**Developmental ecdysteroid titers and DNA puffs in larvae of two sciarid species,
Rhynchosciara americana and *Rhynchosciara milleri* (Diptera: Sciaridae)**

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Abstract: Ecdysteroid titers, developmental landmarks and the presence of prominent amplifying regions (DNA puffs) have been compared during late larval to pupal development in four groups of *Rhynchosciara americana* larvae and in *R. americana* and *Rhynchosciara milleri*. Three prominent DNA puffs (B2, C3 and C8) expand and regress sequentially on the rising phase of the 20-hydroxyecdysone (20E) titer in *R. americana* as a firm, cellular cocoon is being constructed. A sharp rise in 20E coincides with the regression of these puffs. The shape of the 20E curve is similar in *R. milleri*, a species that does not construct a massive cocoon, but the behavior of certain DNA puffs and their temporal relationship to the curve differs. Regions corresponding to B2 and C3 can be identified in *R. milleri* by banding pattern similarity with *R. americana* chromosomes and, in the case of B2, by hybridization to an *R. americana* probe. A B2 puff appears in *R. milleri* as the 20E titer rises but remains small in all gland regions. A puff similar to the *R. americana* C3 puff occurs in posterior gland cells of *R. milleri* (C3^{Rm}) after the B2 puff, but this site did not hybridize to *R. americana* C3 probes. C3^{Rm} incorporated ³H-thymidine above background, but showed less post-puff DNA accumulation than C3 of *R. americana*. *R. americana* C8 probes hybridized to a more distal region of the *R. milleri* C chromosome that did not appear to amplify or form a large puff. These differences can be related to developmental differences, in particular differences in cocoon construction between the two species.

Keywords: sciarid flies, ecdysone, insect metamorphosis, amplification, DNA puffs

Introduction

Species of the sciarid genus *Rhynchosciara* are large-sized flies distributed throughout South and Central America (Breuer 1969; Pavan and Breuer 1955). Each female lays a cluster of eggs that are either male or female and the larvae remain together and develop synchronously through pupation until emergence of the adults. Larvae of the *americana* species group construct massive communal cocoons during the last larvae instar where the final stages of development leading to the adult occur. In contrast, larvae of the *milleri* species group do not spin such massive cocoons (Pavan and Breuer, 1955).

Rhynchosciara is best known as the genus in which gene amplification was first recognized (Breuer and Pavan 1952; 1955). These and other investigators (Ficq and Pavan 1957; Rudkin and Corlette 1957) described a process in the salivary gland polytene chromosomes of *R. americana* during the last larval instar that they termed DNA puffing, which resulted in accumulation of a disproportionate amount of DNA at particular bands. DNA amplification was subsequently studied and confirmed in several other sciarid genera (Crouse and Keyl 1968; reviewed in Monesi et al. 2009).

DNA amplification and puffing and the developmental changes surrounding this phenomenon are under the control of ecdysteroids, the insect molting hormones, or more specifically, the hydroxylated form of ecdysone, 20-hydroxyecdysone (20E) (Alvarenga et al. 1991; Amabis and Simoes 1971; Berendes and Lara 1975; Crouse 1968; Fresquez 1979; Stocker and Pavan 1974). A number of studies have suggested that fluctuations in these hormone levels are necessary to bring about the complete temporal program of DNA puffing as well as the associated developmental changes in sciarids (de Carvalho et al. 2000; Stocker et al. 1984), but the temporal relationships of ecdysteroid titers to puffing patterns during late larval development through pupation have only been investigated in two

sciarid species belonging to different genera, *Rhynchosciara americana* (Stocker et al 1984) and *Bradysia hygida* (Basso et al. 2002). In both studies, changes in DNA puffing inferred from developmental landmarks could be matched to the titer curves. DNA amplification and puffing occurred along a single large ecdysteroid peak in late larvae to prepupae of *B. hygida* (Basso et al. 2002), with some DNA puffs expanding during the rising phase of the curve, while others expanded during the declining phase. Two successive stages of polypeptide synthesis were correlated with these puffs (reviewed in Monesi et al. 2009). *Bradysia* larvae presumably use these polypeptides to build individual pupal cases during the late eyespot stage (Been and Rasch 1972). In contrast to *Bradysia*, DNA puff expansion in *Rhynchosciara americana* seemed to occur along a biphasic curve of ecdysteroid titers (Stocker et al. 1984). Most of cocoon construction and three well-studied DNA puffs (Frydman et al. 1993; Glover et al. 1982; Penalva et al. 1997; Santelli et al. 1991; 2004; Yokosawa et al. 1999) occurred during an initial small ecdysteroid peak which was then followed by an abrupt increase to a high ecdysteroid prepupal peak. Two of these DNA puffs have been correlated with polypeptides produced in the gland (Winter et al. 1977a; de Toledo and Lara 1978) and also present in the salivary secretion of cocoon spinning *R. americana* larvae (Winter et al. 1977b). These same polypeptides were induced by ecdysone treatment (Alvarenga 1980; Alvarenga et al. 1991).

The large size and developmental synchrony of *Rhynchosciara* allow correlations between DNA puffs and developmental ecdysteroid titers in a single group of larvae at carefully staged developmental time points. The developmental timing can then be compared for similarities and differences with other synchronous groups of the same species and also across species of the same genus. For instance, members of the *milleri* species group do not construct an elaborate cocoon, so comparison of DNA puffs and ecdysteroid titers in *R. americana* and *R. milleri* should provide further insights into the role of particular DNA puffs

in cocoon construction. Since *R. milleri* has many developmental similarities to *R. americana*, but pupates in a more individual manner (Pavan and Breuer 1955), we hypothesized that its ecdysteroid titer curve might differ from that of the latter species and be more similar to that of *Bradysia*, which also pupates individually and does not construct a massive cocoon. This would not only contribute information to evolutionary relationships among the Sciaridae, particularly within the genus *Rhynchosciara*, but also reveal life-style associated modifications of hormonal control.

Materials and methods

Animals

Rhynchosciara americana and *Rhynchosciara milleri* larvae were collected at a field site near Mongaguá, State of São Paulo, Brazil. The larvae were maintained in the laboratory at $20\pm 2^{\circ}$ C and fed with fresh, chopped sweet potato leaves. All larvae used in our experiments were female. Four different groups of *R. americana* and two different groups of *R. milleri* were sampled. We hoped to obtain data from additional groups of *R. milleri* but conditions in collecting areas were not favorable during the period when these experiments were being carried out. Nonetheless, we consider that the discrepancy in group size, which is a frequent characteristic of sampling in field sites, would not hinder the conclusions drawn, as we analyzed endocrine and cytological events in a strictly individual and correlative manner. In these *Rhynchosciara* species the adult female, after copulation, deposits a group of eggs which eclose and, develop almost synchronously throughout the larval instars to pupation and adult emergence. Although each egg group is deposited by a single female, there can be some mixing in the field with larvae of the same stage from other groups. However, all larvae in a single group become developmentally synchronous.

