Comparative transcriptomics in Syllidae (Annelida) indicates that posterior regeneration and regular growth are comparable, while anterior regeneration is a distinct process

Rannyele Passos Ribeiro1*, Guillermo Ponz-Segrelles1, Christoph Bleidorn2,3 and Maria Teresa Aguado1,2,4*

Abstract

**Background:** Annelids exhibit remarkable postembryonic developmental abilities. Most annelids grow during their whole life by adding segments through the action of a segment addition zone (SAZ) located in front of the pygidium. In addition, they show an outstanding ability to regenerate their bodies. Experimental evidence and field observations show that many annelids are able to regenerate their posterior bodies, while anterior regeneration is often limited or absent. Syllidae, for instance, usually show high abilities of posterior regeneration, although anterior regeneration varies across species. Some syllids are able to partially restore the anterior end, while others regenerate all lost anterior body after bisection. Here, we used comparative transcriptomics to detect changes in the gene expression profiles during anterior regeneration, posterior regeneration and regular growth of two syllid species: *Sphaerosyllis hystrix* and *Syllis gracilis*; which exhibit limited and complete anterior regeneration, respectively.

**Results:** We detected a high number of genes with differential expression: 4771 genes in *S. hystrix* (limited anterior regeneration) and 1997 genes in *S. gracilis* (complete anterior regeneration). For both species, the comparative transcriptomic analysis showed that gene expression during posterior regeneration and regular growth was very similar, whereas anterior regeneration was characterized by up-regulation of several genes. Among the up-regulated genes, we identified putative homologs of regeneration-related genes associated to cellular proliferation, nervous system development, establishment of body axis, and stem-cellness; such as *rup* and *JNK* (in *S. hystrix*); and *glutamine synthetase*, *elav*, *slit*, *Hox* genes, *β-catenin* and *PL10* (in *S. gracilis*).

**Conclusions:** Posterior regeneration and regular growth show no significant differences in gene expression in the herein investigated syllids. However, anterior regeneration is associated with a clear change in terms of gene expression in both species. Our comparative transcriptomic analysis was able to detect differential expression of some regeneration-related genes, suggesting that syllids share some features of the regenerative mechanisms already known for other annelids and invertebrates.

**Keywords:** Regeneration, Annelida, Syllidae, RNA-seq, Transcriptome, *Hox* genes, *β-Catenin*, JNK, PL10

*Correspondence: rannyele.passos@uam.es; aguadomolina@gwdg.de
1Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain
Full list of author information is available at the end of the article

© The Author(s). 2019 Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.
Background

Growth and regeneration are remarkable developmental abilities of annelids. Most annelids grow during their entire life by addition of segments from a segment addition zone (SAZ) located between the pygidium and the last segment [1–8]. Moreover, nearly all annelid species are able to completely restore the posterior body, while only some groups are able to regenerate the anterior body [6–10]. Whereas several studies describing the process of (anterior and posterior) regeneration are available, the molecular background of this ability remains largely unknown in annelids [6–8, 10].

Studies including molecular data during regeneration in annelids have been published for the clitellates Enchytraeus japonensis Nakamura, 1993 [11–15] and Pristina leidyi Smith, 1896 [1, 16–20]; and the non-clitellates Alitta virens Sars, 1835 [21–24], Capitella teleta Blake, Grassle and Eckelbarger, 2009 [25–29], and Platynereis dumerilii (Audouin and Milne Edwards, 1833) [3, 30–35]. All those species regenerate the posterior body, but only E. japonensis and P. leidyi exhibit anterior regeneration [1, 11–15, 17–20]. Studies on anterior regeneration in non-clitellates have been limited to morphological approaches so far (e.g. [36–44]). Interestingly, some genes that are expressed in the SAZ during regular growth/development have been detected in different stages of posterior regeneration in annelids, for example, Hox genes [21–23, 27, 45], β-catenin [17], and genes of the germ line multipotency program such as piwi, vasa, nanos, and PL10 [27, 46–48].

Within Annelida, Syllidae are known to completely regenerate their tails [8, 49]. However, when dealing with anterior regeneration, many species can only regrow the prostomium and few segments, e.g. Eusyllis blomstrandi Malmgren, 1867 [49–51]; while others additionally regenerate all missing segments and also a characteristic differentiation of the digestive tube called proventricule (e.g. Syllis gracilis Grube, 1840 [37, 52–55]). Interestingly, the molecular background of regeneration in syllids has not been explored.

We used RNA-seq to generate gene expression profiles of the anterior and posterior regeneration processes, as well as the regular posterior growth of two species of syllids: Sphaerosyllis hystrix Claparède, 1863 [56] (Exogoninae), and Syllis gracilis (Syllinae). Our aim was to analyse the changes in gene expression during the first stages of posttraumatic anterior regeneration (AR) and posterior regeneration (PR) by comparing them with the non-regenerating condition (NR) (i.e. intact individuals in regular posterior growth), and between themselves (AR and PR). Additionally, selected genes previously shown to be (highly) expressed during regeneration in other annelids and other invertebrates have been investigated. Finally, we also documented the morphological changes during anterior and posterior regeneration in both species, and identified regeneration-related genes that could be of interest for future studies in syllid regeneration.

Results

Illumina NGS and assembly

We used a comparative transcriptomic approach in order to compare gene expression in three conditions: anterior regeneration (AR), posterior regeneration (PR), and non-regenerating (NR), i.e. intact individuals in regular posterior growth (see Figs. 1, 2 and 3 for experimental design and morphological data). mRNA samples of S. hystrix and S. gracilis were sequenced for each condition using an Illumina sequencing platform. Considering all three conditions, we generated a total of 79.5 GB raw reads for S. hystrix and 74.3 GB for S. gracilis (Table 1). After trimming the reads, 84.0 and 88.3% of reads remained for S. hystrix and S. gracilis, respectively (Table 1). Those cleaned reads were assembled, generating 315,224 contigs for S. hystrix (average length = 733.43, N50 = 1158) and 526,860 contigs for S. gracilis (average length = 626.48, N50 = 858). According to BUSCO [57], both transcriptomes were highly complete 97.8% (S. hystrix) and 98.6% (S. gracilis), despite showing a high level of redundancy with 73.8 and 80.6%, respectively (Table 1). We found 179,841 predicted proteins in the transcriptome of S. hystrix and 309,576 predicted proteins in the one of S. gracilis (Table 1). The raw reads were uploaded at the NCBI Sequence Read Archive (SRA). Assemblies and transdecoder predicted proteins are available under https://github.com/rannypribeiro/Regeneration_transcriptomics.

