas in others it appears to be low, as in bird calls (Horn et al., 1995) and firefly luminescent courtship displays (Lewis and Cratsley, 2008). In addition to courtship, many communication channels can serve an antipredatory (defensive) function under different circumstances. Examples include acoustic signals from birds or insects, chemical signals especially in insects, and visual signals in birds [see Bradbury and Vehrencamp and references therein (Bradbury and Vehrencamp, 2011)]. Both direct and indirect energy costs are required to outwit predators and secure mates, but these costs relative to each other and to an animal's available resources are often unknown (Bradbury and Vehrencamp, 2011). This situation especially holds for organisms that emit visible light as bioluminescence.

The absolute, relative or comparative costs of emitting light have not been extensively examined for most luminescent systems, especially from an evolutionary perspective. In bacteria, however, the cost of inducing luminescence, both in synthesis and enzymatic activity, has been extensively studied, and it has generally been found to be high. Luminescence in bacteria can account for up to 10% of the oxygen consumed and luciferase can constitute 5% or more of the soluble cellular protein (Hastings and Nealson, 1977; Karl and Nealson, 1980; Bose et al., 2008). Recently, Woods et al. (Woods

et al., 2007), based on respiratory measurements, have shown that the cost of intracellular flash production in species of the firefly *Photinus* is relatively small, especially when compared with the costs of walking and flying, or with the comparable energetics of producing acoustic signals by frogs and a variety of insects. However, they also showed that there was a major collateral cost to luminescence in the form of increased predation by congeners who are immune to chemical defenses of the fireflies.

Bioluminescence is biologically produced light that is detected and acted upon by organisms. The receivers of bioluminescence may be the emitter itself, conspecifics or other species, so that the light primarily functions for communication, predation, protection or detection of surroundings (Haddock et al., 2010; Widder, 2010; Hastings and Morin, 1991; Morin, 1983). Furthermore, bioluminescence may serve two or more of these major functions either under different circumstances or concurrently. For instance, bioluminescence in larval fireflies acts as a predator deterrent (deCock and Matthysen, 1999; deCock and Matthysen, 2003), whereas the precise coded signals in adults are used as courtship signals (reviewed by Lewis and Cratsley, 2008). The light that results from the bacterial light organs in the flashlight fish *Photoblepharon steinitzi* (formerly *P. palpebratus*) from the Indian Ocean apparently uses luminescence for multiple purposes to see and attract prey, to confuse and avoid predators, to form conspecific aggregations, and probably courtship (Morin et al., 1975). Because oxygenases are widespread in nature, it is perhaps not surprising that bioluminescence has evolved independently many times and with a diversity of distinct molecules (Rees et al., 1998), in a broad spectrum of organisms, in a range of habitats – both temporally and

spatially where ambient light levels are dim or dark – and for a variety of functions. In the majority of known luminescent systems the light is produced intracellularly, in others it is from bacterial symbionts, but in a few it comes solely from extracellular secretions (Haddock et al., 2010; Widder, 2010; Hastings and Morin, 1991).

One extracellular luminescent system occurs in a clade of marine ostracods in the family Cypridinidae. Unlike the more complexly regulated intracellular luminescent systems, light production in these ostracods results from a relatively simple extracellular reaction involving the union of three components: (1) cypridinid luciferin, a tripeptide substrate, (2) cypridinid luciferase, an oxidative enzyme, and (3) oxygen, which occurs naturally in the seawater. The reaction, which, starting in 1917, has been well studied in the Japanese species *Vargula* (formerly *Cypridina*) *hilgendorffii*, shows simple first-order kinetics and the output (light intensity) is directly proportional to the amount of substrate (luciferin) present [see Harvey (Harvey, 1952), Herring (Herring, 1978) and Shimomura (Shimomura, 2006) and references therein]. During the reaction, cypridinid luciferin, a tripeptide composed of tryptophan, isoleucine and arginine in the form of a fused imidazopyrazinone ring nucleus, is oxidized producing cypridinid oxyluciferin, water, carbon dioxide and light. The luciferase shows a turnover rate of approximately 30 s^{-1} (Shimomura et al., 1969). In nature, the intensity and kinetics of the light emission are influenced primarily by the concentrations (and relative ratios) of the luciferin and luciferase, and the ambient temperature.

Remarkably, despite the relative simplicity of the reaction, Caribbean, and probably all, species can regulate both the intensity and the duration of this external light with surprising precision. All species of light-emitting cypridinid ostracods (approximately 200+ species) appear to produce long-lasting (many seconds) extracellular bursts of very bright luminescence in response to predation attempts in their natural habitat (Morin, 1986; Morin and Cohen, 1991). Such luminescent species occur in virtually every ocean and at all depths (Harvey, 1952; Korniker, 1984; Cohen and Morin, 2003). But in the Caribbean Sea, and apparently only there, a large monophyletic clade of over 60 species can also precisely regulate the duration of the light for sexual courtship. Above reefs and seagrass beds on dark nights, each species produces its own unique temporal and spatial series of distinct extracellular points of light, which we term pulses, that form display trains left behind by rapidly swimming males. Among species, and even within displays, individual light pulses can range in duration from mere milliseconds to several seconds (Morin, 1986; Morin and Cohen, 1991; Morin and Cohen, 2010; Rivers and Morin, 2008; Rivers and Morin, 2009). There is a wide diversity of these patterns, which vary not only in pulse duration but also in train direction, from vertical (up or down), lateral to diagonal; train length, from a few centimeters to many meters; and in pulse number per display train, from as few as two or three pulses to many dozens. Note that ‘courtship display’ henceforth refers to the entire train of pulses that occur as a discrete temporal series. Although all stages in the life cycle including brooded embryos, juveniles and both adult sexes are able to release luminescence in response to predation, only males also utilize light in these complex, species-specific patterns to attract a female in specific microhabitats (Morin, 1986; Morin and Cohen 1991; Morin and Cohen, 2010). There is no luminescent dialog between males and females during courtship such as in fireflies; rather, the female silently approaches the display and, using possible secondary sensory cues (e.g. female pheromones), intercepts the displaying male (T.J.R. and J.G.M., unpublished). However, the relative cost

of using luminescence by ostracods for either defense or these complex courtship displays is unknown.