Hemolymph sampling

In the present work, the ecdysteroid levels were measured from hemolymph of larvae during the final stages of their development. Samples of hemolymph were collected individually from 2-3 female *R. americana* and 2-6 *R. milleri* larvae of each group at different times during development from the late larval period until after pupation. Larvae were placed on ice, a small incision was made in the ventral region of each individual, and the hemolymph was quickly collected with a micropipette and transferred to an Eppendorf tube. The samples from individual larvae were centrifuged at $10,621 \times g$ for 20 min at 4°C and the supernatant collected, quickly frozen in liquid nitrogen and maintained at -20°C until radioimmunoassays could be carried out.

Radioimmunoassay of ecdysteroids

Hemolymph ecdysteroid titers were measured by means of a radioimmunoassay according to the protocol described in Basso et al. (2002). Ecdysteroids were extracted from hemolymph by adding a 100-fold volume of cold methanol (-20°C). The samples were agitated and centrifuged at $5000 \times g$ for 5 min. at 4°C . Three hundred microliters of the supernatant was collected, which corresponds to $3 \mu\text{l}$ of hemolymph, and the solvent was removed by vacuum centrifugation. Radioactively labeled ecdysone [$23,24\text{-}^3\text{H}(\text{N})$]-ecdysone (NEN, specific activity 102 Ci/mmol) was prepared to obtain a radioactivity of $6500 \text{ cpm}/100 \mu\text{l}$ of solution and $100 \mu\text{l}$ of this solution was added to each sample vial and vortexed to mix with sample ecdysteroids. The antiserum, from an H-22 bleed (Warren and Gilbert 1986), was diluted to a concentration of 0.05% in borate buffer (pH 8.4) containing 0.1% BSA (Sigma) and 0.1% rabbit IgG (Sigma), and $100 \mu\text{l}$ of the antiserum solution was added to each of the samples, which were then maintained at 4°C overnight. Antibody-bound ecdysteroids were separated from unbound by adding $200 \mu\text{l}$ of saturated ammonium sulfate solution to each sample. After being kept at 4°C for 30 minutes, they

were then centrifuged at 5000 x g for 15 min. at 4°C. The supernatant was carefully removed, 400 µl of a 50% ammonium sulfate solution was added and the precipitation procedure was repeated. The final pellet was re-suspended in 40 µl of water and Optiphase HiSafe 3 cocktail (Perkin Elmer) was added for liquid scintillation counting. Standard curves covering the range of 25 to 2000 pg were generated using 20-hydroxyecdysone (Simes, Milan) as the non-radioactive ligand. The ecdysteroid content of the samples was therefore expressed as 20-hydroxyecdysone (20E) equivalents.

Chromosome preparations

The same female larvae used to obtain samples of hemolymph were quickly dissected in Ringer solution (NaCl 0.04M, KCl 0.05M, CaCl₂ 0.01M, pH 7.4), and the salivary glands were removed and fixed in a fresh mixture of ethanol/glacial acetic acid (3:1). All salivary gland samples were maintained at -20°C in this fixative until preparation of chromosome squashes. The chromosome preparations were made by removing the salivary glands from the fixative and cutting them into regions. S1 (anterior), S2 (median) and S3 (posterior) gland regions were each placed into a drop of 45% acetic acid on a siliconized coverslip, soaked for 5-10 min and cut into small pieces. S2 is a large region that often is full of secretion, making it difficult to squash. Therefore, large pieces of secretion (along with some cells) were removed from the drop. A small drop of 0.2% lactic acetic orcein was added, the coverslip was picked up with a slide and the chromosomes were squashed. The slides were analyzed under phase contrast optics, major chromosome changes were noted and photographs were taken with an AXIOPHOT microscope (Zeiss) using x20 and x40 objectives.

In situ hybridization

In situ hybridization was carried out for identification of the sites of the B2, C3 and C8 DNA puffs in *Rhynchosciara milleri*. For the first set of hybridizations, the probes for the B2, C3 and C8 DNA puffs as well as the techniques used have been described in Stocker et al. 1993. All probes had been cloned from *R. americana* and hybridized previously to chromosomes of that species. The probe for the B2 DNA puff was a phage clone (λ NM1149) called P-26 containing a 2.5 kb genomic insert from this region (Pirrota et al. 1988). The probe for the C3 DNA puff was a subclone of the yeast plasmid (Yip5) called Y19A, which contained a 6 kb genomic fragment from this region (Millar et al. 1985). A cDNA clone in the Lambda-ZAPTM vector called pRa C-8-22, containing a 1.5 kb segment complementary to the *R. americana* C8 DNA puff gene (Frydman et al. 1993) was used for localization of this region. The clones were labeled by nick translation (ENZO) and hybridized overnight at 37°C to heat denatured polytene chromosomes. The hybridization solution contained 40% formamide, 2XSSC, 5% PEG and 40% probe DNA dissolved in Tris-EDTA buffer. Another set of hybridizations for the transcription units of the C3 and C8 DNA puffs was later carried out using the genomic clones Aj6 for C3 (Yokosawa et al. 1999) and pGem0.9 for C8 (Frydman et al. 1993) from *R. americana*. These were similarly labeled by nick translation (BioNick), but in this case, the hybridization solution contained 40% formamide, 2XSSPE, 0.5µg/µl salmon sperm DNA, 5% dextran sulfate and 20%probe DNA in Tris-EDTA. The location of the probes was visualized by anti-biotin antibodies and secondary antibodies conjugated to FITC (Sigma) or rhodamine (Pierce). In all experiments, *R. americana* chromosomes were hybridized in parallel as controls.