Functional annotation of transcripts and gene ontology

Around 35.7% (S. hystrix) and 31.3% (S. gracilis) of the assembled transcripts were annotated. The annotation results showed hits with human and mouse genes mostly, and less than 1% with known annelid genes (Additional file 1). Within Annelida, most transcripts were annotated with Lumbricus sequences: 38% (S. hystrix) and 28% (S. gracilis) (Additional file 1). Gene ontology (GO) categories were assigned to 28.5 and 24.5% of the transcripts of S. hystrix and S. gracilis, respectively. Our results showed that both species have a similar distribution of genes associated to the categories of cellular component, molecular function and biological process (Additional file 1).

Comparison of gene expression profiles

In order to identify differentially expressed (DE) genes, we compared the transcriptomic profiles of anterior regeneration and posterior regeneration (Axp), anterior regeneration and non-regenerating condition (AxN), and
posterior regeneration and non-regenerating (PxN) of both studied species.

*Sphaerosyllis hystrix*

Considering the overall results, we detected 4771 DE genes in *S. hystrix* (FDR < 0.001) (Fig. 4a; Additional file 2: Tables S1–S4). Analysing the comparisons separately, 108 genes were found to be differentially expressed in AxP, and 4768 genes in AxN. No DE genes were found in PxN. Four thousand six hundred sixty-three of the DE genes were exclusively found in AxN; 105 genes were present in both AxN and AxP; and only 3 genes were exclusive of AxP. Most of the DE genes were up-regulated in AR (4699) rather than in PR (161) or in NR (58) (Fig. 4a). AR up-regulated genes had similar expression levels in both PR and NR (see Additional files 2: Table S1). Gene Ontology analysis showed that 76% of the DE genes were annotated. The most prominent GO terms in AxP and AxN belong to the cellular component category (e.g., secretory granule, zymogen granule membrane, motile cilium, apical lamina of hyaline layer, ribosomal and mitochondrial parts) (Fig. 5a, b; Additional file 2: Tables S5 and S6).

*Syllis gracilis*

The overall results of the differential expression analysis showed 1997 DE genes among the three experimental conditions of *S. gracilis* (FDR < 0.001) (Fig. 4b; Additional file 3: Tables S7–S10). Of those genes, 1863 and 1428 were found in AxN and AxP, respectively. Similarly, to the results obtained for *S. hystrix*, no DE genes were found in PxN (FDR < 0.001). Of the DE genes, 529 were exclusive of AxN; 1334 were present simultaneously in AxN and AxP; and only 134 were exclusively detected in AxP. One thousand nine hundred forty genes were up-regulated in AR rather than in PR (33) or in NR (42) (Fig. 4b). In terms of gene ontology, 86% of genes with differential expression were annotated and the most prominent GO terms in AxP and AxN belong to the cellular component category (e.g., ribosome, intracellular ribonucleoprotein complex, ribosomal unit, macromolecular complex annotated) (Figs. 5c, d; Additional file 3: Tables S11 and S12).

Identification of candidate regeneration genes

In order to identify putative regeneration-related genes in these species, BLAST searches were performed against our transcriptomes using publicly available sequences of those genes that have been previously shown to be (highly) expressed during regeneration in other annelids (Table 2; Additional file 4) [1, 2, 12, 13, 17, 21, 23, 27, 32, 35, 45, 46, 48, 59–63].

A total of 71 regeneration-related candidates were found in the literature. From those, 57 were identified in the transcriptome of *S. hystrix* and 54 in the transcriptome of *S. gracilis*. Multiple gene isoforms were identified after
BLAST searches in *S. hystrix* (e.g. for *paics* and *slit*) and *S. gracilis* (e.g. *even-skipped*, *FGFR*, *gcs1a*, *glutamine synthetase*, *hedgehog*, *JNK*, *Msx*, *piwi1*, *Sfrp1/2/5* and *Wnt*) (Additional file 4), indicating that there might be multiple unique homologs of some of those genes in these species. Of the resulting homologs, *paics* in *S. hystrix*; and *β-catenin*, *cycB3*, *glutamine synthetase*, *paics*, and *PL10* in *S. gracilis* were detected to have differential expression, being all of them up-regulated in AR (FDR < 0.001). If we consider the significance threshold to be FDR < 0.01, the number of candidate regeneration genes with differential expression increases to 14, including *JNK* and *rup2*, in *S. hystrix*; and *brat*, *elav*, *FGFR*, *gcs1a*, *slit*, *Hox7*, *Lox2* in *S. gracilis* (Table 2; Additional file 4). Interestingly, all the *Hox* genes reported to be involved in the regeneration and development of other annelids [2, 3, 23, 46, 64] were found in the transcriptome of *S. hystrix* but none of them presented differential expression in any of the pairwise comparisons. In the case of *S. gracilis*, all *Hox* genes were found in the assembly, except *Hox2* and *Hox3*. Interestingly, *Hox7* and *Lox2* were among differentially expressed genes in the comparisons AxP and AxN, being up-regulated in AR (FDR > 0.01) (Table 2, Additional file 4).
Morphological results of regeneration

The herein studied species exhibited a complete posterior regeneration, but anterior regeneration developed to different degrees. *Sphaerosyllis hystrix* regenerated an incomplete prostomium after 14 dpa and, even in advanced stages (around 50 dpa), they did not restore new segments. Thus, like in many other syllids [51, 65], the anterior regeneration of *S. hystrix* seems to be limited. Regarding *Syllis gracilis*, our own field observations and previous studies provide solid evidence that they are able to restore a complete anterior body with up to 18 segments and all digestive structures [37, 55, 66]. Moreover, specimens of *S. gracilis* from the same area showing advanced anterior regeneration have also been documented in detail by Parapar et al. [55]. *Syllis gracilis* was expected to regenerate the prostomium after 8 dpa, based on previous studies [37, 66]. However, we noticed only a blastema elongation during anterior regeneration after 8 dpa. This observed difference might be a result of the reduced temperature in our study (14 °C) compared to the one used by Boilly and Thibaut [37] (18 °C), as lower temperatures seem to delay the whole regeneration process in syllids [51].