In this paper we ask a simple empirical question about this remarkable system: what are the relative costs of producing both courtship and defensive signals compared with the individual ostracod’s available resources to emit light?

MATERIALS AND METHODS

Collection

Photeros anecoehenae (described by Torres and Morin, 2007) ostracods were collected in baited traps placed in shallow grassbeds or *via* sweep nets during their evening displays (for details, see Rivers and Morin, 2008). This research was completed in accordance with permits received from the Belize Department of Fisheries.

Photomultiplier tube setup and analysis

We used a standard protocol to compare luminescent outputs among: (1) courtship displays, (2) defensive displays and (3) total luminescence. Our experimental setup followed the same methods used in our previous studies to observe individual courtship behavior (Rivers and Morin, 2008; Rivers and Morin, 2009), but with the photomultiplier tube (PMT) replacing the video camera. All light intensities were recorded with an RCA 931-A PMT (Harrison, NJ, USA), covered by an Andover 039FG11-50 3 mm infrared (IR) barrier filter (Salem, NH, USA), and set at a standard distance of 76 cm from the experimental chamber, at which distance an entire display could be observed by the PMT. The PMT was powered by an Emco Ca12N High voltage converter (set to 1000 V; Sutter Creek, CA, USA) and the output was connected to a Dataq Instruments DI-158U analog data acquisition (DAQ) device (Akron, OH, USA) that recorded 240 data points per second using the program WinDAQ (Dataq Instruments) on a Dell Inspiron laptop. We quantified all outputs in relative terms and not in terms of total photons or other energy units. We report all values relative to the mean output of a courtship display, which is the most consistent and least variable of the three types of emissions. Because the three types of outputs differed by over two orders of magnitude, based on experience, the gain was preadjusted for each type to maximize the resolution without saturation. To analyze the relative light output and waveforms, the digital data were converted to a spreadsheet and exported into Excel and the program Origin (Northampton, MA, USA).

Courtship display trains

We used the photometric (PMT) recordings to determine light outputs and waveforms from individual pulses and combined light from courtship displays. As before (Rivers and Morin, 2008), we used five males together per trial. The total relative light emitted per pulse and per courtship display train was calculated from the PMT data. The area under each pulse corresponds to total light emitted (V) of each pulse. The luminescence output per total courtship display was compared with the luminescence output during both defensive displays and when individuals were crushed to produce total available light output from an individual (see below).

Because ostracod luminescence is a simple first-order reaction (Harvey, 1952), rise and decay rates of the luminescent pulses of *P. anecoehenae* are exponential, and the waveforms vary with temperature. The exponential decay rates of individual pulses were calculated using the first-order exponential equation $V(t) = V_0 e^{-\lambda t}$, where $V(t)$ is the voltage at time t , V_0 is the initial voltage at $t=0$, and λ is the exponential rise/decay constant. Based on hundreds of recordings in the laboratory, courtship trains characteristics

are very consistent (Rivers and Morin, 2008). For this study we evaluated 22 representative courtship trains during two separate trials in February 2006 at 26°C for the decay rate constants of the first pulse. We also analyzed 14 second- or third-pulse peaks that had little noise, for a total of 36 pulses. Peaks with high noise to signal ratios (due to low intensity) were not included in our analyses.

Defensive displays

Dusky cardinalfish (*Pheoptyx pigmentaria*), a common nocturnal planktivore known to attack ostracods, were used for studies of predation. Six individuals, ca. 3–4 cm standard length, were collected, using a 20 cm diameter, 20 cm long 500 µm mesh sweep net while snorkeling at night at a patch reef off the southwest tip of Southwater Caye, Belize (16.8118°N, 88.0825°W). To prevent injury, each fish, once netted, was immediately transferred to a 4.5 liter seawater-filled Ziploc plastic bag. In the laboratory, individual fish were kept in opaque, blue 20-liter buckets each with 10 liters of fresh seawater and an opaque 15×15 cm PVC pipe for shelter, and the seawater was replaced daily. After being kept without food for 2 days, we placed two individuals (to maximize predation attempts in a given time period) in a 15×16×30 cm (width × length × height) clear acrylic tank, filled to a depth of 10 cm (~2.4 liters). The seawater was collected off the nearby dock during the day (to minimize potential biases from other luminescent sources). Soon after nightfall, the lights were turned off and the fish were allowed to acclimate for 1 h. Six *P. annecohenae* were gently placed in the tank in the dark, and we recorded the light output (with both the PMT for waveform data and the video camera for fish behavior) that resulted from predatory attacks on the ostracods by the fish. The fish were returned to the field immediately after the trials. The maximum intensities detected and total light produced (area) from 16 defensive episodes were analyzed per defensive display as described above. In addition, the decay rates from 53 clean parts of defensive displays were calculated using the same first-order exponential equation as above. The decay rate constants were log-transformed to satisfy the assumption of equal variance. To determine the differences between the decay rate constants of defensive displays ($N=53$) and courtship displays ($N=36$) at the same temperature (26°C), as well as to determine whether the pulse (or peak) number had any effect on the decay rate constant, we used a random-effects mixed model in SAS.

Total potential luminescence

Seven male and six female *P. annecohenae* were collected *via* traps the night before they were transported in seawater back to Cornell University, where we determined the total available luminescence per ostracod using the same experimental configuration as above. The day after returning to our Cornell laboratory (2 days after collection), individuals were placed in a 5 ml glass tissue homogenizer in 0.5 ml artificial seawater and placed 76 cm from the PMT as above. In a darkened room, 0.5 ml of freshwater was added, which induced luminescence, and subsequently the individual was crushed and ground incrementally. This stepwise procedure was required to allow us to induce the total luminescence from an individual incrementally without exceeding the least sensitive setting of our recording equipment. The data were imported into Origin and the area (total light) per individual was calculated. Because the crushed individuals' luminescence is not under the ostracods' behavioral control, we only compared these results for total relative light output and not kinetics. We then compared this

total light emitted with the light output from both defensive and courtship displays (see below).