DNA replication in *R. milleri* polytene chromosomes

Two microliters of ^3H -thymidine solution, (specific activity $25\text{Ci}/\mu\text{M}$ or $1\mu\text{Ci}/\mu\text{l}$) was injected into the body cavity of female *R. milleri* larvae using a calibrated glass micropipette as described by Amabis (1974) at the time that the "eye spots" were migrating laterally. Eyespot development (formation of the *anlagen* of the adult eye) is a prominent marker in most sciarids (Laicine et al. 1984), including *R. milleri* (Stocker, personal observations). Incorporation was allowed to take place during 20 min before the larvae were sacrificed by dissection in Ringer solution, followed by fixation in 3:1 ethanol/acetic acid. Slides were prepared from S1 (anterior) and S2/3 (posterior) salivary gland regions by squashing in 50% acetic acid under a siliconized coverslip. Coverslips were removed in liquid nitrogen and the slides rinsed for 10 min in 70% ethanol and twice for 10 min each in absolute ethanol before drying. Dried slides were dipped in liquid emulsion (Kodak NTBII) under a safelight and kept at 4°C for two weeks. Autoradiographs were developed with Dektol (Kodak) diluted 1:1 at 15°C for 3 min., fixed in Polymax (Kodak) diluted 1:3 for 5-6 min and stained lightly with Giemsa (Gurr).

Chromosomes of the posterior (S2/3) regions of the gland were analyzed at potential amplifying regions since thymidine incorporation was better in these than in the S1 region. At least 10 labeled sets of chromosomes from five individuals each were examined for amplification at specific chromosome regions. An adjacent chromosome region was designated as background. Relative scores were given: 0 – label not above the chromosome background, 1 – label appearing slightly above chromosome background, 2 – label distinctly above chromosome background but most grains not overlapping, 3 – label heavy with many overlapping grains, appearing black. For labeling of the large puff designated C3^{RM} which formed at this stage, grain density was compared with a puff at A2 that was not believed to amplify. Seven to 15 labeled nuclei from four slides with both regions puffed were examined, and grain densities averaged and compared. Scores were:

0 - C3^{RM} about equal to A2, 1 - C3^{RM} slightly greater than A2, 2 - C3^{RM} distinctly greater than A2, -1 A2 greater than C3^{RM}. Finally, band thickness at these sites was examined in 14 - 17 chromosomes from post-amplification larvae near pupation and compared with the band morphology of pre-amplification feeding larvae.

Results

Ecdysteroid titer and development

Rhynchosciara americana

Although molting hormone titers had previously been measured in *Rhynchosciara americana* (Stocker et al. 1984), these results came from only one group of larvae, and within this group, only one larva per developmental stage. Most of the chromosome comparisons came from other groups, using developmental landmarks for staging. We therefore decided to re-examine the developmental 20E concentration in this species using not only several different groups of larvae, with at least two larvae of the same physical appearance sampled per developmental point, but also carrying out the chromosomal analyses on the same larvae from which the hemolymph samples had been taken. The salivary gland chromosomes of each larva were examined to check the placement of major puffs, in particular DNA puffs, in relation to the hormone titer curves.

The developmental pattern for one of these *R. americana* groups is shown in detail (Table 1), and in Figure 1a-d it is compared graphically with the other three groups. Overall, the hormone titer curves had a similar pattern among these groups, they were even similar with respect to developmental time (Groups 1-3), except for Group 4 which developed somewhat faster. Ecdysteroid levels were low (<50 pg/μg) during the early stages of cocoon spinning. During the construction of this 'covering cocoon' a small bulge could be

seen in all gland regions at the band where the B2 DNA puff will later appear. Larval behavior and the form of this cocoon are described in Table 1. As the 20E concentration increased, the B2 puff became more apparent and amplification was observed at various chromosomal sites. As the 20E titer increased further, the B2 puff enlarged in the proximal part of the gland (S1) only. In all groups, except Group 3, the B2 DNA puff reached maximum size in cells of the S1 region at a 20E concentration of ~100 pg/ μ l. The expansion of the B2 puff to maximum size in S1, which could take a week or more in some groups, thus appears to be closely correlated with the initial gradual increase in the ecdysteroid titer, which can be quite prolonged. In Group 4 (Fig. 1d), where overall developmental time was shorter, this increase in molting hormone levels was also more rapid and, correspondingly, B2 also showed a faster maximal expansion. In contrast, in Group 3, which was the slowest to develop (Fig. 1c), maximal expansion of B2 was not observed. As the B2 puff expanded in the S1 region of the salivary glands, the larvae were spinning the internal fibers of their cocoon, which gives it a spongy consistency. This is the beginning of the individualization of the larvae, when each forms an individual cell and shows a decrease in motility (Table 1).

As B2 reached its maximal expansion, two other puffs, C3 and C8, began their expansion. These two puffs reached maximum expansion rapidly as the 20E titer continued to increase (Table 1, Fig. 1a,b,d). They were particularly large in the posterior region of the gland (S2, S3). Their expansion is correlated with the enclosure of the larvae in firm individual cells and the cocoon becoming leathery. A white material which appears to be CaCO_3 is evacuated from the Malpighian tubules during this time. The C3 and C8 puffs were not observed in group 3 (Fig. 1c). The continued increase in the hemolymph ecdysteroid concentration concomitant with the expansion of C3 and C8 is correlated with the regression of B2 in the S1 region to a smaller puff, similar to its size in the posterior part of

the gland. A subsequent sharp increase in the 20E titer marked the rapid regression of the C3 and C8 puffs and the continued regression of B2 (Table 1, Fig. 1).

As the 20E titer reached its late prepupal peak, migration of the eyespots was observed in some samples (Fig. 1) [it is worthy of note here that the eyespots in *R. americana* are not nearly as prominent as they are in *R. milleri* (Stocker, personal observation)]. A second phase of DNA puffs begins at this stage with B3 being an early example. These puffs have not been well studied. The cocoon has now hardened and the larvae have continued to contract and move into an upright position in their individual cells. As the 20E concentration decreases, the final stage of prepupal development is reached. Some large puffs appeared during this period, several of which may be DNA puffs (Stocker et al. 1984). For groups that were followed just past pupation, another increase in the 20E titer seemed to occur (Fig. 1a,b).

Rhynchosciara milleri

Two groups of *Rhynchosciara milleri* larvae were followed from or just before the first appearance of eyespots until 1-2 days after pupation. In Group 1, two larvae of the same external appearance were sampled for each time point. Eyespot development was slower than normally observed for this species, taking ~5 days for maximum size to be attained at a hemolymph 20E titer of about 100 pg/μl (Table 2, Fig. 2a). However, each pair of larvae sampled in this group had similar 20E levels until after the first peak, which was quite high and sharp (Fig. 2a). This peak coincided with the appearance of a small B2 puff throughout the gland and eyespot migration towards a lateral position. It was followed by the expansion of other puffs as the 20E level dropped abruptly, including a puff that resembled the C3 puff of *R. americana* and is called C3^{Rm}. After this drop, hormone levels became variable

between the pairs of larvae so that it was difficult to define the exact position of a second 20E peak.