**Discussion**

Posterior regeneration resembles regular posterior growth

In this study, we investigate regenerative processes of two species of syllids *Sphaerosyllis hystrix* (Exogoninae) and *Syllis gracilis* (Syllinae). Using comparative

---

**Fig. 3** Light microscopy pictures of the regenerating *Syllis gracilis*. a, b, c, g, h, i anterior regeneration. d, e, f, j, k, l posterior regeneration. Anterior and posterior regeneration of *S. gracilis* were observed during 8 dpa. The wound is completely healed after 2 dpa and a blastema develops during the following days in both anterior and posterior regeneration. After 8 dpa, the blastema was still elongating during anterior regeneration (a-c, g-i). Regarding posterior regeneration, the blastema differentiated between 4 and 7 dpa; after 8 dpa a pygidium bearing three short cirri was restored (d-f, j-l). All pictures are in dorsal view. Scale bar 0.2 mm. White dashed lines show amputation region.
transcriptomics, we analyse three conditions: anterior regeneration, posterior regeneration, and regular growth. In both investigated species, our analyses revealed no differentially expressed (DE) genes between posterior regeneration (PR) and regular growth (NR); whereas the anterior regeneration (AR) significantly differed from those other conditions by having a high number of up-regulated genes. The absence of DE genes in the PxN comparisons of both species indicates that genes in PR and NR have similar expression levels. This result suggests that the genetic mechanisms behind the posterior regeneration and regular growth are similar in syllids with lifelong growth.

Previous studies provided similar results indicating that several genes expressed in the SAZ are also expressed in the blastema during posterior regeneration in annelids [3, 5, 21–23, 29, 46]. These two regions contain undifferentiated cells (blastema) and pluripotent cells (teloblasts in the SAZ), which require the activity of certain genes linked to stem-cellness, differentiation, re-establishment of antero-posterior and dorso-ventral axes, and elongation of the nervous system, among other processes [1, 3, 4, 7, 27, 46, 59]. Those processes are present during regeneration, growth, and homeostasis in planarians and acelos, and have been shown to be regulated by similar genetic pathways, e.g. Wnt and FGFRL signalling, TOR (target of rapamycin) control, and germ-line multipotency program activity [67–71]. Body growth and regeneration, therefore, are somehow similar programs in animals with high regenerative capacity.

### Table 1

| Parameters                          | Sphaerosyllis hystrix | Syllis gracilis |
|-------------------------------------|-----------------------|-----------------|
| Raw reads**                         | 79.5 GB               | 74.3 GB         |
| Total assembled bases               | 2,131,967,267         | 3,300,068,885   |
| Total number of reads               | 122,278,261           | 113,602,020     |
| Number of clean reads               | 102,763,252           | 100,322,750     |
| Median contig length (nucleotides) | 405                   | 377             |
| Average contig length (nucleotides) | 733.43                | 626.48          |
| N50 value (nucleotides)             | 1,115                 | 858             |
| Total number of transcripts         | 315,224               | 526,860         |
| Average length of transcripts (nucleotides) | 642.64            | 546.32          |
| Transcripts with GO annotation      | 90,058                | 128,997         |
| Predicted proteins                  | 125,040               | 184,632         |
| Trinity ‘genes’                     | 179,841               | 309,576         |
| Completeness                        | 97.8%                 | 98.6%           |
| Duplicated copies                   | 73.8%                 | 80.6%           |
| Single copies                       | 24.0%                 | 18.0%           |
| Fragmented copies                   | 1.9%                  | 1.3%            |

**Sum of raw reads of all sequenced libraries

transcriptomics, we analyse three conditions: anterior regeneration, posterior regeneration, and regular growth. In both investigated species, our analyses revealed no differentially expressed (DE) genes between posterior regeneration (PR) and regular growth (NR); whereas the anterior regeneration (AR) significantly differed from those other conditions by having a high number of up-regulated genes. The absence of DE genes in the PxN comparisons of both species indicates that genes in PR and NR have similar expression levels. This result suggests that the genetic mechanisms behind the posterior regeneration and regular growth are similar in syllids with lifelong growth.

Previous studies provided similar results indicating that several genes expressed in the SAZ are also expressed in the blastema during posterior regeneration in annelids [3, 5, 21–23, 29, 46]. These two regions contain undifferentiated cells (blastema) and pluripotent cells (teloblasts in the SAZ), which require the activity of certain genes linked to stem-cellness, differentiation, re-establishment of antero-posterior and dorso-ventral axes, and elongation of the nervous system, among other processes [1, 3, 4, 7, 27, 46, 59]. Those processes are present during regeneration, growth, and homeostasis in planarians and acelos, and have been shown to be regulated by similar genetic pathways, e.g. Wnt and FGFRL signalling, TOR (target of rapamycin) control, and germ-line multipotency program activity [67–71]. Body growth and regeneration, therefore, are somehow similar programs in animals with high regenerative capacity.

### Table 1

| Parameters                          | Sphaerosyllis hystrix | Syllis gracilis |
|-------------------------------------|-----------------------|-----------------|
| Raw reads**                         | 79.5 GB               | 74.3 GB         |
| Total assembled bases               | 2,131,967,267         | 3,300,068,885   |
| Total number of reads               | 122,278,261           | 113,602,020     |
| Number of clean reads               | 102,763,252           | 100,322,750     |
| Median contig length (nucleotides) | 405                   | 377             |
| Average contig length (nucleotides) | 733.43                | 626.48          |
| N50 value (nucleotides)             | 1,115                 | 858             |
| Total number of transcripts         | 315,224               | 526,860         |
| Average length of transcripts (nucleotides) | 642.64            | 546.32          |
| Transcripts with GO annotation      | 90,058                | 128,997         |
| Predicted proteins                  | 125,040               | 184,632         |
| Trinity ‘genes’                     | 179,841               | 309,576         |
| Completeness                        | 97.8%                 | 98.6%           |
| Duplicated copies                   | 73.8%                 | 80.6%           |
| Single copies                       | 24.0%                 | 18.0%           |
| Fragmented copies                   | 1.9%                  | 1.3%            |