Comparisons of the types of luminescence

To determine whether we could pool the total light emitted from males and females, we log-transformed the areas and ran a general linear model (GLM) in SAS 9.1 (SAS Institute, Cary, NC, USA). Because there was no significant difference between the males and females ($F_{1,15}=0.11$, $P=0.7448$), we pooled the results. The light emitted from courtship displays, defensive displays and the pooled total body stores of *P. annecohenae* were log-transformed to obtain equal variance between treatments, and a GLM in SAS 9.1 was used to compare treatments.

RESULTS

Courtship displays and defensive signals

Males of the Caribbean cypridinid ostracod *P. annecohenae* have been shown to produce highly visible trains of short extracellular pulses of light in temporally and spatially precise patterns (Fig. 1A, supplementary material Movie 1) above shallow seagrass beds at nightfall when no moon is present in an attempt to attract sexually receptive females (Rivers and Morin, 2008; Rivers and Morin, 2009). Rarely (ca. once every few nights) individuals (males, females or juveniles) were observed to be preyed upon by nocturnal planktivores, especially small fishes such as cardinalfish, usually just prior to the commencement of the courtship period near the

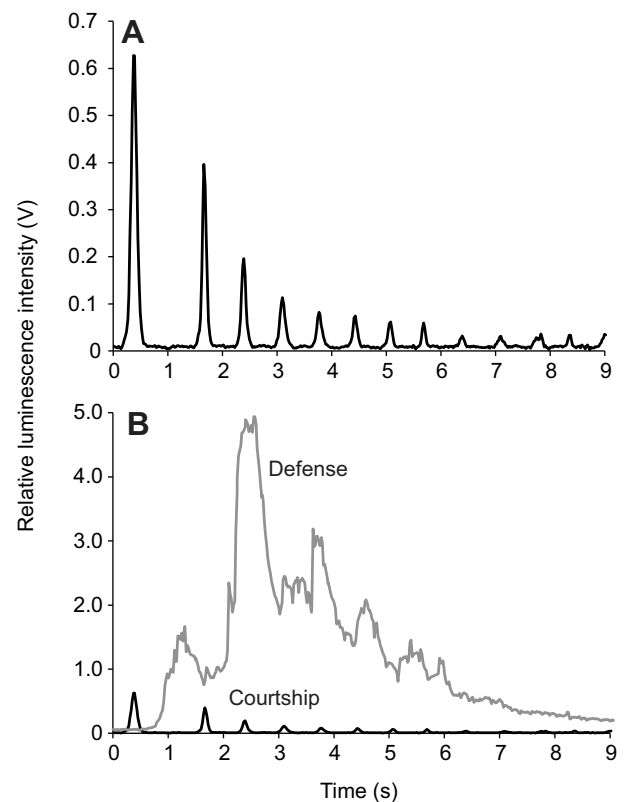


Fig. 1. Relative light output recordings of courtship and antipredation displays in the cypridinid ostracod *Photeros annecohenae*. (A) Courtship behavior only. (B) Antipredation behavior (gray line) interposed on the courtship display shown in A. The variable peaks in the antipredation luminescence are due to the ostracod releasing luminescence in pulses as it is disturbed, and also to the fish predator pumping its gills and ejecting the luminescence from its mouth and behind its gill opercles.

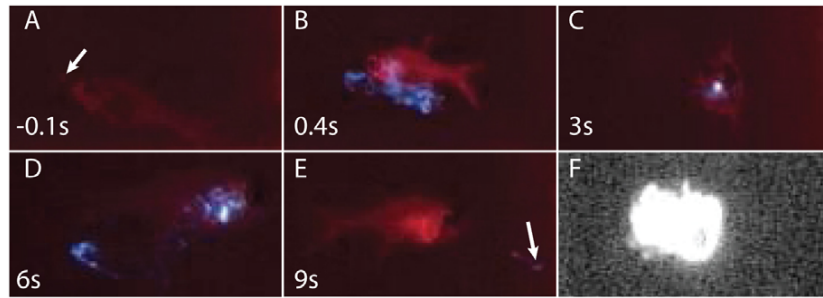


Fig. 2. Attempted predation on *Photeros annecohenae* by a dusky cardinalfish, *Phaeoptyx pigmentaria*. (A–E) Single-frame images taken from a video made with a light source from a dim red light but no light amplification, with elapsed time of attack shown. (A) Arrow indicates the ca. 2 mm ostracod with the cardinalfish approaching 0.1 s prior to attack. (B) Almost immediately after being taken into the fish's mouth, the ostracod releases copious quantities of luminescence, which is being pumped from the gill opercles. (C) Even when the fish is not pumping luminescence through its opercles, some luminescence is apparent within the buccal cavity. (D) Once the fish pumps its gills, more luminescence is released into the water. (E) The fish expectorates the ostracod (arrow); the trail to the upper left of the ostracod is luminescence left behind as the ostracod swims away apparently unharmed. (F) A low-light CCD still image of another antipredation episode at its brightest (the cardinalfish is in the center of the luminescent spot); in this image there was no external light source.

end of nautical twilight (ca. 45–50 min post-sunset). During a predation attempt, an ostracod releases copious quantities of luminescence that appears as bright, enveloping, swirling clouds of light, which coincides with the pumping frequency of the fish's gills (Fig. 1B). As a result of the gill pulsations, we do not know whether the ostracods release a continuous stream of luminescence (Fig. 2, supplementary material Movie 2) and, because of the absorptive opacity of some of the fish tissues, the actual amount of emitted light will be somewhat higher than the recorded levels. Our feeding experiments in the laboratory, either during the day or at night, resulted in the ostracod *P. annecohenae* almost always being expectorated by the cardinalfish, in most cases alive, and with the ostracod swimming away rapidly. During each 5 min tank trial, whether in the dark or light, there were approximately three to four predation attempts on *P. annecohenae* by the cardinalfish, each of which resulted in expectoration. A fish would take an ostracod into its buccal cavity, sometimes retaining it in for a number of seconds without swallowing, before releasing it (Fig. 2).