For Group 2, more larvae were sampled for most points. In this group, eyespots reached maximum size in less than two days (Table 3, Fig. 2b). However, as for Group 1, the 20E concentration at which this occurred was about 100 pg/ μ l and the ecdysteroid concentration then rose abruptly. The peak attained by this increase was not as high as that observed in Group 1, and when post peak variability is considered, it could be considered a plateau or shoulder. Nonetheless, for both groups this first increase coincided with the formation of a small B2 puff. Eyespot migration was observed after this increase in Group 2 and numerous other puffs including C3^{Rm} began to expand. In this group, another increase in 20E concentration occurred just before pupa formation (Table 3, Fig.2b).

For both groups, larval movements became slower after the first 20E increase, the larvae began to shorten and seemed to be covered with a sticky secretion. Subsequently, during the decline (or plateau) phase of the 20E concentration, the groups began spinning threads of a very sparse cocoon which connected but never completely covered or enclosed them. During this period, B2 was a small to medium puff in all gland regions and numerous other puffs expanded, particularly in the posterior gland region (Table 3, Fig. 2b).

Amplifying regions

Most chromosome regions of *R. milleri* can be homologized with those of *R. americana*. The A and X chromosomes have almost identical banding patterns in the two species. The distal part of the B chromosome, site of the B2 and B3 DNA puffs, is also very similar in the two species (Fig 3a,b). The similarity breaks down at about region 5, which forms a large RNA puff in *R. milleri* but not in *R. americana*. Although the C chromosome, site of the C3 (Penalva et al. 1997) and C8 (Frydman et al. 1993) DNA puffs in *R. americana*, appears to

differ at several regions in *R. milleri*, most regions can still be homologized through banding similarities at certain developmental stages (Stocker, unpublished observations). A region in the *R. milleri* C chromosome that looks similar to the *R. americana* C3 region was observed at a more central position in *R. milleri* (Fig. 4a,b). A large puff that resembles the *R. americana* C3 puff formed in this region in chromosomes of the posterior salivary gland of *R. milleri* after the first 20E peak. It was therefore designated as C3^{Rm} (Fig. 4e). It is the largest puff on the *R. milleri* C chromosome in S3 cells, less prominent in S2 cells and small in S1 cells.

The location of the C8 region in *R. milleri* was not clear upon initial observations. Therefore, we hybridized *in situ* probes for the B2, C3 and C8 DNA puff regions of *R. americana* to the polytene chromosomes of *R. milleri*. The B2 probe hybridized to the structurally homologous B2 band in *R. milleri*, the C3 probe did not hybridize to any region, and the C8 probe hybridized to a site nearer the telomere of the C chromosome (Tables 2 and 3 for characteristics, Fig. 4 for location of the C8 probe). Although small puffs are observed in this region of the C chromosome, they did not appear to coincide with the site of the C8 probe. At least one other amplified band could be observed proximal to C3^{Rm} (Fig. 4). It may be homologous with the C5 amplifying site of *R. americana* and has, therefore, been labeled C5^{Rm}.

B2, C3^{Rm}, C8 and several other sites, two of which were designated as possible DNA amplifying regions in *R. americana* (Machado-Santelli and Basile 1974; Stocker et al. 1984), were examined cytologically for evidence of amplification in *R. milleri*. Radioactive labeling patterns were analyzed in amplification stage larvae (Fig. 5, Table 4). As stated previously, chromosomes from posterior gland cells were used because of their stronger labeling compared with chromosomes of anterior gland cells. B2 and the C3^{Rm} site showed somewhat higher grain density than background regions while the C8 site did not show any

increased grain density. Nonetheless, other chromosome regions such as A3, A13b and B3 showed higher grain density than B2 or C3^{Rm} at this stage (Table 4, Fig.5). In many cells, B2 and C3^{Rm} were puffed at the stage that labeling was carried out. The B2 puff was small but the C3^{Rm} puff was quite large in some cells. Since grain density can appear higher when a site is puffed, C3^{Rm} was compared with A2. This region also forms a large puff but is not as strongly labeled as C3^{Rm} although its grain density was higher than background (Table 4). Its level of amplification has not been measured. Grain density was about 1.4 times greater in the C3 puff compared with the A2 puff (Table 4), suggesting some level of amplification in posterior gland chromosomes at the region designated as C3^{Rm}. However, the amount of labeling at the C3^{Rm} site does not look very high when compared with labeling at A3, A13b and B3.

Band thickness in late prepupae of *R. milleri* was examined in S2 and S3 chromosomes (Table 4), since a site that amplified would be expected to have a thicker band at the conclusion of amplification. Considerable cell to cell variability was observed (Table 4). In some cells, little or no amplification seemed to have occurred while in others, condensed bands were clearly evident. The highest level of amplification, using band thickness as criterion was in S3 cells. The most consistent evidence of amplification among the sites examined was at A13b, B2 and B3 (Table 4). The latter two sites were the only ones that showed a distinguishable increase in band thickness in the S1 gland region, although a large puff was never seen at B2. Accumulation at A3 and A13b bands was most obvious in S3 cells and did not seem to have occurred in S1. The C3^{Rm} region of posterior gland cells sometimes showed a thicker, more prominent post-puff band (Fig 4g, Fig. 6e), but the increased thickness appeared minimal in many cells, contributing to a low band thickness average (Table 4). C3 did not seem to have amplified in S1 (Fig. 6b) and a large puff was not observed in this region. Comparison of C3^{Rm} accumulation in *R. milleri* with that of C3

accumulation in *R. americana* shows a much thicker post-puff band in anterior and posterior gland chromosomes of the latter species (Fig. 6).

We conclude that three of the major DNA puff regions in *R. americana* do not appear to be major amplification and transcription regions in *R. milleri*. B2 amplifies in all gland regions of *R. milleri* but the large puff formed in S1 cells of *R. americana* is not developed. The region that looks like C3 amplifies and puffs in only some cells of *R. milleri* but its visual signs of DNA accumulation appear low. It expands after B2, but only when the 20E concentration has declined. C8 does not form a large puff in *R. milleri* and does not appear to amplify.