**Sum of raw reads of all sequenced libraries

### Fig. 4

Heatmaps of differentially expressed genes during regeneration (FDR < 0.001). a Sphaerosyllis hystrix results. b Syllis gracilis results. Note that some of the genes can be up-regulated in more than one condition. Values in centred log 2(fpkms + 1). AR: anterior regeneration, PR: posterior regeneration, NR: non-regenerating. See Additional file 2: Table S1 and Additional file 3: Table S7 for detailed results.
Gene up-regulation in the anterior regeneration

The high number of up-regulated genes in AR may be due to the combination of two different factors: First, the presence of two proliferative zones acting at the same time (the SAZ and the blastema of anterior regeneration (see Fig. 1)). Second, as suggested by a previous study in flatworms [72], some DE genes in AR might be involved in the reestablishment of anterior identity and the regeneration of anterior-specific structures, such as the brain. The presence of two proliferative zones in AR implies the
existence of a higher number of cells simultaneously expressing certain genes involved in regeneration and growth. Therefore, the overall number of reads of transcripts related to these processes might be higher in AR than in PR or NR. The GO annotation showed that most of the DE genes were assigned to the cellular component category, thus suggesting functions related to cellular proliferation.

However, many of the up-regulated genes in AR of both species could not be identified and, hence, their functions remain unknown. This is probably due to the scarcity of annelid genomic data in the databases used for annotation. In addition, the generated assemblies had high duplication levels, which resulted in artificially large transcriptomes. These high duplication levels were probably a consequence of pooling different individuals for each sequencing library, which can introduce allelic variation, splicing differences, and assembly artefacts [73]. Nevertheless, our transcriptomic analyses relied on highly complete assemblies based on BUSCO criteria and the comparison with other annelid assemblies [57, 74, 75]; and, since we performed the differential expression analyses at the level of Trinity ‘genes’ (which sums up the expression values of all isoforms of a ‘gene’), this redundancy does not affect our results.

Regeneration-related genes

Using BLAST searches, we were able to identify regeneration-related candidates among the DE genes of our analysis. Those candidates have been associated to regenerative processes such as wound healing, blastema formation, stem cell regulation, cell proliferation, segmentation, and morphogenesis by several studies in annelids [1, 2, 12, 13, 17, 21, 23, 27, 32, 35, 45, 46, 48, 59–63]. Among the regeneration-related genes explored in this study, we found 12 DE genes in S. gracilis and 3 DE genes in S. hystrix; all of them were up-regulated in AR (FDR > 0.01, see Table 2).

Some of the DE genes are associated to cell proliferation and nervous system elongation, processes that support the two-proliferation-zones hypothesis suggested above; they are paics, JNK, PL10, slit, elav, glutamine synthetase (gs), andrup [29, 46, 76, 77]. Of these candidate genes, only paics (phosphoribosylaminomimidazolone) was differentially expressed in both species (Table 2). paics is required for de novo biosynthesis of purines during cellular proliferation, and it has been reported to be highly expressed during regeneration in the clittellate Enchytraeus japonensis [12]. Similarly, JNK (up-regulated homolog in AR of S. hystrix) translates signals into apoptotic cell death and controls cell proliferation and differentiation to coordinate regeneration in planarians [78, 79]. Also, a homolog of PL10 was differentially expressed in S. gracilis results, up-regulated in AR (AxN comparison). PL10, like vasa, piwi, and nanos, is one of the germline multipotency program genes [80]. These genes are linked to somatic differentiation and stem-cellness, and can be considered conserved markers of the SAZ in annelids [14, 26, 34, 48, 62, 74, 80, 81].

The genes gs, elav and slit play an important role in nervous system regeneration and growth in annelids [12, 13, 46]. Homologs of those genes were found to be up-regulated in AR (AxP and AxN comparisons of S. gracilis). The enzyme glutamine synthetase (encoded by gs) plays a role in cell metabolism, ammonia detoxification, glutamate transmitter degradation in the nervous system, and was found to be expressed in early stages of regeneration in Enchytraeus japonensis [12, 13, 82]. The genes slit and elav, on the other hand, encode signalling and a RNA-binding proteins, respectively [83, 84]. They are expressed, for example, in the ventral midline cells (slit) and differentiating neurons (elav) during posterior regeneration in Platynereis dumerilii [46], and are evolutionary conserved across animal evolution [83, 84].

Interestingly, in both transcriptomes we identified homologs of Ej-rup 1–5 (E. japonensis regeneration up-regulated genes 1–5), regeneration-related genes previously reported for the clittellate E. japonensis (Table 2) [12]. However, only in S. hystrix one of them (Shy-rup2) was up-regulated in AR (AxN comparison). The function of this gene is not clear, but Ejrup2 was detected in epidermal cells of the blastema during anterior regeneration and might be a regeneration-specific gene [12].

### Table 2 Results of BLAST searches for candidate regeneration genes

| Sphaerosyllis hystrix | Syllis gracilis |
|-----------------------|----------------|
| Trinity gene ID AxP AxN | Trinity gene ID AxP AxN |
|                     |                     |
| β-catenin         | TR65158|c1_g2 – – | TR89060|c2_g1 + + |
| Brat              | TR63166|c4_g3 – – | TR74232|c0_g1 + + |
| cycB3            | TR69437|c2_g1 – – | TR101261|c2_g1 + + |
| Elav             | TR886647|c2_g1 – – | TR79253|c1_g1 + + |
| FGFR             | TR95577|c0_g2 – – | TR64245|c1_g1 + + |
| Gs               | TR72222|c3_g2 – – | TR76174|c0_g1 + + |
| gcs1a           | TR38757|c0_g2 – – | TR89735|c2_g1 + + |
| Hex7            | TR74688|c1_g5 – – | TR50489|c0_g1 + + |
| JNK             | TR19529|c0_g1 – + | TR151703|c0_g1 – – |
| Lox2            | TR72209|c0_g3 – + | TR122252|c3_g1 + + |
| Paics           | TR52155|c0_g2 + + | TR87989|c0_g1 + + |
| PL10           | TR60633|c1_g1 – – | TR99989|c1_g1 – – |
| rup2           | TR83599|c2_g1 + + |                 |
| Slit            | TR63077|c0_g1 – – | TR107009|c0_g1 + + |