Overall light output comparisons

Our data on luminescent outputs show that although the courtship display trains are highly visible to an observer several meters away and are repeated over and over by sexually active males on any given night in the field, they constitute only a small fraction of the luminescence that is spewed out when an ostracod is attacked by a predator (Fig. 1, Fig. 3A, supplementary material Table S1). Furthermore, the defensive (antipredatory) displays themselves represent only a fraction of the light available from the total stores available within an individual (Fig. 3A). There were significant differences in total luminescence between courtship displays, defensive displays and crushed individual (total) luminescence (GLM, $F_{1,36}=77.56$, $P<0.0001$; Fig. 3A, supplementary material Table S1). Crushed individuals released such large quantities of luminescence in the tissue grinder that the light was visible to the dark-adapted human eye for well over 3 min. The brightest (i.e. maximum) pulse from an average defensive display was 13 times brighter in intensity than the brightest pulse of an average courtship display train, and showed much more variation between peak intensities (Fig. 1, Fig. 3B).

Courtship display light budgets

There are two distinct phases of courtship displays (Figs 1, 4, supplementary material Table S1), which differ in their luminescence

output (Rivers and Morin, 2008). The first (stationary) phase contains mostly three, but occasionally four, brighter, longer-duration pulses followed by a second (helical) phase consisting of, on average, nine but up to 16 dimmer, more uniform pulses for a total of 12 (to 19) pulses per train (Rivers and Morin, 2008). In the laboratory, spatial constraints of our tanks and/or the later pulses becoming indistinguishable from the PMT noise allowed us to analyze only a maximum of nine pulses per courtship display from our PMT recordings. A previous study (Rivers and Morin, 2008) has shown that the final helical pulses ($N=9-16$) were nearly equal in intensity and waveform. Thus, we extrapolated our data to predict the luminescent percentages found in both the mean ($N=12$ pulses) and maximum ($N=19$ pulses) display trains (supplementary material Table S1). A typical train of 12 pulses observed in the field utilized only 0.22% of the mean total stores in the animal. In other words, the average individual has enough stores for over 450 displays. Furthermore, within a 12-pulse display, 62% of the luminescence occurs in the stationary phase (the first three pulses of the display). A display consisting of 19 pulses would utilize only 0.29% of the mean total stores with 52% of this luminescence occurring during the stationary phase. Thus, an average individual male producing 19 pulses would have enough stores for nearly 350 displays. The first pulse of a display, which is always the brightest, utilized 0.08% of the total stores on average, but the brightest helical pulse utilized only 0.01%. The maximum recorded luminescent output of a display released nearly twice the amount of luminescence as an average display (supplementary material Table S1).

Defense light budgets

In defensive responses induced by fish predators in the dark (Fig. 1B), the light rises quickly and peaks within approximately 3–4 s and subsequently gradually diminishes in intensity over the next several seconds. As noted above, because of partial shielding by fish tissue while the ostracod is in the buccal chamber, the light levels represent a lower limit of the actual output and probably contribute to some of the variability. The average defensive episode comprised approximately one-tenth of the total luminescent stores found in crushed individuals, but released approximately 50 times more luminescence than a typical 12-pulse courtship display train (Fig. 2, supplementary material Table S1). There was large variation in the amount of luminescence per defensive display, with the maximum recorded display releasing nearly five times the average

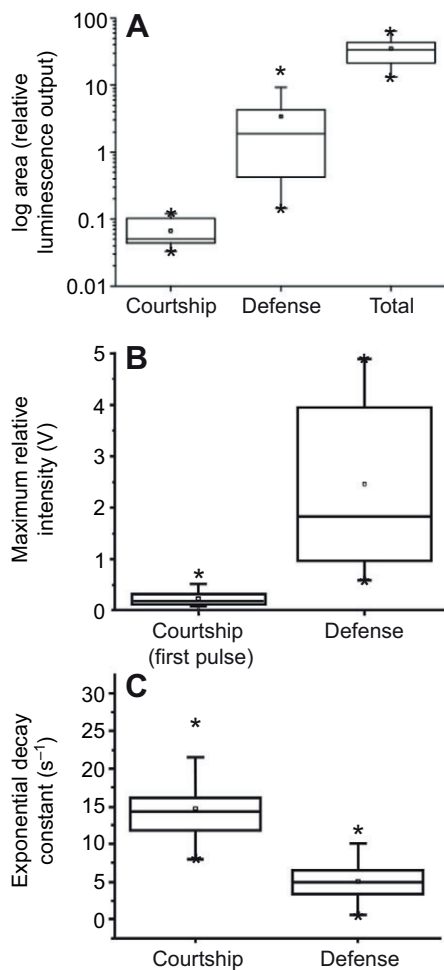


Fig. 3. Comparisons of relative luminescence between courtship, defense and total animal outputs. (A) Log comparisons of the relative total luminescence per ostracod from individual luminescent courtship displays, antipredation events and the total animal. (B) Maximum relative intensity comparisons between antipredation and courtship display behaviors only. Data were log-transformed to satisfy the assumptions of linear regression analysis; these raw data show the wide variation in luminescence intensities during an antipredation episode *versus* the narrow range during courtship displays. (C) Decay constant (s^{-1}) comparisons between courtship and antipredation luminescence. Each box encompasses the middle 75% of the recorded areas, the horizontal line is the median, the small square is the mean, and the arms extend to 95% of the range. Asterisks represent the maximum and minimum values recorded.

amount (supplementary material Table S1). The maximum defensive display we recorded represented 46% of the mean individual stores, or 25% of the maximum recorded stores from an individual.