Discussion

In all four groups of *Rhynchosciara americana* sampled for this study, the curves for the hemolymph 20E concentrations exhibited a similar shape and relationship to developmental markers. However some differences were observed at the times leading to the prepupal peak. In Groups 1 and 4 this part of the curve appeared as a broad shoulder, showing a gradual increase in 20E before the abrupt rise to a high and pronounced prepupal peak. In Groups 2 and 3, in which development was somewhat slower, fluctuations in 20E were observed in this part of the curve. These fluctuations are similar to the small first peak in the 20E curves of our earlier study (Stocker et al. 1984, Fig. 4), and also to the suggestion of a plateau in the 20E titer curve presented in a recent study on cell death in *R. americana* that emphasized post-pupal ecdysone changes (dos Santos Brandão et al. 2014). They probably represent developmental variation within and/or between groups. None of our *R. americana* 20E titer curves clearly show the separate low ‘commitment’ peak observed in *Bradysia hygida* (Basso et al., 2002) and present in other dipteran taxa (Richards, 1981) which corresponds to the wandering stage of the final larval instar. There is, instead, a gradual rise in 20E concentration corresponding to the initiation of the B2 DNA puff. The

low 'commitment' peak may have occurred earlier in *R. americana* or may not be discernible above background 20E levels. It could correspond to the peak seen early in the fourth instar of the *R. americana* curve presented by dos Santos Brandão et al. (2014).

Distinct from our earlier investigation, we have compared here four different groups of larvae and also performed chromosomal analyses on the same larvae that had their hormone levels studied. This allowed a much more precise placement of prominent DNA puffs and of their temporal profiles of expansion and regression and gave the results more certainty. The B2, C3 and C8 DNA puffs, whose amplification levels had previously been measured (Glover et al. 1982; Frydman et al. 1993; Penalva et al. 1997; Santelli et al., 2004; Yokosawa et al. 1999), were given particular attention. In all four of our *R. americana* groups, the B2 puff started to expand when the 20E levels were beginning to increase. In the proximal gland cells only (S1 region) it became a large puff when the 20E concentration reached about 100 pg/μl. B2 is thought to be responsible for the production of peptides involved in the early stages of cocoon construction (Alvarenga et al. 1991; Winter et al. 1977a,b;). However, a loosely formed covering cocoon was already apparent before we observed puffing at B2. Some groups of larvae left and rebuilt this covering cocoon several times before constructing their final cocoon. This is similar to the behavior reported for the moth, *Ephestia kuehniella*. This moth can restart outer envelope spinning several times if the process is interrupted. However, if removed from its cocoon while constructing the inner envelope, it will not restart spinning (Giebultowicz et al., 1980). The spinning of *E. kuehniella*'s outer cocoon was not induced by the concentration of 20E injected in this study. It was shown in subsequent experiments to depend on various environmental stimuli but its presence appeared to stimulate ecdysteroid release and inner cocoon spinning (Giebultowicz et al. 1984). Early cocoon formation in *R. americana* may also be induced by factors other than 20E or it may be the effect of initial low levels of this hormone on non-

amplified genes. It is also possible that some B2 messenger RNA is being produced before the appearance of an obvious puff. Clarification of these questions requires further study of the initiation of cocoon construction in *Rhynchosciara*.

As the 20E levels continued to increase in *R. americana* and B2 expanded to its maximum size in the proximal part of the gland (S1), the larvae were constructing the inner fibers of the cocoon, giving this structure a spongy consistency. Construction of the inner fibers of the cocoon appears to commit the larvae to pupation. As the 20E concentration increased further, the B2 puff began to regress and C3 expanded rapidly. C8 reached maximal expansion at about the same time as C3, but is known to already produce RNA earlier (Santelli et al. 1991). Regression of these three puffs accelerated as the 20E titer increased rapidly to form the prepupal peak.

Differing from the other groups, for the larvae sampled from Group 3, B2 was not fully enlarged and expansion of C3 and C8 was not observed although amplification did occur at these sites. This could be due either to a higher level of asynchrony among larvae in this group, or to the steeper increase of the 20E levels to the prepupal peak which caused these puffs to regress prematurely. The cocoon was not so well-formed in this group.

In any case, the temporal correlation between physiological hormone levels and expansion and regression of these early DNA puffs in *R. americana* salivary glands suggests that these amplifying regions are functionally controlled by the circulating 20E levels. A certain (threshold) concentration of this hormone apparently needs to be reached, and perhaps maintained, for these DNA puffs to amplify and achieve full expansion. A rapid increase in hormone level would then cause the regression of these early DNA puffs and the expansion of later ones. Such a model, in which different ecdysteroid levels induce the expression of different sets of DNA puffs has been presented by Monesi et al. (2009), based on studies in

Bradysia hygida (Basso et al. 2002; de Carvalho et al. 2000). However, earlier investigations where salivary glands of young and old *Rhynchosciara* larvae were reciprocally transplanted (Amabis 1974; Amabis and Simoes 1971) suggest that despite the close temporal correlation between 20E levels and expansion and regression of particular DNA puffs, these events are not so tightly linked. For example, when B2 has regressed in the host larva, this DNA puff was still in the process of expansion in the implanted glands. Therefore, in addition to the circulating hormone levels, certain cell-intrinsic control mechanisms must also be operating. Studies of DNA puffs in several sciarid species indicate that DNA puff development is a late response to ecdysone (Crouse 1968; Stocker and Pavan 1974), and RNA and protein synthesis are prerequisites for DNA puffing (Amabis and Amabis 1984a,b; Basso et al. 2006; Berendes and Lara 1975; Foulk et al. 2006). Cell-specific production of these secondary proteins may, thus, explain the divergent responses obtained in transplantation experiments. These proteins may, furthermore, be important in generating modulated chromosomal puffing patterns seen here in the comparisons across the faster and slower developing groups of sciarid larvae.

Increasing evidence has now been presented to support the view that ecdysteroids interact directly with DNA puff genes to initiate amplification. Specifically, the presence of the ecdysone receptor protein, EcR, was revealed by anti-EcR antibody labeling of DNA puff sites in the sciarids *Trichosia pubescens* (Stocker et al. 1997), *Bradysia hygida* (Candido-Silva et al. 2008) and *Sciara (Bradysia) coprophila* (Foulk et al. 2013; Liew et al. 2013). Ongoing investigations in *Sciara coprophila* are especially relevant in explaining the probable mechanism of this interaction through potential ecdysone response elements (EcREs) near the origin of replication site in the promoter of a DNA puff gene (Foulk et al. 2006; Foulk et al. 2013; Liew et al. 2013). It is, thus, probable that the control of DNA

amplification and chromosomal puffing by ecdysone involves a complex assortment of direct and indirect interactions.

The 20E titer curves for the two groups of *R. milleri* that we examined indicated more variability between larvae of this species than for *R. americana*. A more distinct peak or plateau preceding the prepupal peak was also present. Interestingly, the shape of the *R. milleri* Group 2 curve resembled the 20E curves of *R. americana* shown in Fig. 4 of Stocker et al. (1984) and recently in dos Santos Brandão et al. (2014). It seems that a certain amount of variation in ecdysteroid levels is compatible with the completion of development in *Rhynchosciara* species. This variation may be more tolerable in a species such as *R. milleri* which is not obliged to construct a communal cocoon.