Plus signs indicate statistically significant differential expression results (FDR < 0.01). AxP: anterior regeneration versus posterior regeneration. AxN: anterior regeneration versus non-regenerating individual. NxP: non-regenerating individual versus posterior regeneration (see Additional files 2, 3 and 4 for complete results)
Supporting the hypothesis of AR gene up-regulation being related to the reestablishment of anterior identity and structures, we detected some DE genes probably related to the reestablishment of anterior identity and regular posterior growth, but are required during anterior regeneration of *S. gracilis*. In studies on the annelids *P. dumerilii* and *A. virens* (which cannot regenerate anteriorly), the expression of *Hox7* and *Lox2* was detected during larval development, growth and posterior regeneration [21–23, 64]. In addition, we detected up-regulation of a homolog of β-catenin in AR of *S. gracilis* (AxP, AxN comparisons; FDR > 0.01), which means that they are expressed in similar levels during tail regeneration and regular posterior growth, but are required during anterior regeneration of *S. gracilis*. In studies on the annelids *P. dumerilii* and *A. virens* (which cannot regenerate anteriorly), the expression of *Hox7* and *Lox2* was detected during larval development, growth and posterior regeneration [21–23, 64]. In addition, we detected up-regulation of a homolog of β-catenin in AR of *S. gracilis* (AxP, AxN comparisons; FDR > 0.001). In annelids, for example, β-catenin expression has been found in the blastema of *P. leidyi* during anterior and posterior regeneration, and in fission zones during asexual reproduction [17]. Additionally, Demilly et al. [59] suggested that the Wnt/β-catenin pathway is involved in neural cell proliferation/differentiation in *P. dumerilii*. In planarians, Wnt/β-catenin signalling is known to be required for the establishment of the antero-posterior axis during regeneration, promoting homeostasis and proper brain regeneration [67, 69, 85].

**Conclusions**

We studied the regenerative abilities of two syllid species. Both species can completely regenerate the posterior body after one to 2 weeks post amputation. However, only *Syllis gracilis* is able to regenerate the entire anterior body and, in contrast, *Sphaerosyllis hystrix* has a limited anterior regeneration. By using RNA-seq, we found that, for both species, individuals in posterior regeneration and intact individuals have comparable gene expression profiles. On the other hand, anterior regeneration shows a significant up-regulation of DE genes, including some candidate regeneration genes related to cellular proliferation (*paic* and *JNK*), nervous system development (*gs, elav, slit*), stem-cellness (PL10), and reestablishment of antero-posterior axis (*Hox* genes and β-catenin). Those results lead to two main conclusions, first that posterior regeneration is similar to the postembryonic process of growth in annelids, while anterior regeneration is markedly different from both; and second, that syllids regenerate using common genetic pathways (regeneration-related genes) already described for other annelids and other groups of invertebrates, supporting the importance of comparative studies to illuminate the evolution of regeneration in Metazoa.

**Methods**

**Sampling**

Animals were collected in intertidal rocky shores from Ferrol, Galicia, Spain (43°27′17.0″N; 8°18′39.8″W) during third quarter moon in April 2017. Specimens of *Syllis gracilis* and *Sphaerosyllis hystrix* were sorted for regeneration experiments. *Syllis gracilis* is a species complex with eight recognized lineages, from which a specimen from Galicia, Spain has been shown to belong to lineage 8′ [87]. We confirmed that our specimens also belong to this lineage through phylogenetic analysis using sequences from the transcriptome assembly and those provided by a previous study [87] (see Additional files 5 and 6). For each molecular marker (COI, 16S, 18S, 28S), alignments were performed using MAFFT version 7 [88] (G-INS-I iterative method), and the datasets produced were concatenated using FASconCAT-G version 1.02 [89]. Then, a maximum likelihood analysis was conducted using RAxML, with 1000 bootstrap pseudoreplicates, and a partition scheme allowing for optimization of the three genes separately [90, 91].

**Experimental procedures**

Intact non-reproducing adults were selected for regeneration experiments. The animals were anesthetized in a 3.5% MgCl₂ solution dissolved in seawater. Bisection was performed in the midbody of 48 individuals of *S. hystrix* (after chaetigers 13–18), and of 30 individuals of *Syllis gracilis* (after chaetigers 25–48) (Fig. 1). The animals were kept in one-litre aquariums with flowing filtered natural seawater at 14 °C for up to 14 dpa. Since there were only few specimens of *S. gracilis*, and some of them died during experimentation, the regeneration process could only be followed until 8 dpa. Anterior and posterior amputees were separated in different aquaria and kept in starvation during the experiment. Two amputees of each condition were fixed in 4% PFA every day for morphological observations (up to 14 dpa in *S. hystrix* and 8 dpa in *S. gracilis*, see Fig. 1). Optical microscopy images of fixed animals were taken to document morphological changes using an Olympus CX31 microscope and a BQ Aquaris V. For transcriptome sequencing, amputees were fixed in RNA later (Ambion, Darmstadt, Germany) to represent four stages of regeneration: stage 1 (healing response/cicatrisation); stage 2 (early blastema development); stage 3 (late blastema development); stage 4 (patterning/cap regeneration), only observed during posterior regeneration (see Fig. 1). Two amputees of *S. hystrix* were fixed per stage: 1 dpa (stage 1), 5 dpa (stage 2), 9 dpa (stage 3) and 12 dpa (stage 3/ stage 4), summing up a total of 16 amputees; i.e. 8 for anterior regeneration (AR) and 8 for posterior regeneration (PR). Additionally, five intact individuals of *S. hystrix* were fixed as non-regenerating condition (NR). For *S. gracilis*, one amputee of each regenerative condition (AR, PR) was fixed in 1 dpa (stage 1), 3 dpa (stage 2), 6 dpa (stage 3) and 8 dpa (stage 3/ stage 4), and two whole animals were fixed for NR (Fig. 1). All experimental procedures were conducted in April
Illumina sequencing and de novo assembly

Three libraries were prepared for each of the two species: anterior regeneration (AR), posterior regeneration (PR), and non-regenerating adults (NR), i.e. intact individuals in regular posterior growth. RNA extraction was conducted by pooling together all individuals belonging to the same condition and species, i.e. amputees in different stages were pooled for each regenerative condition (AR, PR) and intact specimens were pooled together to prepare the non-regenerating condition (NR). Considering that the sequences represent samples of pooled individuals, there were no biological replicates from which to estimate inter-individual variability in gene expression. Although replicates provide a robust statistical support in differential expression analyses [92, 93], pooling samples for transcriptomic sequencing can be a useful strategy to establish a good framework of DE genes from small animals and neglected organisms [94].