Decay constant comparisons

The decay constants of both courtship and defensive displays follow first-order kinetics (Fig. 3C). Defensive display luminescence decreased almost three times slower than courtship display luminescence and with large variation. The mean decay constant of a defensive episode was $5.47 \pm 3.83 s^{-1}$ (time to half decay $\sim 0.13 s$) compared with a mean courtship display constant of $14.64 \pm 3.65 s^{-1}$ (time to half decay $\sim 0.05 s$; Fig. 3C). Courtship display decay constants were consistent across pulses. At $26^{\circ}C$, the mean decay

constant for pulse number 1 was $14.23 \pm 3.39 s^{-1}$, virtually identical to that of subsequent pulses ($15.07 \pm 4.30 s^{-1}$). Using a random-effects mixed model to compare courtship and defensive display decay constants, we found that neither the pulse number ($F_{1,75.9} = 0.12$, $P = 0.7266$) nor the interaction between treatment (courtship or defense) and pulse number ($F_{1,75.9} = 1.45$, $P = 0.2321$) had any significant effect on decay rate. However, we did find that courtship display luminescence had a significantly higher decay constant than that of defense ($F_{1,89.6} = 21.0$, $P < 0.0001$).

DISCUSSION

Luminescent budgets

All animals invest heavily in acquiring food, deterring predators and attempting to reproduce. Feeding mechanisms are often structurally elaborate and temporally active, both of which utilize significant resources. Furthermore, many animals allocate much of their resources into building fortified skeletons, protective weaponry or chemical defenses to thwart predators. Still others invest in camouflage or speed for protection. Additionally, procuring a mate, often involving some form of communication, also uses valuable resources (Bradbury and Vehrencamp, 2011). In the case of the Caribbean ostracod *P. annectoehanae*, both predation defense and courtship behaviors utilize bioluminescence. This species is capable of secreting an astonishing amount of extracellular luminescence from its stores. These ostracods have a surprisingly long lifespan for such tiny organisms: the maximum adult lifespan of *P. annectoehanae* observed in culture is approximately 6 months (Gerrish and Morin, 2008). Furthermore, females can produce multiple broods even from a single insemination, males probably display nightly throughout their adult lives, and both sexes are continuously capable of producing and replenishing their luminescent capabilities during this entire time. Therefore, they are probably income breeders (*sensu* Jönsson, 1997).

In these ostracods, very long secretory cells, 94% of the length of which is packed with secretory vesicles, extend the entire length of the light organ (ca. $400 \mu m$) and terminate at a series of nozzles on the upper lip (Huvard, 1993). How much of this vesicle store is readily available to produce luminescence is unknown. However, if we start with the assumption that an ostracod has a full store of luminescent compounds (cypridinid luciferin and luciferase and mucus) in separate secretory cells that is available to be secreted, then a typical male has the potential to produce over 450 average 12-pulse courtship display trains, 10 average defensive displays or four maximum defensive displays before depleting the luminescent potential it has stored at a given time (supplementary material Table S1). Within a single typical male courtship display train, the first pulse contains over one-third of the total luminescence of the total train, whereas a typical pulse in the terminal helical phase contains only approximately 5% of the output of that single courtship display train. We use these calculations in determining the nightly luminescent budget and luminescent compound regeneration for an ostracod to remain in a steady state, and in considering the behavioral strategies used by males participating in courtship and defensive displays.

Defense

A single defensive episode depletes an average of approximately 10% of the luminescent potential, but maximum displays can deplete approximately 25% of the maximum luminescent potential of the individual (supplementary material Table S1). These displays are thus costly, and are rarely observed in the field. Although we have observed tens of thousands of courtship displays during our observations between dusk and the end of a display period, on any

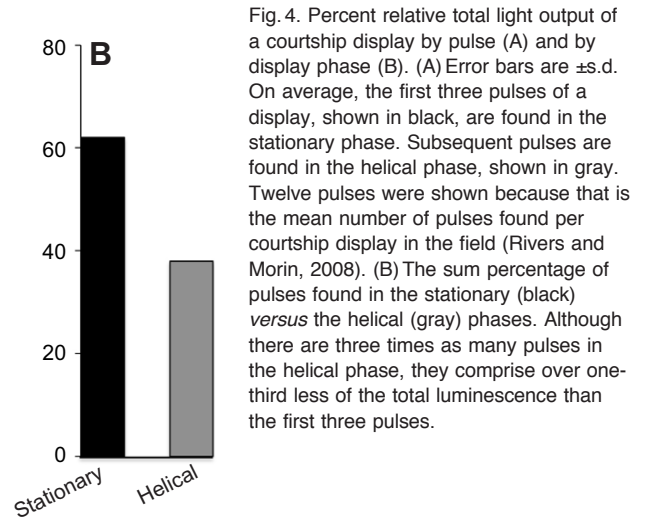
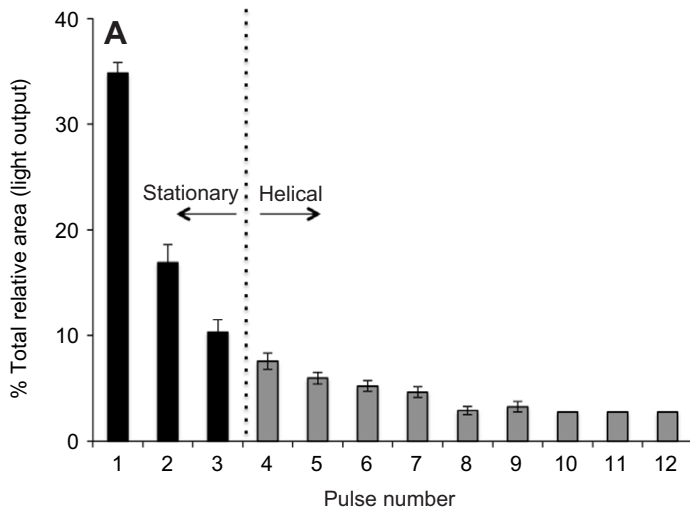


Fig. 4. Percent relative total light output of a courtship display by pulse (A) and by display phase (B). (A) Error bars are \pm s.d. On average, the first three pulses of a display, shown in black, are found in the stationary phase. Subsequent pulses are found in the helical phase, shown in gray. Twelve pulses were shown because that is the mean number of pulses found per courtship display in the field (Rivers and Morin, 2008). (B) The sum percentage of pulses found in the stationary (black) versus the helical (gray) phases. Although there are three times as many pulses in the helical phase, they comprise over one-third less of the total luminescence than the first three pulses.

given night we almost never observe even one defensive event in the same area. When defensive displays are observed, they almost inevitably occur earlier in twilight during nautical twilight (20–50 min post-sunset), when there is more ambient light and the courtship displays have not commenced (Morin, 1986; Morin and Cohen, 1991; J.G.M. and T.J.R., personal observation). Even so, despite their rarity, based on our comparisons between luminescent reserves and defensive displays, a single ostracod still contains enough components for between four and 10 more defensive displays before requiring actual synthesis of new compounds. We have never observed this rate of attacks in the field.