The position of certain developmental landmarks on the 20E titer curves differed between *R. americana* and *R. milleri*. Even though eyespots are not easily visible in *R. americana*, eyespot migration could be observed near the prepupal 20E peak, when the early group of DNA puffs (B2 in S1, C3, C8) had regressed. As in other sciarid genera, eyespots are prominent in *R. milleri* and eyespot migration occurs at or just after the first major increase in 20E, when a small B2 puff appears throughout the gland. The development of this puff is more rapid in *R. milleri* than in *R. americana* but unlike members of the *americana* species group, B2 does not become large in the S1 gland region of *R. milleri*.

A homolog of the *R. americana* C3 gene could not be identified by *in situ* hybridization in *R. milleri*, although a region with similar banding morphology at a different position on the C chromosome gave rise to a puff that, like the C3 puff, is the largest one on the C chromosome (Fig 4). In *R. milleri*, this puff expanded to maximal size after B2, but only as the 20E level dropped or reached a plateau, whereas in *R. americana* the C3 puff expanded as the level of 20E increased. Like C3 of *R. americana*, the C3^{Rm} puff of *R. milleri*

is predominately a puff of the posterior part of the salivary gland (regions S2, S3), but it does not appear to be highly amplified. There may be some banding differences at this region between the chromosomes of these two species and it is possible that C3^{Rm} originates from a more chromocenter-proximal band in *R. milleri* (Stocker, personal observations).

In situ hybridization with an *R. americana* C8 probe identified a site on the C chromosome of *R. milleri*, but this site did not seem to amplify or form a prominent puff. Therefore, the early group of *R. americana* DNA puffs, which have been hypothesized to be important in cocoon construction in that species (Winter et al. 1977a,b), are not major DNA puffs in *R. milleri*. Other DNA puffs, some showing heavy incorporation of ³H-thymidine, are present in *R. milleri* around the same time as C3^{Rm}, when the 20E titer drops or stays at a plateau.

R. milleri larvae spin their sparse cocoon during this period, rather than initiating it before the appearance of the B2 puff, as observed in *R. americana*. This timing in *R. milleri* may correspond to the spinning of the definitive inner fibers of the cocoon in *R. americana*. Differences in size and position of the B2, C3 and C8 DNA puff homologs, and absence of an elaborate cocoon in *R. milleri* when compared with *R. americana*, thus lend support to suggestions from earlier studies (Alvarenga 1980; Alvarenga et al. 1991; Winter et al. 1977a,b; de Toledo and Lara 1978) that these puffs are involved in the formation of the massive cocoon type constructed by *R. americana* and larvae of other species in the *americana* group (Stocker et al., 1993; Stocker and Gorab, 2000).

One idea at the inception of this study was that a species like *R. milleri* might be evolutionarily intermediate between the majority of sciarid species studied that do not have gregarious larval development and the *Rhynchosciara* species that have both gregarious development and build communal cocoons. If this were the case, we thought we might see

some indication in the shape of the 20E titer curve of *R. milleri* since ecdysone along with juvenile hormone has a central role in the control of developmental events in insects (Edgar 2006; Richards 1981). It had been suggested earlier that some of the changes in cocoon building behavior within the *Lepidoptera* could result from the timing of hormone release (Giebultowicz et al. 1980; Sehnal et al. 1981).

An obvious developmental difference between *R. americana* and *R. milleri* is the long early period of cocoon construction in the former species which for much of its time course coincides with the slow expansion of the B2 DNA puff. In *R. milleri* this puff appears and expands more quickly, never reaching a large size in any gland region and cocoon spinning occurs later in development, after the B2 puff has appeared. The cocoons of non-gregarious sciarids such as *Bradysia hygida* (reviewed in Monesi et al. 2009) and *Trichosia pubescens* (Amabis 1983) are also constructed later in development. However, unlike these latter species, the *R. milleri* cocoon is web-like, similar to the early cocoon spun in *R. americana*. Additionally, the 20E curves of *R. milleri* look more similar to the *R. americana* curves (see also dos Santos Brandão et al. 2014 and Stocker et al. 1984) than to the *Bradysia hygida* curve (Basso et al. 2002). Although the relationship of B2 and other DNA puffs to the 20E titer curve differs in *R. americana* and *R. milleri*, the sequence of these puffs is quite similar in the two species. In particular, it seems that the B2 DNA amplifying region is very old in this genus, as it has maintained its position in the genome and its activity in two different species groups (Stocker and Gorab 2000; Stocker et al. 1993) although its behaviour has changed between the *americana*-like and *milleri* species. In the *americana* species group, B2 expands from a small to a very large puff only in the proximal region of the gland when the inner fibers of the cocoon are being constructed (spongy cocoon). The completion of cell construction and the hardening of the cocoon correspond to the expansion of the C3 DNA puff in *R. americana*. Since *R. milleri* larvae do not form a

cellular cocoon and do not have a discernible C3 gene homolog, this gene may be involved the late stages of cocoon construction in *R. americana* while C3^{Rm} may have a different function.

A logical path in the evolution of *Rhynchosciara* species that form a massive common cocoon would seem to be from a sciarid like *R. milleri*, that had already evolved gregarious behavior and the production of same sex progeny. Gregarious behavior is not uncommon in the Sciaridae (Pratt 1899; Steffan 1974; Eberhard and Flores 2002; Shin et al. 2013; Sutou et al. 2011). According to Shin et al. 2013, it is most frequently observed in larvae of the Sciarinae and Chaetosciara groups. However, *Phytosciara vulcanata*, placed in the Megalosphyinae group, was described as gregarious by Steffan (1974) and *Bradysia impatiens* was reported to become gregarious under dry conditions (Eberhard and Flores 2002). Gregarious behavior was determined to have evolved independently on several occasions in *Heliconius* butterflies (Beltrán et al. 2007) and this is probably also the case for the Sciaridae. Many female sciarids lay their eggs within a short time as a single mass (Steffan 197; Eberhard and Flores 2002) and this appears to predispose to the development of larval gregariousness (summarized in Sillén-Tillberg 1988). A large mass of larvae would be susceptible to predation unless they had evolved unpalatable features. *Heliconius* butterflies that are gregarious were shown to have warning coloration (as the criterion of unpalatability) that preceded gregarious behavior (Sillén-Tullberg 1988). *Hybosciara gigantea* was demonstrated to have a disagreeable odor and to be avoided by some predators (Eberhard and Flores 2002). *Rhynchosciara* species are also colored but their palatability has not yet been tested.