RNA was isolated using NZYTech’s Total RNA isolation kit and the pure RNA was eluted in a final volume of 30 μL. Quality and quantity of RNA were checked in an Agilent 2100 Bioanalyzer using Agilent RNA 6000 kit. To prepare the libraries, we used Illumina’s TruSeq Stranded mRNA Library Prep Kit following manufacturer’s instructions. Fragment size distribution and concentration were checked in the Agilent Bioanalyzer. Qubit dsDNA BR Assay kit (Thermo Fisher Scientific) was used to quantify the libraries, which were then sequenced in an Illumina HiSeq 4000 PE100 lane. All procedures of RNA isolation, library construction, and sequencing were performed by AllGenetics & Biology SL (A Coruña, Spain).

We used FastQC v0.11 (http://bioinformatics.babraham.ac.uk/projects/fastqc/) to assess sequence quality, and Trimmomatic v0.33 [95] to trim the raw sequences based on quality results with options HEADCROP:10 LEADING:20 SLIDINGWINDOW:5:20 MINLEN:70. De novo transcriptome assembly was performed using Trinity v2.3.2 [96, 97] and transcripts with ≥200 bases were kept. Assembly statistics were obtained using the TrinityStats tool of Trinity, and BUSCO [57] was used to estimate transcriptome completeness.

Functional annotation and gene ontology

Transcripts were annotated following the Trinotate pipeline (https://github.com/Trinotate/Trinotate.github.io/wiki). For that, TransDecoder v3.0.1 (https://transdecoder.github.io/) was used to predict protein sequences from the assembled transcripts. Then, both the assembled transcripts and the predicted proteins were used for functional annotation, which was performed using BLAST v2.5.0 [98], HMMER v3.1b2 (http://hmmer.org), signalp v4.1 [99], RNAmer v1.2 [100], and tmHMM v2.0c [101] to find known sequences and domains. Gene ontology (GO) terms analysis was used to classify the functions of the predicted genes. Trinotate v3.0.1 (http://trinotate.github.io) and WEGO Web Server [102] were used to summarise the results of gene ontology (GO).

Differential expression analyses

Using the Differential Expression module of Trinity v2.3.2 (https://github.com/trinityrnaseq/trinityrnaseq/wiki/Trinity-Differential-Expression), we performed pairwise comparisons at the Trinity ‘gene’ level between each condition of our experiment: anterior regeneration against posterior regeneration (AxP), anterior regeneration against non-regenerating condition (AxN), and posterior regeneration against non-regenerating (PxN). We ran RSEM [103] to estimate per-condition transcript abundance, and edgeR [104, 105] to perform the differential expression analyses. At this point, we tried several values for the dispersion parameter of edgeR, and concluded that 0.5 was the best fitting value for our data.

After that, we used the analyze_diff_expr.pl script of Trinity to create differential expression subsets for each pairwise comparison, and included the examine_GO_enrichment flag that, by combining the results of differential expression and the functional annotation, can inform which Gene Ontology categories are enriched or depleted in each experimental condition for each pairwise comparison. We used GOplot to illustrate the results of gene ontology (GO) enrichment analyses [58]. The outputs files of the differential expression analyses and the functional enrichment analyses are available under https://github.com/rannypribeiro/Regeneration_transcriptomics. Overall results are summarised in Additional files 2 and 3.

Identification of candidate regeneration genes

BLASTn and BLASTp searches were used to detect homologs of genes related to animal regeneration that had been previously reported in the literature. The top hits in the BLAST results were analysed via a reciprocal BLASTn against the nr/nt database (NCBI) to verify the putative identity of candidate regeneration genes in the transcriptomes of S. hystrix and S. gracilis.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12864-019-6223-y.

Additional file 1. Results of functional annotation of the transcriptomes of Sphaerosyllis hystrix and Syllis gracilis. a Results against all metazoan database. b Results within Annelida. c Gene ontology distribution of the annotated genes grouped in the three main functional categories.
Additional file 2. Differential gene expression and functional enrichment results for Sphaerosyllis hystrix.

Additional file 3. Differential gene expression and functional enrichment results for Syllis gracilis.

Additional file 4. BLAST results of candidate genes including differential gene expression results. ns: not significant.

Additional file 5. Syllis gracilis phylogeny including sequences identified from the transcriptome assembly of this study. The lineages are in agreement with previous study [87].

Additional file 6. GenBank accession numbers of sequences used for phylogenetic reconstruction. Codes used for S. gracilis sequences by Alvarez-Campos et al. [87] were maintained here.

Abbreviations

AR: Anterior regeneration, as experimental condition; AxN: Anterior regeneration versus non-regenerating; AxP: Anterior regeneration versus posterior regeneration; BP: Biological process; BUSCO: Benchmarking Universal Single-Copy Orthologs; CC: Cellular component; DE: differential expression; GS: Glutamine synthetase; GSK3: glycogen synthase kinase-3; JNK: c-Jun N-terminal kinase; MF: Molecular function; NR: Non-regenerating, as experimental condition; px: Multifunctional protein ADE2; PR: Posterior regeneration, as experimental condition; PA: Posterior regeneration versus non-regenerating.

Acknowledgements

We thank Guillermo Díaz-Agras, Marcos Abad, Maria Candás, and Xela Cunha-Wei for helping in sampling and for providing facilities to perform the experiments at the Marine Biological Station of A Graña (Ferrol, Spain); Alexa Bely and one anonymous reviewer for their valuable suggestions to improve this manuscript.

Authors’ contributions

RPR, CB, and MTA conceived and designed experiments. RPR, GPS and CB collected the specimens. RPR conducted regeneration experiments and CB, RPR, and MTA collected the specimens. RPR, CB, and MTA conceived and designed experiments. RPR, GPS and CB collected the specimens. RPR wrote the first draft of the manuscript. MTA supervised the whole study. All authors have written, reviewed, and approved the final manuscript.