If a male does produce a defensive display, with a possible depletion of up to a quarter of its stores from a single attack, this could severely lower a male’s ability to produce courtship displays in order to be sexually competitive. With an operational sex ratio of approximately 178 males for every available female, competition among males is high (Rivers and Morin, 2008; Rivers and Morin, 2009). Because luminescence is important in *P. annecohenae* courtship, variations in display ability may affect variation in mating success for males. Assuming that 25% of their luminescent potential is close to the maximum amount from storage readily available to males, and if males vary significantly in their rates of synthesis of luminescent compounds (due to intrinsic or metabolic differences), there may be significant variation in the frequencies or intensities of courtship displays males are able to exhibit each night depending on attack history. But the rarity of observed attacks and high male densities suggests that defensive displays may have little or no effect on overall individual display variation.

Courtship

Compared with defensive luminescence, courtship displays are relatively inexpensive, but are regularly and abundantly produced. A male would have to display, on average, 50 times to equal the luminescent output of a single, average defensive display, and 200 times to equal that of the maximum observed defensive display. Courtship displays in an area of 1 m² above shallow seagrass beds in Belize occur at an average of approximately once every 9 s (7 displays m⁻¹ m⁻²), and it is highly unlikely that a single male is responsible for all of these (Rivers and Morin, 2008; Rivers and Morin, 2009). When tracking individual male behavior in the laboratory (Rivers and Morin, 2008), we found the highest courtship frequency for a single male to be approximately 1.5 displays per

minute (T.J.R. and J.G.M., unpublished). Extrapolating this individual courtship display frequency to encompass an entire hourlong courtship period gives the male a maximum of approximately 90 courtship displays per night. This number is probably somewhat higher than the frequency of an actual typical displaying male, as in no case in the laboratory has one male been shown to display consistently over that length of time. Rather, a male will display several times, and then sneak or entrain on other courtship displays (Rivers and Morin, 2009). In addition, we have also seen activity stop completely in both the field and laboratory, often over 5 min in duration, before commencing again (T.J.R. and J.G.M., personal observation). However, this number of 90 courtship displays per night provides a plausible high-end maximum frequency for our estimations. As a single courtship display train of 12 pulses [the mean in both the laboratory and the field (Rivers and Morin, 2008)] uses only approximately 0.22% of the total stores available, a male that produces 90 courtship displays per night would exhaust only approximately one-fifth of its total stores per night. Accordingly, it would take over five nights to completely exhaust his existing stores *via* displaying without replenishment, or a regeneration rate of approximately 20% per day to completely restore the previous night’s usage.

If two males had equal courtship display frequencies but varied significantly in the intensity of each display, then the rate of regeneration for full luminescence recovery would necessarily vary. In an earlier study in the laboratory we discovered that, among individual males, the brightest pulse of the brightest courtship display was 85% brighter than the brightest pulse of the dimmest luminescent courtship display train, and 50% brighter than the brightest pulse of a mean courtship display train (Rivers and Morin, 2008). Therefore, some males are expending more energy per display than others and, as a result, probably release more of their luminescent components per night than others, thus requiring a higher rate of synthesis to replenish their stores. Furthermore, there are also differences in the frequency of displays among males (Rivers and Morin, 2009). If males with brighter courtship displays have a lower frequency of displays per night, there may be two separate behavioral strategies being utilized by males with the same luminescent budget. Either males could invest more heavily in brighter displays but at a lower frequency over the night, thus hoping to attract a female by being much brighter than other displays, or

they could display more dimly but more often, giving more opportunities for a receptive female to notice them.

From an examination of the differential allocation of luminescence between the stationary (alerting) phase and the final helical (orienting) phase within a single courtship display train, we can make predictions about the costliness of different potential courtship display strategies of equal pulse intensities. In our analysis of the light budgets, 50–60% of all the light produced in a train occurs during the initial stationary phase (first three to four pulses) whereas the succeeding nine to 16 pulses of the helical phase contain less than half the light. However, once a female is focused on a signaling male, it is this last half that is crucial for allowing a receptive female to align with and intercept the signaling male (T.J.R. and J.G.M., unpublished). Adding successive pulses in the helical phase, as they are all quite similar and each is an expenditure of only approximately 5% of a typical train, does not represent much of a drain on the male (less than a 0.07% decrease of a male's total stores by increasing the train length from 12 to 19 pulses). Therefore, in addition to providing an approaching female more cues for successful interception and copulation (T.J.R. and J.G.M., unpublished), it would seem to be relatively more efficient to have fewer, many-pulsed courtship display trains rather than more, fewer-pulsed trains. However, if a female is easily distracted by the brighter pulses in the stationary phase of a nearby competing courtship display, although more energetically costly and providing fewer interception targets to the female, the original male may be more successful at regaining her attention (with the much brighter luminescence of the stationary phase) by abandoning his current display and starting again. Both of these competing factors may be acting at the same time upon displaying males in the field. The most successful courtship display train length, therefore, may be a trade-off with the average closer to the mean of 12 pulses that we see in the field.