According to the cladogram presented by Vilkamaa and Hippa (2004) based on 138 morphological characters, the genera *Rhynchosciara* and *Hybosciara* are closely related, being parallel branches from an ancestral lineage. *Hybosciara gigantea* larvae exhibit

synchronous behavior and pupate in a manner similar to *Rhynchosciara milleri* (Eberhard and Flores 2002). Since groups of *H. gigantea* contained individuals of both sexes, this species appeared to be a bisexual producer. However, the groups of larvae and pupae examined by Eberhard and Flores (2002) were collected in nature so the investigators could not be certain that each group originated from the eggs of one female. The features shared by the *Hybosciara* and *Rhynchosciara* lineages suggest that the *ancestral* lineage leading to the genus *Rhynchosciara* had behavioral characteristics similar to *R. milleri* and that the construction of a more elaborate cocoon was a later evolutionary development which enhanced the survival of some *Rhynchosciara* species.

At the field site in the Atlantic Rain Forest biome where we have collected the two species, *R. americana* is found later in the spring than *R. milleri* and develops more slowly. Its pupal case is softer so that *R. americana* pupae removed from the cocoon tend to dry up or are attacked by fungi. In *R. americana* the cells of the cocoon are coated with a substance excreted from the Malpighian tubules which appears to be CaCO_3 . The Malpighian tubules of *R. milleri* do not have noticeable amounts of CaCO_3 . The CaCO_3 'cement' may help maintain an optimal humidity for the development of *R. americana* pupae within their cells. The cocoon can also be attached to the base of a tree to improve the chances of larval survival during storms. These and other possible cocoon functions are discussed by Danks (2004) and Gross (1993). Obviously, a more thorough understanding of the relationship between *R. americana* and *R. milleri* and their position within the Sciaridae will depend on future genetic studies, in particular genomic comparisons. Nonetheless, our correlative analyses of developmental timing, morphogenetic hormone titers and chromosomal puffing patterns revealed both similarities and differences among larval sibling groups within and across two *Rhynchosciara* species. This emphasizes the need for a comparative intra- and interspecific approach whenever one is interested in understanding the intricate relationship

between relatively stereotyped behaviors, the endocrine milieu and genomic responses related to variation in life histories and life styles. It would also be interesting to have some developmental knowledge about other species in the genus *Rhynchosciara*, in particular for Group III, the *mathildae*-like *Rhynchosciara* species, which are only known from adult holotypes (Breuer 1969).

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Table1. Developmental landmarks and mean ecdysteroid titer (20E equivalents pg/ μ l hemolymph) for *R. americana* group 1

Days of development	Characteristics of larvae sampled	Characteristics of chromosomes	Mean ecdysteroid titer (pg/ μ l hemolymph $\pm$ standard error)	Number of larvae in sample
1 (PM)	Covering cocoon, Motile larvae	No large puffs	19.79 $\pm$ 0.33	2
2 (PM)	Left cocoon	As above, S2/3 chromos larger	8.15 $\pm$ 8.18	2
4 (PM)	Covering cocoon	Small B2 bulge	24.56 $\pm$ 11.21	2
6	Left cocoon			No sample
7	Covering cocoon			No sample
8 (PM)	Spongy Cocoon , begin cells, "kinked" larvae	Amplification, B2 bulge obvious all regions	55.13 $\pm$ 12.51	2
9 (noon)	Cocoon firmer, posterior gut emptying	B2 $\frac{1}{3}$ - $\frac{1}{2}$ S1, C3 amplification obvious S2/3	82.23 $\pm$ 8.05	2
9 (PM)	As above	B2 $\frac{1}{2}$ - $\frac{3}{4}$ S1, C3 amplifying	87.2 $\pm$ 5.21	2
10 (noon)	Larvae curved, not very mobile, gut as above	B2 $\frac{1}{2}$ -full S1, C3/8 amplifying S2/3	102.9 $\pm$ 19.89	2
11 (PM)	Cocoon leathery, cells, gut ~empty, Malphigian tubules evacuating	B2~max $\rightarrow$ regressing S1, C3/8 enlarging, ~maximum in S3	170.2 $\pm$ 1.7	2
12 (AM)	Short curved larvae in cells coated with CaCO ₂ , eyespots migrating	B2 regressing/regressed, C3/8 regressing, B3 expanding	524.0 $\pm$ 130.3	2
12 (PM)	Short, straight larvae, heads up in cells of hard cocoon, eyespots ~lateral	B2, C3,C8 regressed, amplified, B3 maximum, A2/4, B10 expanding	525.1 $\pm$ 3.41	2
13 (AM)	Heads up, bean shape, begin thorax "cincture", leg eversion	A2/4 large, B10 large	384.7 $\pm$ 1.55	2
13 (PM)	Apolysis beginning, wing eversion	A2 region puffs large, C2 puff	387,6 $\pm$ 53.86	2
14 (AM)	Young pupae*, glands still present.	A few RNA puffs	210.5 $\pm$ 2.40	2
14 (PM)	Older pupae, glands not found		260.0 $\pm$ 18,19	2

Table 2. Developmental landmarks and mean ecdysteroid titer (20E equivalents pg/μl hemolymph) for *R. milleri* group 1