Funding

This research was supported by MINECO/FEDER, UE funds (Grant: CGL2015–63593-P, “Macroevolutionary transitions in Syllidae” project, PI: MTA). RPR is supported by the program “Contratos predoctorales para Formación de Personal Investigador, FPI-UAM,” Universidad Autónoma de Madrid. GPS is supported by the “Contratos Predoctorales para la Formación de Doctores 2016” program of the MINECO, Spain (code: BES-2016-076419), cofinanced by the European Social Found. We acknowledge support by German Research Foundation and the Open Access Publication Funds of the Georg-August-Universität Göttingen.

Availability of data and materials

The raw reads are available at the NCBI Sequence Read Archive (SRA) for Sphaerosyllis hystrix (BioProject ID PRJNA517681, SRX5314036–38) and Syllis gracilis (BioProject ID PRJNA517687, SRX5314271–73). Assemblies, transdecoder predicted proteins, and differential gene expression results generated in this study can be accessed in https://github.com/ranynpribeiro/Regeneration_transcriptomics.

Ethics approval and consent to participate

Sampling was conducted under the supervision of the staff at Estación de Bioxoxía Marinha da Graña, USC, and with permission and in agreement with the requirements by the Galician regional government (Consellería de Medio Ambiente, Territorio e Vivenda; Xunta de Galicia).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

1Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain. 2Animal Evolution & Biodiversity, Georg-August-Universität Göttingen, 37073 Göttingen, Germany. 3German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, 04103 Leipzig, Germany. 4Centro de Investigación en Biodiversidad y Cambio Global (CIBC-UAM), Universidad Autónoma de Madrid, Madrid 28049, España.

Received: 5 April 2019 Accepted: 24 October 2019
Published online: 14 November 2019

References

1. Bely AE, Wray GA. Evolution of regeneration and fission in annelids: insights from engrafted- and orthodenticle-class gene expression. Development. 2001;128:2791–91.

2. Pfeifer K, Dorrersteijn AWC, Fröblus AC. Activation of Hox genes during caudal regeneration of the polychaete annelid Platynereis dumerilii. Dev Genes Evol. 2012;222:165–79.

3. Gazzave E, Béhague J, Laplanche L, Guillou A, Préau L, Demilly A, et al. Posterior elongation in the annelid Platynereis dumerilii involves stem cells molecularly related to primordial germ cells. Dev Biol. 2013;382:246–67.

4. Balavoine G. Segment formation in annelids: patterns, processes and evolution. Int J Dev Biol. 2014;58:469–83.

5. Bely AE, Zattara EE, Sires JM. Regeneration in spiralian: evolutionary patterns and developmental processes. Int J Dev Biol. 2014;58:223–34.

6. Zattara EE, Bely AE. Phylogenetic distribution of regeneration and asexual reproduction in Annelida: regeneration is ancestral and fission evolves in regenerative clades. Invertebr Biol. 2016;135:400–14.

7. Özpolat BD, Bely AE. Developmental and molecular biology of annelid regeneration: a comparative review of recent studies. Curr Opin Genet Dev. 2016;40:144–53.

8. Ribeiro RP, Bleidorn C, Aguado MT. Regeneration mechanisms in Syllidae (Annelida). Regeneration. 2018;5:26–42.

9. Bely AE. Early events in annelid regeneration: a cellular perspective. Integr Comp Biol. 2014;54:688–99.

10. Boilly B, Boilly-Marer Y, Bely AE. Regulation of dorso-ventral polarity by the nerve cord during annelid regeneration: a review of experimental evidence. Regeneration. 2017;4:54–68.

11. Nakamura Y. A new fragmenting entchytraeid species, Enchytraeus japonensis from a cropped Kurobokosu soil in Fukushima, northern Japan (entchytraeids in Japan 3). Edaphologia. 1993;50:17–9.

12. Myohara M, Niva CC, Lee JM. Molecular approach to annelid regeneration: cDNA subtraction cloning reveals various novel genes that are upregulated during the large-scale regeneration of the oligochaete, Enchytraeus japonensis. Dev Dyn. 2006;235:2051–70.

13. Niva CC, Lee JM, Myohara M. Glutamine synthetase gene expression during the regeneration of the annelid Enchytraeus japonensis. Dev Genes Evol. 2008;218:39–46.

14. Takodoro R, Sugio M, Kutsuna J, Tochinai S, Takahashi Y. Early segregation of germ and somatic lineages during gonadal regeneration in the annelid Enchytraeus japonensis. Curr Biol. 2006;16:1012–7.

15. Takeo M, Yoshida-Noro C, Tochinal S. Morphallactic regeneration as revealed by region-specific gene expression in the digestive tract of Enchytraeus japonensis (Oligochaeta, Annelida). Dev Dyn. 2008;237:1284–94.

16. Smith F. Notes on species of north American Oligochaeta. Ill. Illinois Nat Hist Surv Bull. 1896;4:396–413.

17. Nyberg KG, Conte MA, Kostyun J, Forde A, Bely AE. Transcriptome characterization via 454 pyrosequencing of the annelid Pristina leidyi, an emerging model for studying the evolution of regeneration. BMC Genomics. 2012;13:287.

18. Zattara EE, Bely AE. Evolution of a novel developmental trajectory: fission is distinct from regeneration in the annelid Pristina leidyi. Evol Dev. 2011;13:80–95.

19. Zattara EE, Bely AE. Investment choices in post-embryonic development: quantifying interactions among growth, regeneration, and asexual...
reproduction in the annelid Pristina leidyi. J Exp Zool B Mol Dev Evol. 2013;320:471–88.

20. Zatta EA, Turlington KW, Bely AE. Long-term time-lapse live imaging reveals extensive cell migration during anterior regeneration. BMC Dev Biol. 2016;16:66.

21. Kalužová M, Bakalenko N, Novikova E, Cokk CE, Eliseeva E, Steinmetz PRH, et al. Hox gene expression in larval development of the polychaete Nereis virens and Platynereis dumerillii (Annelida, Lophotrochozoa). Dev Genes Evol. 2007;217:39–54.