Kinetics

Cypridinid luminescence produced with purified extracts (mostly of *Vargula hilgendorffii*) in laboratory settings with known concentrations of enzymes and substrates in predetermined volumes and no extraneous compounds always follows first-order kinetics (Amberson, 1922; Stevens, 1927; Chance et al., 1940; Harvey, 1952; Chase, 1948; Tsuji et al., 1970; Shimomura, 2006). The decay rate is logarithmic and depends directly on enzyme (luciferase) concentration; hence, it is a simple unimolecular process. In these reactions the intensity and total light produced are a function of the absolute concentrations of luciferin and luciferase released, and the rate of the reaction is a function of the relative ratios of luciferase to luciferin produced; higher luciferin to luciferase ratios yield slower kinetics. Luminescent cypridinid ostracods in nature appear to be able to dramatically control the duration of their extracellular secretions over several orders of magnitude between courtship and defensive displays, yet all are consistent with simple first-order kinetics. Determining enzyme:substrate ratios and concentrations in the water column from ostracods that are actively regulating the release of luminescent constituents as well as other nonluminescent compounds makes the determination of the exact kinetic interactions much more difficult. However, as both defensive and courtship displays follow first-order kinetics, this still suggests that simple unimolecular processes occur during both behaviors.

The defensive displays produced by luminescent Caribbean ostracods in response to rare predator attacks appear similar among species (Morin, 1986), tend to be exceedingly bright and can last many seconds. Our records show that the defensive displays in *P.*

annecohenae are composed of complex waveforms, but it is uncertain whether these resulted from the actions of the fish or successive expulsions by the ostracods, or both. Preliminary data indicate that an ostracod can produce multiple expulsions of luminescence as a result of a single electrical stimulus (T.J.R. and J.G.M., unpublished). In all cases, the most rapid defensive flashes to fish within these clouds were much brighter, slower and more variable in duration than courtship pulses, but still demonstrated first-order kinetics.

Similarly, Tsuji et al. (Tsuji et al., 1970) reported that *Cypridina serrata*, a west Pacific species, produced luminous defensive clouds in the sea that took 3–4 s to decay in response to stimulation by a flashlight and brilliant secreted luminescence that lasted many seconds from individuals that were placed in buckets of seawater and mechanically disturbed. In the only record of spontaneous, unstimulated luminescence by ostracods other than Caribbean species, they further reported that two to four individuals placed in 10 ml vials with seawater showed that the pulses yielded first-order decay kinetics with a decay constant that varied from approximately 0.8 to 1.4 s⁻¹, a time to half decay of ~0.35–0.4 s, and a duration of approximately 1.5–2.5 s. By comparison, *P. annecohenae* (Fig. 2) defensive flashes within the longer clouds were only slightly faster (rate constants of 5.5 s⁻¹, but with much variation, and time to half decay of ~0.13 s) but with similar combined durations of sometimes many seconds, and courtship pulses were much faster [decay constants of ~14.6 s⁻¹, time to half decay of ~0.05 s and durations of 0.1–0.4 s (see Rivers and Morin, 2008)].

Because defensive displays are almost always much brighter and have slower decay constants than courtship displays (Fig. 1B, Fig. 3C), this suggests that there is more luciferase and luciferin released and there is relatively more luciferase than luciferin in defensive displays compared with courtship displays. It also clearly indicates that individual ostracods have control over both the amount and proportion of each constituent they secrete into the water. Moreover, other signaling ostracod species demonstrate decidedly different pulse characteristics in their courtship displays [pulse durations among species vary over almost two orders of magnitude from as short as 50 ms to over 10 s (Morin and Cohen, 2010)]. In contrast, the consistency of decay constants among pulses within and between courtship displays in *P. annecohenae* (Fig. 3C), even though intensities and total light vary, suggests that they are secreting a constant ratio of luciferin to luciferase but with different equal molar amounts.

The morphology of the upper lip and light organ (Huvard, 1993) suggest a mechanism for both the secretion of the constituents and their independent regulation that leads to the control of both intensity and duration of pulses. There are three distinct zones of the upper lip where nozzles are found, from which the luciferin (the tripeptide), luciferase (the enzyme) and mucus are separately secreted. Huvard's (Huvard, 1993) data suggest that the luciferin is secreted from the center (medial) row of nozzles, the luciferase is secreted from nozzles distributed along a pair of lateral rows, each flanking the medial row and the tusks, and mucus is secreted from the tusks. A fourth cell type of unknown function also terminates at the lateral nozzles. The secretions of these materials into the water column are postulated to occur by the 'hydrostatic pressure for opening and elastic recoil for closing' hypothesis and to be controlled by the action of several different muscles that surround and penetrate the light organ, and the associated surrounding sclerites (Huvard, 1993), and act much like a hand squeezing a tube of toothpaste. Luminescent cypridinid ostracods probably can individually control the different muscle bands whose precise placement around the

luminescent organ indicates that each could control secretion from a separate type of cell (Huvar, 1993).

The external luminescent reaction itself is postulated to occur in a kind of containment chamber or reactive core (bolus) consisting of luciferase surrounding the luciferin, which is enclosed in a cohesive rind of mucus; this model is consistent with the distribution of the secretory cells (Huvar, 1993). In this model, by restricting the diffusion of the enzyme and substrate into the surrounding seawater, the mucus could increase the encounter rate of the two constituents, thus maximizing the reaction rate. Alternatively, if the mucus is pervasive throughout the bolus, the mucus could actually slow the diffusion of the luciferin and luciferase to a point that it reduces the reaction rate more than if it were in only seawater. Regardless of its actual effect on the reaction rate, the lack of variation between pulses during a courtship display indicates that the mucus secretion and the luminescent reaction are tightly controlled. The most parsimonious hypothesis is that the intensity of the reaction is controlled by varying the absolute amounts of the substrates while the kinetics is regulated by the ratios among the constituents. If the luciferin and luciferase are in approximately equal molar concentrations, then the reaction will occur quickly and coincide with the first-order kinetics where the luciferin is limiting. However, longer pulses could be produced by providing much higher concentrations of the luciferin relative to the luciferase so that the enzyme remains saturated, thus extending the duration.