Days of development	Characteristics of larvae sampled	Characteristics of chromosomes	Mean ecdysteroid titer (pg/μl hemolymph ± standard error)	Number of larvae in sample
1 (AM)	Feeding, intense body movement, eye spots not present	RNA puffs, most prominent in A & proximal B chromosomes	11.99±2.83	2
2 (AM)	As above, larvae larger	As above	19.07±0.52	2
2 (PM)	As above	As above	15.62±4.39	2
3 (AM)	Larvae a bit less motile	As above	23.81±1.42	2
3 (PM)	As above	As above	36.11±16.28	2
4 (AM)	As above	As above	40.94±5.96	2
5 (AM)	Begin eyespots, larvae less motile, lateral discs larger, intestine full	Some RNA puff changes	25.80±2.51	2
5 (PM)	Eyespots larger	RNA puffs	77.0±61.72	2
6 (AM)	Larvae much less motile	As above, chromosome A puffs	28.96±7.30	2
6 (PM)	As above	As above	25.2±1.01	2
7 (AM)	Larvae with little movement, eyespots enlarging	As above	65.52±5.05	2
7 (PM)	As above	As above	52.43±0	1
8 (AM)	Eyespots larger, darker	As above	63.84±10.23	2
8 (PM)	Eyespots large & central, lateral discs large	RNA puff changes	85.12±2.40	2
9 (AM)	As above	Chromosomes larger	94.22±26.46	2
9 (PM)	Eyespots large, dark and central, intestine emptying	C3 ^{Rm} region slight bulge	116.66±14.65	2
10 (AM)	Eyespots migrating, intestine emptying, lateral discs v. prominent	Small B2 puff, increase C3 ^{Rm} , other puff changes, amplification S3	626.15±88.18	2
10 (PM)	Eyespots migrating, almost lateral, intestine almost empty, spinning cocoon	A2 puffs large, amplification/ puffing A3, A13, B3 S2/3, small B2 puff, C3 ^{Rm} large in S2/3	383.56±25.20	2
11 (AM)	Short larvae, lateral eyespots, intestine empty, strands of cocoon	As above but B3 small puff, C3 ^{Rm} regressing,	370.81±181.78	2
11 (PM)	Bean-shaped larvae, near molt	A2&other puffs regressing, large B5 puff, C2 puff	366.34±165.89	2
12 (AM)	Pupae: head, wings, legs	C2, B5 puffs, signs of amplification evident	526.34±283.5	2
12 (PM)	Older pupae, histolysis	B5 puff	326.90±151.43	2

Table 3. Developmental landmarks and mean ecdysteroid titer (20E equivalents pg/ μ l hemolymph) for *R. milleri* group 2

Days of development	Characteristics of larvae sampled	Characteristics of chromosomes	Mean ecdysteroid titer (pg/ μ l hemolymph $\pm$ standard error)	Number of larvae in sample
1(AM)	Intense body movement, appearance of eyespots	Poor slides, no information	19.72 $\pm$ 8.88	2
1(PM)	Intense body movement, lateral discs enlarging, darker eyespots	A few RNA puffs all chromosomes especially A & B, none major	42.63 $\pm$ 11.36	6
2(AM)	Slower body movement, intestine beginning to empty, prominent eyespots	As above	81.50 $\pm$ 39.52	2
2(PM)	As above, very slow body movement, prominent eye spots	B2 puff begins, C3 ^{Rm} bulges, especially in S2/3, amplification S2/3	235.68 $\pm$ 42.39	2
3(AM)	Little body movement, body shorter, intestine emptying, eye spots migrating laterally, begin spinning	A2 puffs, A3, A13 B2, B3, C3 ^{Rm} puffs, especially in S3, B5 puff starting	198.17 $\pm$ 88.94	2
3(PM) early	Eye spots migrating laterally, sparse covering cocoon, larvae "stick together"	A2 puffs, A3, A13, B2 amplification/ puffing S2/3, large C3 ^{Rm} puff S2/3, B5 puff variable	195.86 $\pm$ 79.17	4
3(PM) late	Very little body movement, intestine emptying, sparse cocoon, lateral eye spots	B2 puff; A2 large, A3, A13, B3, C3 ^{Rm} puffs S2/3	204.48 $\pm$ 64.34	4
4(AM)	Shorter body but some movement, intestine partially empty	A2 large all regions, B2 small puff or regressed, B5 puff variable, other regions regressing	331.31 $\pm$ 87.53	4
4(PM) early	Short body, curved head, v. little movement, intestine almost empty, lateral (almost ventral) eye spots	A2 large S2/3, B5 large, other regions regressing	365.14 $\pm$ 102.92	4
4(PM) late	pupa day 1, no movement, salivary gland still normal	A2, B2 small/ regressed, B5&C2 puffed, amplification	166.08 $\pm$ 51.34	4
5(AM)	pupa day 2, salivary gland histolyzing	B5, C2 puffs, signs of amplification espec. S2/3	229.88	1

Table 4. Amplification at certain chromosome sites in *R. milleri*

Site	Relative grain density* ¹	Relative band thickness* ² post puff
A2	1.1	0.4
A3	2,7	2.4
A13b	2,8	1.9
B2	1.0	1.3
B3	2.2	2.3
C3	1.5	1.1
C8	0.3	0.0
C3puffed/A2puffed	1.4	

*¹ Relative to general grain density over other chromosome regions. 0 = no grain density difference, 1 = slight density difference, 2 = very definite density but most grains don't overlap, 3 = region "black" with overlapping grains. Unless specifically stated, site scored was not always puffed.

*² Relative to band in pre-amplification larvae. 1 = appears somewhat thicker, but does not project from chromosome, 2 = definitely thicker, usually appears as slight "bulge" in chromosome or is refractile. 3. = thick band that projects from chromosome and is usually refractile

Figure legends

Figure 1. Developmental ecdysteroid titer curves (20E equivalents pg/μl hemolymph) for four female groups of *Rhynchosciara americana* larvae. Important developmental landmarks are indicated for each group. For details of Group 1 see Table 1. Each point usually represents the mean of two larvae; vertical lines shown indicate the respective standard error of the mean. Note that one of the hemolymph samples was lost for days 16, 17 and 18 in Group 3. pupa * = after ecdysis of larval cutical

Figure 2. Developmental ecdysteroid titer curves (20E equivalents pg/μl hemolymph) in 2 female groups of *Rhynchosciara milleri* larvae. See Tables 2 and 3 for details.

Figure 3. Chromosome B preparation from S1 region salivary gland cells of *R. americana* (a) and *R. milleri* (b) at the stage of maximal expansion of the B2 DNA puff. B3, another band that undergoes amplification, is indicated. B5 forms a large RNA puff in *R. milleri*.

Figure 4. C chromosome preparations from the S3 salivary gland region for (a) *R. americana* and (b) *R. milleri* pre-amplification stage larvae; preparations for various later developmental stages of *R. milleri* are shown in (d-f). The C8 site is also indicated in both species. Another region that appears to amplify in *R. milleri* and may be homologous to C5 of *R. americana* is indicated as C5^{Rm}. C2 is an RNA puff that appears in both species just before pupation.

Figure 5. ³H-thymidine incorporation into a set of chromosomes from the S3 region of the *R. milleri* salivary gland a day after the appearance of the B2 DNA puff. C3^{Rm} forms a large puff at this time. Other sites mentioned in the text are labeled.

Figure 6. Comparison of DNA accumulation at C3 of *R. americana* Group 4 (a,c,d) and C3^{Rm} of *R. milleri* Group 2 (b,e). S1 salivary gland region (a,b). S3 salivary gland region

(c,d,e). c = pre-puff amplification in *R. americana*; other chromosomes are from larvae near pupation.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

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