22. Bakalenko N, Novikova EL, Nesterenko NY, Kulakova MA. Hox gene expression during postlarval development of the polychaete Alitta vira. Evodevo. 2013;4:13.

23. Novikova EL, Bakalenko NI, Nesterenko AK, Kulakova MA. Expression of Hox genes during regeneration of nereid polychaete Alitta vira (Annelida, Lophotrochozoa). Evodevo. 2013;4:21.

24. Sars M. Beskrivelser og Iagttagelser over nogle moerkelige eller nye i Havet i Norden. Helsink: Ex Officina Frenckelliana; 1863.

25. Blake JA, Grassle JP, Eckelbarger KJ. Tagging and age-structure of Cornelia thea, a new species of the polychaete family Syllidae. J Exp Mar Biol Ecol. 2008;358:144–53.

26. Dill KK, Seaver EC. A stable thoracic Hox code and epimorphosis characterize posterior regeneration in Capitella teleta. PLoS One. 2011;6:e19472.

27. de Jong DM, Seaver EC. Investigation into the cellular origins of posterior regeneration in the annelid Platynereis dumerillii. J Exp Mar Biol Ecol. 2006;325:39–53.

28. Neal S, de Jong M, Seaver EC. CRISPR/CAS9 mutagenesis of a single r-opsin gene in the polychaete Capitella teleta. PLoS One. 2016;11:e014972.

29. de Jong M, Seaver EC. CRISPR/CAS9 mutagenesis of a single r-opsin gene in the polychaete Capitella teleta. J Exp Mar Biol Ecol. 2016;378:54–61.

30. Audouin JV, Milne EH. Classification des Annélides et description de celles qui habitent les côtes de la France. Ann des Sci Nat Paris. 1833;1:195–269.

31. Prud’homme B, Rosa R, Arendt D, Julien JF, Papajt R, Doresteijn AWC, et al. Arthropod-like expression patterns of engrailed and wingless in the annelid Platynereis dumerili suggest a role in segment formation. Curr Biol. 2003;13:1876–81.

32. de Rosa R, Prud’homme B, Balavoine G. Caudal and even-skipped in the polychaete annelid Nereis virens. J Exp Zool B Mol Dev Evol. 2014;275:1418–27.

33. Kulakova MA, Bakalenko NI, Kulakova MA. Expression during larval regeneration of Nereis virens (Annelida, Lophotrochozoa). BMC Dev Biol. 2008;8:1–18.

34. Planqué A, Malem J, Pararaj J, Veroort M, Gazave E. Morphological, cellular and molecular characterization of posterior regeneration in the marine annelid Platynereis dumerillii. Dev Biol. 2019;455:189–210.

35. Özpolat BD, Sloane ES, Zattara EE, Bely AE. Plasticity and regeneration of gonads in the annelid Pristina leidyi. Evodevo. 2016;7:22.

36. Okada YK. Regeneration and fragmentation in the sydillidan polychaetes. Wilhelm Roux Arch Entwickl Mech. 1929;115:542–600.

37. Novikova EL, Bakalenko NI, Nesterenko AK, Kulakova MA. Hox gene expression in larval and postlarval development of the polychaete Nereis virens (Annelida, Lophotrochozoa). BMC Dev Biol. 2008;8:1–18.

38. Ribeiro et al. BMC Genomics (2019) 20:855
69. Gurley KA, Rink JC, Alvarado AS. β-catenin defines head versus tail identity during planarian regeneration and homeostasis. Science. 2008;319:233–7.

70. Hill EM, Petersen CP. Positional information specifies the site of organ regeneration and not tissue maintenance in planarians. eLife. 2018;7:e3680.

71. Peiris TH, Weckerle F, Ozamoto E, Ramirez D, Bartscherer K, Salò E, Adell T. JNK controls the onset of mitosis in planarian stem cells and triggers apoptotic cell death required for regeneration and remodeling. PLoS Genet. 2014;10:e1004400.

72. Juliano CE, Swartz SZ, Wessel GM. A conserved germline multipotency program. Development. 2010;137:415–26.

73. De novo transcriptome assembly and development of molecular specific gene expression of Pygospio elegans. Evol Dev. 2017;19:205–17.

74. Boilly B. Étude ultrastructurale de l’évolution des tissus impliqués dans la régénération céphalique et caudale de Syllis amica Q. (Anellida, Syllidae). I. La dédifférenciation. J Microsc. 1968;110:75–86.

75. Boilly B. Étude ultrastructurale de l’évolution des tissus impliqués dans la régénération céphalique et caudale de Syllis amica Q. (Anellida, Syllidae). II. L’activation et la différenciation. J Microsc. 1968;110:775–97.

76. Tasaki J, Shibata N, Sakurai T, Agata K, Umesono Y. Role of c-Jun N-terminal kinase activation in blastema formation during planarian regeneration. Develop Growth Differ. 2011;53:389–400.

77. Almuedo-Castillo M, Crespo X, Seebek F, Bartscherer K, Salò E, Adell T. The transcription factor GIN2 controls the onset of mitosis in planarian stem cells and triggers apoptotic cell death required for regeneration and remodeling. Planaria. 2014;10:e1004400.

78. Giani VC, Yamaguchi E, Boyle MJ, Seaver EC. Somatic and germline expression of piwi– mediates the generation of an alternatively spliced neural protein isoform. Cell. 1999;96:795–806.

79. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215:403–10.

80. Petersen TN, Brunak S, Heijne G, Nielsen H. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods. 2011;8:785–6.

81. Lagesen K, Hallin P,Radland EA, Stærkelid HH, Rognes T, Ussery DW. RNaMMer: consistent and rapid annotation of ribosomal RNA genes. Nucleic Acids Res. 2007;35:3190–8.

82. Krogh A, Larsson B, von Heijne G, Sonnhammer EL. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol. 2001;305:567–80.

83. Ye J, Fang L, Zheng H, Zhang Y, Chen J, Zhang Z, et al. WEGO: a web tool for plotting GO annotations. Nucleic Acids Res. 2006;34:293–7.

84. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics. 2011;12:323.

85. McCarthy DJ, Chen Y, Smyth GK. Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. Nucleic Acids Res. 2012;40:4288–97.

86. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010;26:139–40.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.