It is unknown whether there are differences in the concentrations between the molecules released during courtship displays and defensive displays; thus the variation between the two types could also be attributed to other factors. The slower decay rates in defensive displays may be a result of not only a difference in luciferin:luciferase or mucus:luciferin:luciferase ratios, but also differences in the volume in which the reaction occurs, with a larger volume corresponding to a slower rate of decay, because the encounter rate of enzyme to substrate would be lower. Indeed, defensive episodes typically occupy much larger and variable luminescent volumes, especially after being dispersed by the pumping action of the fish's gills. Only by knowing the exact volume in which the defensive display occurs can we determine whether the majority of the difference between courtship and defensive display decay rates are explained by this effect. Furthermore, the presence or absence of mucus in defensive displays and their probable effect on the diffusion rate of luminescence compounds may also contribute to the differences.

The relationship of predator deterrence to stimulated luminescence is complex. Diurnal planktivorous fishes tested in the laboratory during daylight hours, when luminescence is masked by ambient light, also almost always reject these live, but not dead, ostracods (J.G.M. et al., unpublished). These ostracods therefore appear to be directly unprofitable to predators by being chemically (via one or more of the secreted molecules, or constitutively), mechanically or behaviorally defended. Triggered luminescence therefore is likely to represent a synergistic effect by conferring further protection to the ostracod. It has been proposed that the bright pulse of light startles a predator ('boo effect') and/or renders the predator vulnerable to attack from its own predators by revealing the location of an attack ('burglar alarm effect') (Morin, 1983; Morin, 1986; Haddock et al., 2010), but neither of these hypotheses has been experimentally tested in this system.

Comparison to firefly mating and defensive luminescence

In our analysis we only considered the relative usage costs of defensive and courtship signals in cypridinid ostracods and not total

costs because of the inherent difficulties in determining the developmental and maintenance costs. The usage cost of luminescent courtship signals in these ostracods is cheap compared with both their defensive signals and total reserves. Interestingly, the observed rarity of antipredatory displays strongly indicates that luminescent ostracods are virtually immune to any predation during their very obvious courtship display periods. Similarly, the usage costs of emitting courtship flashes by *Photinus* male fireflies is low (Woods et al., 2007) and they also similarly experience low predation risks to generalist predators because of an unpalatability (aposematic) association with these courtship signals (reviewed in Lewis and Cratsley, 2008). It should be noted that neither Woods et al. (Woods et al., 2007) nor we considered the hidden developmental and maintenance costs in our analyses. Further, compared with other components involved in firefly courtship (flying and walking, etc.), the usage cost to generate firefly courtship signals is also relatively low. However, Woods et al. (Woods et al., 2007) found that these low-cost sexual signals from *Photinus* fireflies carried a high collateral cost because of secondary receiver interception by visually orienting specialized predators (female *Photuris* fireflies), which are immune to the general chemical defenses of *Photinus* species. In contrast, in ostracods we have not observed any comparable specialized predators that are able to target them through secondary receiver interception, and thus ostracods appear to be able to signal openly without fear of predation. In fact, it is quite possible that during their signaling periods on the display grounds above the reefs of the Caribbean, other similar-sized, non-luminescent demersal plankton might benefit from the presence of signaling ostracods because planktivorous predators might be wary of inadvertently capturing a plankton that is potentially dangerous to eat.

Conclusions

Although the cypridinid ostracod luminescent system is one of the most thoroughly studied in the laboratory [see Harvey (Harvey, 1952), Herring (Herring, 1978) and Shimomura (Shimomura, 2006) and references therein], *P. annecohenae* is the first ostracod species in which the kinetics of two separate behaviors from the field have been documented. Individual ostracods (male and female, juvenile to adult) are capable of releasing surprising quantities of luminescence into the water column as a predator deterrent and males also exhibit regulated control over the release of luminescence in discrete, complex patterns. The stores of luminescent materials a male has readily available may impact which mating tactics he uses, therefore potentially influencing his mating success. Ostracods may have evolved predator deterrence from a preexisting set of molecules that were involved in oxidative processes (oxygenases) that emitted light as chemiluminescence (*sensu* Rees et al., 1998) and which were already costly but important. Our data suggest that adding courtship displays as a separate function is relatively cheap by comparison. It is therefore surprising that courtship displays do not appear to have evolved in luminescent cypridinids anywhere except the Caribbean. However, the luminescent repertoire of these tiny, 2 mm crustaceans is impressive by any measure.

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Table S1. Comparison of relative luminescence between pulse courtship displays, defensive displays and total available stores as percentage of total of the mean for each category

A	Mean total	Mean defense	Mean courtship	Mean pulse 1	Mean train pulse
Mean total	100	10.14	0.22/0.29	0.08	0.01
Mean defense		100	2.27/2.95	0.83	0.1
Mean courtship			100	36.24/27.9	4.27/3.29
Mean pulse 1				100	11.79
Mean train pulse					100
B	Max. total	Max. defense	Max. courtship	Max. pulse 1	Max. train pulse
Max. total	100	25.36	0.24/0.33	0.09	0.01
Max. defense		100	0.93/1.32	0.35	0.06
Max. courtship			100	37.3/26.34	5.95/4.2
Max. pulse 1				100	15.94
Max. train pulse					100
C	Max. total	Max. defense	Max. courtship	Max. pulse 1	Max. train pulse
Mean total	179.53	45.53	0.42/0.6	0.16	0.03
Mean defense		466.54	4.34/6.15	1.62	0.26
Mean courtship			191.51/208.81	71.44/55	11.39/8.77
Mean pulse 1				197.14	31.43
Mean train pulse					266.67
D	Mean total	Mean defense	Mean courtship	Mean pulse 1	Mean train pulse
Max. total	55.70	5.44	0.12/0.16	0.04	0.01
Max. defense		21.43	0.49/0.63	0.18	0.02
Max. courtship			52.22/47.89	18.92/13.36	2.23/1.57
Max. pulse 1				50.72	5.98
Max. train pulse					37.50

The first of the two numbers in the courtship rows and columns correspond to the output of a 12-pulse display (the mean found in the laboratory and field), and the second a 19-pulse display (the maximum observed in the field). (A) Comparisons among mean outputs. (B) Comparisons among maximum values recorded. (C) Comparisons between maximum outputs recorded and mean values. (D) Comparisons between mean outputs and maximum outputs recorded.